Clot Architecture Is Altered in Abdominal Aortic Aneurysms and Correlates With Aneurysm Size


Objective—Abdominal aortic aneurysm (AAA) is characterized by widening of the aorta. Once the aneurysm exceeds 5.5 cm, there is a 10% risk of death due to rupture. AAA is also associated with mortality due to other cardiovascular disease. Our aim was to investigate clot structure in AAA and its relationship to aneurysm size.

Methods and Results—Plasma was obtained from 49 controls, 40 patients with small AAA, and 42 patients with large AAA. Clot formation was studied by turbidity, fibrin pore structure by permeation, and time to half lysis by turbidity with tissue plasminogen activator. Plasma clot pore size showed a stepwise reduction from controls to small to large AAA. Lag phase for plasma clot formation and time to half lysis were prolonged, with smaller AAA samples showing intermediate response. Clot structure was normal in clots made with fibrinogen purified from patients compared with controls, suggesting a role for other plasma factors. Endogenous thrombin potential and turbidity using tissue factor indicated that the effects were independent of changes in thrombin generation.

Conclusion—Patients with AAA form denser, smaller pored plasma clots that are more resistant to fibrinolysis, and these characteristics correlate with aneurysm size. Clot structure may play a role in AAA development and concomitant cardiovascular disease. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: aortic diseases ■ blood coagulation ■ fibrin ■ fibrinolysis ■ thrombosis
structure in first-degree relatives of patients with coronary artery disease, suggesting a role of fibrin in predisposition to thrombosis. Based on the common role of thrombosis in cardiovascular disease and AAA, we tested the hypothesis that patients with AAA produce clots with altered structure. The objectives of this study were to investigate (1) clot structure in patients with AAA compared with controls, and (2) association of clot structure with aneurysm size. We found that plasma clots were denser and more resistant to lysis in patients with AAA and that this was particularly evident in patients with large aneurysms.

Patients and Methods

Subjects

Eighty-two patients with AAA were enrolled as part of the Leeds Aneurysm Development Study in the vascular outpatient department, Leeds Teaching Hospitals. All subjects were white, northern European males over the age of 50. Forty of these patients had small AAA, with an aortic diameter ranging from 3.0 to 5.4 cm. Forty-two patients had large AAA (≥5.5 cm). Large and small AAA patients were age matched with each other and with 49 male controls, recruited at random from cardiology and vascular outpatients. Control subjects all underwent ultrasound assessment and had aortic diameters ≤2.9 cm. Subjects who were on oral anticoagulation, had underlying malignancy, had had surgery in the last 3 months, or had active inflammatory conditions were excluded. Written informed consent was obtained. Ethical approval was provided by the Leeds Teaching Hospitals Research Ethics Committee.

Clinical Data

Subjects were fasted and asked to refrain from smoking for 10 hours overnight. A detailed clinical history was obtained by questionnaire. Blood pressure, body mass index, and waist:hip ratio measurements were obtained using standard protocols. A 12-lead ECG was performed to analyze ischemic changes indicative of angina. Maximal infrarenal anteroposterior aortic diameter (inner to inner) was determined by a departmental ultrasound scan.

Blood Sampling

Blood was drawn from the antecubital vein with minimal stasis. The first few milliliters were discarded. Blood was collected on 0.1 mol/L sodium citrate (9 parts blood per 1 part citrate) and centrifuged at 2400 g for 20 minutes within 2 hours to separate platelet-poor plasma. Samples were stored at -20°C.

Hemostasis Measurements

Fibrinogen was determined according to Claus on an Amelung KC10 coagulometer (Lemgo, Germany). Factor VII clotting activity was measured on an ACL300 Plus (Instrumentation Laboratories, Warrington, United Kingdom) using factor VII–deficient plasma (Sigma-Aldrich) and recombinant rabbit thromboplastin (Instrumentation Laboratories). Factor XIII A subunit was measured by in-house ELISA. Tissue plasminogen activator (tPA) (Imulys, Biopool International, Umea, Sweden), plasminogen activator inhibitor-1 (PAI-1) (Imulys, Biopool), thrombin-antithrombin complex (Dade-Behring, Marburg, Germany), and D-dimer (TintElize, Biopool) were measured by ELISA.

Clot Permeation

Ten µL of activation mixture (0.11 mol/L calcium chloride, 11 U/mL human thrombin [Calbiochem, San Diego, CA]) in permeation buffer (0.05 mol/L Tris, 0.10 mol/L NaCl, pH 7.5) was mixed with 100 µL of plasma. The mixture was carefully transferred into the tip of a 1-mL pipette and placed in a humidity chamber at room temperature for 2 hours. Tips were connected to a reservoir containing permeation buffer, with a 4-cm pressure drop. Six drops were allowed to permeate before measurements. A small container was attached to the tip to collect the percolate. At 30-minute intervals, the container was changed and weighed for percolate volume. Percolate measurements were performed a minimum of 3 times per clot. The permeation coefficient (Ks), representing the clot surface allowing flow through the network (pore size), was calculated as described. Samples were analyzed in duplicate.

Turbidity Lysis

Plasma (25 µL) was loaded into 96-well plates, and 75 µL of permeation buffer was added. Fifty µL of activation mix (22.5 mmol/L calcium chloride and 0.5 U/mL human thrombin in permeation buffer) was added. The plate was immediately placed in a Bio-Tek ELX 808 reader. Absorbency was read every 12 seconds at 350 nm for 60 minutes. For turbidity lysis with purified samples, 0.5 mg/mL fibrinogen was incubated with 0.1 U/mL thrombin and 2.5 mg/mL calcium chloride (final concentrations) in permeation buffer (150 µL, final volume).

Lysis was measured in a parallel plate. tPA (Technoclone, Vienna, Austria) was diluted to 170 ng/mL in permeation buffer, of which 75 µL was added to the plasma instead of buffer in the above turbidity assay. Activation mix was added as above. Absorbency at 350 nm was read every 12 seconds for 1 hour and then every 2 minutes for 9 hours. Data were analyzed using custom-written software, which is available from the authors on request. Samples were analyzed in duplicate. Turbidity and lysis was also performed in all samples with platelet-poor plasma tissue factor (TF) (endothelial thrombin potential [ETP] reagent) at a final concentration of 1.25 pmol/L (correlating with 5 pmol/L in undiluted plasma) instead of thrombin.

ETP

The ETP was performed according to the manufacturer’s instructions (Thrombinoscope, Maastricht, the Netherlands), using 5 pmol/L TF.

Fibrinogen Purification

Fibrinogen was purified from 4 controls, 5 patients with small AAA, and 5 patients with large AAA as previously described, selected on the basis of representative clot permeation characteristics. In brief, plasma collected in heparin was applied to affinity chromatography with a calcium-dependent antibody (Ig-F). Fibrinogen was eluted with EDTA and dialyzed extensively against permeation buffer. Purity of the samples was tested by SDS-PAGE.

Confocal Microscopy

Plasma (30 µL) was diluted with 25 µL of permeation buffer and 5 µL of Alexa Fluor 488 fibrinogen (5% final concentration, Molecular Probes, Leiden, the Netherlands) and placed in a µ-slide VI (Ibidi, Munich, Germany). Six µL of activation mix (5 U/mL thrombin, 100 mmol/L CaCl in permeation buffer) was added. Clots were left in a humidity chamber overnight. A Zeiss LSM510META Axioplan2 confocal microscope fitted with a ×63 oil immersion objective was used for imaging. Scan format was 512×512 pixels. Ten scans of 230/230/20 µm (x/y/z) were taken to visualize the fibrin network and to analyze fiber density per cubic area. The 3D information was converted into 2D images by projection using the Zeiss LSM software (version 4.20.0.121).

Statistical Analysis

Based on an average Ks of 4.7, a dispersion of 0.75, and an α-value of 0.05, a minimum of 36 subjects were needed in each group to detect an average difference of 0.5 with 80% power. Nonparametric data were described with median and interquartile ranges (IQRs). Variables with normal distribution were described with mean and standard deviation. A Kruskal-Wallis test compared Ks and turbidity across the 3 groups, followed by a Mann-Whitney U test for comparison between 2 groups. Spearman’s rank was used to correlate continuous variables. We used SPSS, version 12.0 (SPSS, Chicago, IL), and significance was defined as P<0.05. Multiple linear regression to test for independent covariates of Ks and lysis was performed using R, version 2.13.
Results

Clinical Characteristics

Subjects in the control, small AAA, and large AAA groups were men who were matched for age (Table 1). Aortic diameter was 2.0 cm (IQR 1.9–2.3) in controls, 4.2 cm (3.8–4.6) in small AAA patients, and 6.5 cm (6.0–7.0) in large AAA patients. There were no differences in current smoking, angina, or body mass index. There was a higher prevalence of ever smoking, hypertension, and alcohol consumption in AAA, the latter only in the group of large AAA patients. Use of statins and aspirin was significantly higher in the patients, particularly with large AAA. A small but significant decrease in total and low-density lipoprotein cholesterol was observed in large AAA patients, a likely reflection of the increased use of statins in this group. There were no differences in triglyceride levels.

There were no differences in fibrinogen, factor VII (not shown), or factor XIII level (Table 1).
antithrombin, a marker of thrombin generation, was increased in AAA patients. Fibrin degradation product D-dimer was also increased. ETP, a measure of how much thrombin can be generated in plasma, was reduced in AAA patients. ETP peak height showed a similar reduction in AAA patients, and ETP lag-time was prolonged. PAI-1 and tPA were not different (not shown).

Clot Structure
Permeation showed a decrease in average plasma clot pore area, Ks, from $4.7 \times 10^{-9}$ cm$^2$ (IQR 4.1–5.5) in controls, $4.0 \times 10^{-9}$ cm$^2$ (3.4–4.3) in small AAA, and $3.4 \times 10^{-9}$ cm$^2$ (2.5–4.1) in large AAA ($P<0.0001$; Figure 1). Ks was $3.6 \times 10^{-9}$ cm$^2$ (2.8–4.3) in all patients combined ($P<0.0001$ versus controls). There was no correlation between Ks and aspirin or statins. These results indicate that patients with AAA show a propensity to form clots with more densely packed fibers and smaller pores than controls and that this correlates with aneurysm size. Differences in Ks were also significant when comparing subgroups with each other: control versus small AAA ($P=0.001$), controls versus large AAA ($P<0.0001$), and small versus large AAA ($P=0.045$).

Turbidity lag phase increased in small (497 seconds [IQR 409–687], $P=0.001$) and large AAA (484 seconds [IQR 425–555], $P<0.0001$) compared with controls (399 seconds [IQR 227–477], $P<0.0001$). A similar turbidity lag-phase increase was observed with TF instead of thrombin as trigger (controls versus small versus large: 400 seconds [IQR 355–437] versus 498 seconds [447–588] versus 433 seconds [390–479], $P<0.0001$). There was no difference in lag phase between patients with small and large AAA. Maximum absorbency was not different in patients with AAA when compared with controls, either with thrombin or with TF as trigger (data not shown).

Confocal microscopy was used to study plasma clot ultrastructure in large AAA, small AAA, and controls. Clots from large AAA samples consistently demonstrated a more densely packed fibrin structure compared with small AAA and controls, in agreement with the observed differences in plasma clot pore structure by permeation analysis (Figure 2). There was an increase in fiber density comparing controls with small and large AAA (mean±SD: 75±7.7, 114±9.7, and 159±10±10/105 μm$^3$, respectively, $P<0.0001$).

Fibrinolysis
We next investigated whether differences in plasma clot structure in AAA influenced susceptibility to fibrinolysis. Lysis rates were analyzed using turbidity with tPA, and time to 50% lysis was recorded. As with clot pore size, fibrinolysis rates showed a stepwise change from controls to patients with small and large AAA (Figure 3). Lysis times were shorter in clots from controls (1892 seconds [IQR 1758–2107]), intermediate in patients with small (2101 seconds [IQR 1785–2285]) and longer in patients with large AAA (2195 seconds [IQR 2041–2640], $P<0.0001$). The difference in lysis times between controls and patients with small AAA was not significant ($P=0.054$), but the differences between controls and patients with large AAA and between patients with small

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** Clot pore structure in abdominal aortic aneurysm (AAA). Clot permeation showed a stepwise decrease in average pore size (permeation coefficient [Ks], $10^{-9}$ cm$^2$) comparing controls with small and large AAA patients. Data represent median (thick line), interquartile range (box), and 95th percentiles (error bars). Individual outliers are shown by circles and asterisks (*).

![Figure 2](http://atvb.ahajournals.org/)

**Figure 2.** Clot structure in abdominal aortic aneurysm (AAA) imaged by confocal microscopy. Representative clots from control subjects (A), patients with small AAA (B), and patients with large AAA (C). Images are 2D projections of 10 z-stacks obtained over 230/230/20 μm (x/y/z).
and large AAA were significant \((P<0.0001\text{ and }P=0.007,\text{ respectively})\). In all patients combined, lysis was 2192 seconds \((IQR 1933–2580)\) \((P<0.0001\text{ versus controls})\), PAI-1 and tPA were not different between patients and controls (not shown). Also, when triggering plasma clot formation with TF, lysis was significantly prolonged in patients with AAA \((P<0.0001\text{ versus controls})\). Previous studies have shown that clot architecture is dependent on fibrinogen absorbency. In our study, the association between plasma clot structure and lysis was analyzed by turbidity with tissue plasminogen activator. Data represent median (thick line), interquartile range (box), and 95th percentiles (error bars). Individual outliers are shown by circles and asterisks (\(*\)).

**Clot Structure and Cardiovascular Risk**

In all, 65 subjects had a history of cardiovascular disease. As cardiovascular disease has previously been associated with dense clot structure,13,14 we analyzed plasma clot structure in the absence of this confounder. When we excluded cases with all cardiovascular disease, Ks also decreased in patients with the absence of this confounder. When we excluded cases with all cardiovascular disease, Ks also decreased in patients with the absence of this confounder. When we excluded cases with all cardiovascular disease, Ks also decreased in patients with the absence of this confounder. When we excluded cases with all cardiovascular disease, Ks also decreased in patients with the absence of this confounder. When we excluded cases with all cardiovascular disease, Ks also decreased in patients with the absence of this confounder. When we excluded cases with all cardiovascular disease, Ks also decreased in patients with the absence of this confounder.

Figure 3. Fibrinolysis in abdominal aortic aneurysm (AAA). Clots from patients with large AAA showed longer lysis times than clots from those with small AAA, which in turn were longer than control. Time to 50% lysis was analyzed by turbidity with tissue plasminogen activator. Data represent median (thick line), interquartile range (box), and 95th percentiles (error bars). Individual outliers are shown by circles and asterisks (\(*\)).

In an attempt to further investigate mechanisms underpinning changes in clot structure and lysis, we purified fibrinogen from 4 controls, 5 patients with small AAA, and 5 patients with large AAA. There were no differences in fibrin clot structure by turbidity or in fibrinolysis rates in clots produced from these fibrinogens (data not shown).

### Discussion

Our study shows significant differences in plasma clot structure in patients with AAA. Patients with large AAA produced denser, less porous clots compared with patients with small AAA who in turn produced denser clots than control subjects. This stepwise decrease in porosity was associated with increased lysis times, showing that clots from patients with larger aneurysms are more difficult to lyse than those of patients with small aneurysms and controls. These cross-sectional data suggest that clot structure may be involved in AAA development and progressive dilation of the aorta, however, prospective studies will be required to test this hypothesis further.

Formation of fibrin is the final step in coagulation. Studies have shown that clot architecture is dependent on fibrinogen and thrombin levels.24 Fibrinogen levels are high in patients with cardiovascular disease and high fibrinogen associates with clots of reduced permeability and increased maximum absorbency. In our study, the association between plasma clot structure and AAA cannot, however, be explained by changes in fibrinogen levels, as these were comparable between the 2 AAA groups and the controls. Previous studies have shown that dense clot structures are caused by high levels of thrombin generation.24 To elucidate whether thrombin gener-

### Table 2. Covariates of Permeation Coefficient in a Multiple Linear Regression Model

<table>
<thead>
<tr>
<th>Covariate/Factor</th>
<th>Coefficient</th>
<th>95% CI</th>
<th>(P) Value</th>
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<tbody>
<tr>
<td>Age</td>
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<td>–0.077, 0.034</td>
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<tr>
<td>All CVD</td>
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<td>–1.150, 0.304</td>
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<td>Smoking</td>
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<td>–0.895, 0.748</td>
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<td>Statins</td>
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<td>–0.534, 0.813</td>
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<td>Aspirin</td>
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<td>0.95</td>
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<tr>
<td>Fibrinogen</td>
<td>–0.271</td>
<td>–0.689, 0.147</td>
<td>0.20</td>
</tr>
<tr>
<td>ETP</td>
<td>–0.000</td>
<td>–0.001, 0.000</td>
<td>0.14</td>
</tr>
<tr>
<td>Small AAA</td>
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<td>–1.523, –0.227</td>
<td>0.009</td>
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<tr>
<td>Large AAA</td>
<td>–2.310</td>
<td>–3.551, –1.069</td>
<td>0.0001</td>
</tr>
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</table>

Regression was performed using R, version 2.13. CI: confidence interval. All CVD was as defined in Table 1. CVD indicates cardiovascular disease; ETP, endogenous thrombin potential; AAA, abdominal aortic aneurysm.
ation was responsible for our findings, we performed thrombin generation experiments on the plasma samples. However, the ETP, a direct measure of how much thrombin can be generated in plasma, was not increased but slightly decreased. Furthermore, fibrin polymerization studies using TF instead of thrombin (and therefore incorporating effects of endogenous thrombin generation) showed similar differences between AAA patients and controls, thereby reflecting that overall thrombin generation within the plasma samples was not responsible for our findings.

In an attempt to further elucidate a mechanism for the observed changes, we purified fibrinogen from a limited number of controls and AAA patients. Clots made from purified fibrinogen samples did not show a difference in structure, unlike those from plasma samples. Together with the thrombin generation data and the turbidity data using TF, these results suggest that the alterations in clot structure in AAA were not caused by changes in fibrinogen levels, thrombin generation, or modifications of the fibrinogen molecule itself but that they may be caused by another, unknown factor. However, although the calcium-dependent IF-1 antibody, which we used to purify fibrinogen from plasma, is known to bind a large number of different fibrinogen variants (including proteins with mutations, deletions, splice variations, glycation, and acetylation), we cannot exclude that this procedure failed to isolate a subpopulation of abnormal fibrinogen. There are several candidate factors that may provide a mechanism responsible for changes in clot structure in AAA. Factors that change clot structure independently of thrombin generation include factor XIIa, C3a, and lipoprotein(a). Future studies are required to determine whether these or other factors could be responsible for the observed effects.

Smoking is one of the strongest risk factors for AAA. Undas et al showed that current smoking in healthy subjects reduces clot permeability and prolongs lysis times. Another recent study showed that cigarette smoke has direct and immediate effects on clot structure, leading to thinner fibers, smaller pores, and increased stiffness. Passive exposure to secondhand smoke has also been shown to influence clot structure and stiffness. In our study, the number of current smokers did not differ between the groups, which indicates that smoking was not responsible for the observed differences in plasma clot structure. We did observe a higher number of ever smokers in patients with AAA, particularly those with large AAA, in agreement with previous studies.

We found a stepwise increase in lysis from the controls to small and large AAA patients, which agrees with the differences in plasma clot structure. Statins have previously been shown to produce beneficial effects on clot structure by increasing pore size and enhancing fibrinolysis rates. However, we found a decrease in plasma clot porosity in patients with large AAA, despite the fact that a larger proportion of these patients were treated with statins. These data suggest that statins may be insufficient in these patients to counteract changes in clot structure.

There are several possible consequences of altered clot structure in AAA. It has been suggested that larger aneurysms are associated with increased convection flow, which, in the context of an aorta with normally very fast-flowing blood, leads to local pockets of reduced flow and shear stress. This reduced flow, in combination with the propensity of patients with large aneurysms to form denser clots that are slower to lyse, could significantly increase the prothrombotic burden in the aneurysmal aorta. Furthermore, it is possible that a more densely packed clot may trap and accumulate a greater number of polymorphonuclear leukocytes, which in turn release biologically active proteases, causing AAA dilatation. This is supported by recent studies reporting that ILT leads to increased release of pro–matrix metalloproteinase-9 and other proteases, causing degradation of the extracellular matrix. On the other hand, a recent report has suggested that ILT composed of dense fibrin may deprive the thrombus of cells, thereby reducing proteolysis. Finally, altered clot structure may influence the elastic properties of the thrombus, with potential implications for AAA extension and growth. Additional studies will be required to investigate the effects of a densely packed thrombus and clot elasticity on the vascular wall.

Fibrin polymerization showed no differences in maximum absorbency in AAA. It has been reported that maximum absorbency reflects average fiber thickness. The reason for a lack of significance in maximum absorbency is not clear, but it may reflect the small study size. Overall, our data suggest that maximum absorbency may not be a strong determinant of clot structure. This is supported by Collet et al, who reported that tighter fiber arrangement is more important than fiber diameter in determining fibrinolysis speed. We observed a prolongation of the lag phase by turbidity in AAA. Lag phase represents rate of lateral aggregation of fibrin protofibrils. The prolonged lag phase in AAA therefore suggests a decrease in lateral fiber aggregation, which is in agreement with reduced porosity for the fibrin network.

The abnormal plasma clot structure in AAA appears similar to those previously described for other cardiovascular disease, both in terms of type and magnitude of the changes. Studies in patients with coronary artery disease have found denser clots, with increased stiffness, decreased permeability, and decreased susceptibility to lysis. Changes in clot structure in patients with acute coronary disease are associated with markers of inflammation and oxidative stress. Clot structure also show similar changes in patients with venous thrombosis and their first-degree relatives, suggesting that clot structure provides a common mechanism for venous and arterial thrombosis. Our current data suggest that clot structure may represent an important common link between other cardiovascular disease and AAA. These findings further suggest that drugs that counteract abnormal clot structure may be beneficial to reduce the risk for other cardiovascular events in patients with AAA. Aspirin and statins have been shown to change clot structure beneficially, however, new drugs may be required to address this issue without incurring a bleeding risk.

In conclusion, large AAA patients produce clots with lower permeability than small AAA and controls. Lysis time was increased in large AAA patients compared with small AAA and controls. These results support the following conclusions: (1) plasma samples from large AAA produce denser clots with smaller pores that are more resistant to fibrinolysis than...
small AAA; (2) the propensity to form dense clots resistant to fibrinolysis is associated with aortic diameter; and (3) altered clot structure may represent a common mechanism that links AAA and cardiovascular disease.

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Disclosures
None.

References
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