Clinical and Population Studies

Dilation-Dependent Activation of Platelets and Prothrombin in Human Thoracic Ascending Aortic Aneurysm

Ziad Touat, Laurent Lepage, Véronique Ollivier, Patrick Nataf, Ulrich Hvass, Julien Labreuche, Martine Jandrot-Perrus, Jean-Baptiste Michel, Guillaume Jondeau

Objectives—The purpose of this study was to investigate whether thoracic ascending aortic aneurysm (TAAA) induces platelet activation as mural thrombus participates in aortic dilatation in abdominal aortic aneurysm and TAAA are associated with rheological factors favoring coagulation activation.

Methods and Results—We studied the relation between coagulation activation and aortic diameter in Marfan patients (MFS) with various aortic diameters (n=52). We then studied patients presenting large aneurysms associated with bicuspid aortic valve (BAV) and degenerative form. Lastly, we used immunochemistry and biochemistry to investigate prothrombin/thrombin retention within the aortic wall. Microparticles, sGPV, tissue factor, and TAT complexes were increased in plasma from MFS with large aneurysms (≥45 mm) compared to MFS with limited aortic dilatation (<45 mm). Similar elevations were observed in all patients with large aortic aneurysms, regardless of the etiology, the site of maximal aortic dilation, associated valvulopathy, risk factors, or treatments. P-selectin and platelet-bound fibrinogen were also increased, demonstrating platelet activation in large aneurysms. Significant increase in sCD146 plasma concentration suggested alteration of endothelium.

Conclusions—Platelet activation occurs in patients with large aneurysms of the ascending aorta, is dependent on aortic dilation, and is associated with thrombin generation, part of which appears to be retained in mucoid degeneration areas.

Key Words: thoracic aortic aneurysm ■ coagulation ■ Marfan ■ platelets

Thoracic ascending aortic aneurysms (TAAA) are defined by an enlargement of the ascending aorta. TAAA can be observed in old patients without clear etiologic factors, in whom it represents a primary degenerative pathology of the ascending aorta.1 But it can also occur in the younger population; it is then usually linked to a genetic or congenital disease. Marfan syndrome represents the majority of these cases. This is usually related to mutations in fibrillin2 and rarely to mutations in transforming growth factor (TGF)R, which can also be responsible for Loeys-Dietz Syndrome and familial aortic aneurysms.3–5 Other molecular abnormalities have been implicated, such as mutations in myosin,6 TGFβR,7 ACTA2,8 or other currently unknown genes.8 Moreover, bicuspid aortic valves (BAV), which may be familial,9 are associated with a high risk of aneurysm of the ascending aorta.10

In contrast to their etiologic diversity, all localized aortic dilations share common rheological characteristics. In normal human aorta, the regular geometry (tubular shape and parallel walls) results in laminar blood flow and in a homogeneous shear rate, maintaining a physiological steady-state among the blood components and between the circulating blood and arterial wall.11 Changes in arterial geometry considerably modify rheological parameters and therefore influence interactions between blood components.11,12 The modulations by shear stress of platelet interactions with von Willebrand factor (vWF) exemplify the role of rheology in hemostasis.13,14 In this respect, an aneurysm of the ascending aorta induces rheological perturbations, characterized by vortices (low wall shear rate).15

Rheological disturbances have been suggested to play a role in mural thrombus formation associated with cerebral artery and abdominal aortic aneurysms, and the luminal thrombus appears to play a role in the progression of aortic dilation.16 In contrast with this localization, TAAAs are characterized by the absence of any mural thrombus formation (Figure 1) even in older patients with diffuse ath erosclerosis. Therefore, TAAA offers a unique opportunity to test the hypothesis that platelets and the coagulation cascade could be activated by dilation of the ascending aorta, and could be associated with this pathology independently of a mural thrombus formation. There have been no clinical studies focusing on the hemostasis cascade in patients with TAAA. For this purpose, we explored markers of activation of both platelets and prothrombin in patients with TAAA. In a
first step, we compared Marfan patients with various aortic diameters and matched controls. In a second step, we studied platelets and prothrombin activation in patients presenting TAAA of other aetiologies (BAV and degenerative).

## Materials and Methods

### Population

One hundred fifty-six patients were divided into 4 groups (Table 1): Healthy individuals (Controls, n=66); Patients fulfilling international criteria for Marfan syndrome (MFS).17,18 They were subdivided into 2 groups: MFS patients with limited dilation (maximal aortic diameter <45 mm) (n=28), and MFS patients with large aneurysm19 (aortic diameter ≥45 mm) (n=24); TAAA patients scheduled for surgery because of aortic dilation. Here, large aneurysms were associated with bicuspid aortic valves (BAV) (n=15), or were isolated with no sign of genetic disease (Degenerative; n=23). For more details, please see the supplemental data section, available online at http://atvb.ahajournals.org.

### Results

#### Patient Groups

Clinical and echocardiographic data are summarized in Table 1. MFS patients were significantly younger (P<0.01) than those of the BAV and degenerative groups. Patients from the degenerative group were the oldest with, as expected, more cardiovascular risk factors (χ²=10.5, P<0.01), mainly hypertension, a higher frequency of medications, and a higher incidence of significant aortic valve regurgitation (P<0.01).

Aortic valve stenosis was present more frequently in the BAV group (χ²=7.8, P<0.01). In this group, maximal aortic dilation was observed above the sinotubular junction (χ²=20, P<0.0001), whereas it was maximal at the level of the Valsalva sinuses in MFS patients, and the patients with degenerative aortic aneurysm.

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#### Marfan and Controls: Relation of Platelet Activation to Aortic Diameter

No increase in plasma markers of platelet activation was observed in the group of MFS with limited dilatation (ie, maximal diameter <45 mm), when compared to age- and sex-matched control: microparticle count (Annexin-V positive) and concentrations of sGPV, Tissue factor and TAT were similar in the 2 groups (Table 2). In contrast, all these markers were significantly higher in plasma from MFS patients with large aneurysms (≥45 mm) (Table 2). The increase in microparticle concentration (Figure 1) was correlated to aortic diameter (P<0.05, r²=0.38) in the Marfan group with and without large aneurysms. Similar results were found for sGPV concentration (P<0.001, r²=0.56), and these 2 markers of platelet activation were highly correlated in the whole Marfan group (P<0.0001, r²=0.65).

#### Extension to Other Aetiologies of TAAA

##### Microparticles

Microparticle counts were increased in plasma of patients with large aneurysms of all etiologies (Marfan, BAV-associated or degenerative) when compared to controls (P<0.0001) (Table 2). However, plasma levels of microparticles were similar, regardless of etiology (Table 2) of large aneurysms. 93.2±3.9% of microparticles were positive for CD41 antibody (integrin GPIIb/IIIa) in patients with large aneurysms and 90.9±6.4 in controls (NS) demonstrating their platelet origin in both cases.

##### Plasma Markers

Other markers for platelet activation (sGPV, sCD40L) were also significantly (P<0.0001) higher in plasma of patients with large aneurysms than in that from controls, with no
difference between etiologies (Table 2). In contrast, the plasma concentration of sP-selectin was not increased in association with large aneurysms compared to controls (36.2±1.7 ng/mL versus 34.2±1.8 ng/mL).

We observed no difference in these plasma markers when patients were under low doses of aspirin. Aspirin blocks thromboxane A2 synthesis and platelet recruitment by this secreted TxA2. It thus limits the secondary progression of platelet activation, but does not block primary platelet activation.20

Platelet Markers
Platelet membrane expression of P-selectin, measured with FACs on whole blood, was low, but significantly higher in large aneurysms compared to controls (4.08±0.53% positive versus 2.91±0.56% in controls, P<0.05). Similar but more significant results were obtained by analyzing the curve shift induced by the specific antibody as compared to a nonrelevant antibody (Overton method) (large aneurysms versus controls: 14.6±2.1% versus 7.8±0.6%, P<0.01) (Figure 1A and 1B). P-selectin expression on platelets did not differ with regard to etiology.

Conversely, platelet-bound fibrinogen was not significantly different in large aneurysm patients compared to controls when measured by the classical analysis method (0.96±0.29% versus 0.89±0.21%, ns). However, curve shift analysis (Overton) revealed a small significant shift of the signal to the right in platelets originating from patients with large aneurysms (TAAA: 7.34±1.3% versus controls: 3.9±0.5%, P<0.05, Figure 1B).

Markers of Prothrombin Activation
In plasma of patients with large aneurysms, TF antigen concentration was significantly higher than in control plasma, regardless of etiology (Table 2). The 1-step recalcification clotting time was reduced for plasma of patients with large aneurysms compared to controls (175.8±8.0 seconds versus 210.7±7.9 seconds, P<0.001; Figure 2). The involvement of TF activity in the shortening of the clotting time was demonstrated by adding a blocking anti-TF antibody that prolonged the clotting time of patients with large aneurysms by 35.9±5.8%, to reach a value similar to that of controls. Plasma thrombin generation, measured using a global assay, showed an increased “endogenous thrombin potential” (ETP) for large aneurysms relative to controls (1902.3±74 nM.min−1 for TAAA versus 1712.8±63.7 nM.min−1 for controls, P<0.05; Figure 2) and an increased maximal thrombin generation (peak height) for the large aneurysm group (335.3±13.2 nmol/L versus 284.5±10.6 nmol/L for controls, P<0.01; Figure 2). Interestingly, there was a significant correlation between sGPV and maximal thrombin generation (r²=0.49, P<0.01) in plasma of patients with large aneurysms. Furthermore, TAT complexes were also slightly but significantly increased in the plasma of patients with large aneurysms (P<0.001, Table 2). These results were statistically similar for all aetiologies.

Figure 1. Microparticles in relation to aortic diameter. Number of circulating microparticles, in plasma from Marfan patients with various degrees of dilation and matched healthy individuals, was increased only when the dilation was superior to 45 mm.
Endothelial Plasma Markers
Soluble vascular cell adhesion molecule (VCAM)-1 was not statistically different in large aneurysm and control groups (377.6±48.4 ng/mL versus 293.3±36.1 ng/mL, P=0.08). In contrast, sCD146 plasma levels were significantly higher in patients with large aneurysm than in controls, with no significant differences whatever the etiology of the TAAA (Table 2).

Inflammatory Markers
Fibrinogen and hsCRP levels were similar in large aneurysm patients and controls (2.8±0.1 g/L versus 2.6±0.2 g/L and 1.88±0.3 mg/L versus 1.47±0.2 mg/L, respectively). These markers were slightly, but not significantly, superior in degenerative group. This is consistent with greater age and the presence of more risk factors in this group.

Aortic Tissue Samples and Conditioned Media
In control aortic tissue, immunoreactivity for prothrombin/thrombin (II/IIa) was negative (Figure 3A). In contrast, aortic sections from patients with surgically removed large aneurysms, whatever the etiology, showed prominent immunoreactivity for factor II and IIa in the mucoid areas, which were localized in serial sections stained with Alcian blue (Figure 3A). These results were confirmed on conditioned media of aortic samples, where factor II and IIa were detected by immunoblotting (Figure 3B). Conditioned media from large aneurysms expressed larger bands of prothrombin and thrombin than conditioned media from controls. By ELISA, a higher concentration of TAT was detected in conditioned media of large aneurysms, regardless the etiology as compared to controls, supporting the immunohisto-logical and immunoblot data (Figure 3C).

Discussion
Our main result is the presence of biological signs of in vivo activation of both platelets and prothrombin in patients with thoracic ascending aortic aneurysm. This activation is only present when maximal aortic diameter is greater than 45 mm in Marfan patients of young age, devoid or risk factors, but not observed in MFS patients when maximal aortic diameter is less than 45 mm. It is also present in patients presenting large aneurysms of other aetiologies (BAV, Degenerative), and is not dependent on the location of the aneurysm (maximal dilation of the aortic root at the level of sinuses of Valsalva or maximal dilation of the ascending aorta above the sinotubular junction).

In contrast to abdominal aortic aneurysm (AAA), aneu-rysms of the ascending aorta are not associated with a mural thrombus, which could readily explain the presence of circulating markers of platelet and prothrombin activation. Also in contrast with AAA, inflammation, which predominates in the adventitia of AAA, is absent in the aortic wall obtained from TAAA, including the adventitia. Our peripheral measurements of plasma CRP and fibrinogen are in keeping with this observation.

It is possible that activated platelets and coagulation factors are more rapidly diluted and washed away by the particular flow conditions in the ascending aorta. Indeed, it appears that the triggering event is sufficient to initiate thrombin genera-
tion and platelet activation but insufficient to permit fibrinogen clotting and platelet aggregation. Because elevation of P-selectin, a highly procoagulant mediator, is only minimal, it could also limit the onset of coagulation.

The absence of any thrombus or sign of significant inflammation in TAAA raises the question of the origin of the prothrombotic markers. Our most clear-cut observation was related to the activation of platelets and prothrombin. The main trigger of the thrombin generation cascade is tissue factor (TF), which is constitutively expressed in tissues. Plasma TF concentrations were found to be increased in patients with large aneurysms compared to controls. This signifies that higher amounts of active TF are circulating in blood from patients with large aneurysms compared to controls. This signifies that higher amounts of active TF are circulating in blood from patients with large aneurysms than in blood from controls, but that TF activity is not sufficient to generate a clot, as there was no evidence of macroscopic or microscopic thrombus in TAAA patients. The increased level of TAT is in favor of in vivo thrombin generation, which is further supported by the elevated level of sGPV, a marker of platelet activation by thrombin.

The rate of prothrombin activation is dependent on the assembly of tenase and prothrombinase complexes, which in turn depends on the presence of surfaces enriched in negatively-charged phospholipids. The increased number of microparticles of platelet origin observed in association with large aneurysms is likely to provide the phosphatidylserine-enriched membranes required to favor thrombin generation. Microparticles are the main link between platelet activation and thrombin generation. Elevated levels of sGPV and platelet-derived microparticles in vivo indicate platelet activation by thrombin in TAAA. Elevated soluble CD40L (CD154), a transmembrane protein released by proteolytic cleavage in response to various stimuli, in the plasma of TAAA patients also suggests platelet activation. sCD40L release is directly related to platelet activation, depends on metalloproteinase activities and triggers TF expression on binding to CD40 on monocytes. Nevertheless, because CD40L can also be released by leukocytes and endothelial cells, a nonplatelet origin of this marker cannot be completely excluded in our patients. The slight but significant increase in P-selectin expression on circulating platelets provides additional evidence for a platelet activation state in TAAA. However, when compared to the situation in AAA patients, a lower level of proteolytic shedding probably occurs in TAAA patients, because plasma levels of soluble P-selectin were not significantly different from control levels.

Figure 3. Prothrombin/thrombin in the aortic wall. A, Immunohistochemistry of prothrombin/thrombin (control and pathological aortic media). B, Immunoblot of prothrombin/thrombin (conditioned media). C, Western-blot anti-prothrombin (II) / thrombin (IIa)
In older patients (degenerative group) or BAV patients with associated valve disease, the activation detected may not be solely related to the ascending aortic aneurysm. Indeed, in the absence of cases of ascending aortic aneurysm of diameter below 45 mm in these groups, we cannot definitively rule out the presence of platelet activation even when aortic dilatation is limited. However, this activation was not observed in the control group, nor was it related to the presence of risk factors in multivariate analysis. In contrast, Marfan patients are young and without cardiovascular risk factors or valve disease. Our MFS patients were divided according to the maximal aortic diameter (≥45 mm) in 2 subgroups of similar age, sex, and both without risk factors or valvulopathy. The only difference between these 2 subgroups was the value of the dilation of the Valsalva sinuses, suggesting that activation of platelets and prothrombin was directly dependent on the magnitude of TAAA dilation.

The ascending aortic aneurysm location is interesting, because the normal dilation of Valsalva sinuses induces a particular, but physiological, disturbed flow. A significant dilation in this area should strongly increase these perturbations. Vortices have also been observed in vivo by magnetic resonance imaging in aneurysms of the human ascending aorta. An abrupt increase in lumen dimension leads to localized vortex phenomena characterized by a stagnation point and a recirculation zone in vitro.11,37 Jou et al reported the correlation between changes in luminal geometry and hemodynamics in fusiform intracranial aneurysms.38

The importance of rheological factors in platelet biology and hemostatic status is now well established.11,39 It was also demonstrated earlier that dilation-induced vortical flow could lead to platelet activation, via the interaction of GPIIbIIIa and fibrinogen.11,40 This phenomenon could participate in the poststenotic accumulation of platelets.11 In addition to platelets, vortices also influence red blood cell morphology and aggregate formation.11,42 In particular, red blood cells enhance platelet reactivity43 and release ADP at low shear rates,44 which could participate in platelet activation.45,46 Therefore, this relationship between arterial wall dilation, rheological disturbance, and platelet activation could account for the observed increase in plasma markers of platelet and prothrombin activation in patients with TAAA, regardless of their localization.16,47

Thrombin is able to increase endothelial cell monolayer dysfunction and permeability.48 The elevation of soluble CD146, a protein involved in endothelial-cell-cell interactions49 in patients with TAA, suggests endothelial alteration.50 This is compatible with an elevation of endothelial permeability to which in vivo thrombin generation could contribute. The increase in endothelial permeability could allow convection of plasma prothrombin/thrombin into the aortic wall from the blood compartment. Actually, we evidenced the presence of thrombin inside the pathological aortic wall using different approaches. Indeed, TAAAs are characterized histopathologically by mucoid degeneration associated with vacuole formation within the aortic media, whatever their etiology. The initial pathological process probably associates smooth muscle cell disappearance, mucoid degeneration, and extracellular matrix breakdown not linked to atherosclerosis. The release of TAT into the conditioned media indicated that active thrombin was present within the aortic media (Figure 3) and could play a role in thoracic ascending aortic aneurysm pathology.

In conclusion, large aneurysms of the ascending aorta are associated with a significant level of platelet activation and thrombin generation which can be detected via a peripheral approach. This activation process is related to the magnitude of the aortic dilation, but independent of the etiology of the aneurysm. The generation of thrombin could have a direct effect on endothelial cells and on smooth muscle cells, because we found thrombin within the ascending aortic medial layer. Exploring the role of this protease in aneurysmal progression requires further studies, and may open up the perspective of new therapeutic targets in patients with aneurysm of the ascending aorta.

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Disclosures
None.

References
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Materials and methods

Population
156 patients consulting at Bichat hospital, Ambroise Paré hospital and the Marfan Clinic, of ages ranging from 19 to 81, without aortic dissections or coronary artery disease and not on anticoagulant treatment, were approached for study participation. They represented different sub-populations of recruited patients:

- Healthy individuals without dilation or genetic disease, coming to the Marfan clinic to exclude the syndrome or because they were parents of Marfan patients (Controls, n=66)
- Patients fulfilling international criteria for Marfan syndrome (MFS)¹, recruited because of scheduled surgery or because of referral to the reference center for Marfan syndrome because of diagnostic uncertainty. They were subdivided into 2 groups:
  - MFS patients with limited dilation (maximal aortic diameter <45mm) (n=28)
  - MFS patients with large aneurysm (aortic diameter ≥45mm) (n=24)
This cut-off at 45 mm was chosen (i) because a maximal aortic diameter above 45mm is an important criteria indicating surgery²,(ii) it represents the median diameter in the Marfan group and (iii) for the other aetiologies, the minimum diameters were within the range of 45 - 50 mm.
- TAAA patients scheduled for surgery because of aortic dilatation. Here, large aneurysms were associated with bicuspid aortic valves (BAV) (n=15), or were isolated with no sign of genetic disease (Degenerative) (n=23). In contrast to MFS patients, the recruitment of these patients occurred when surgery was scheduled. Thus, we do not have cases of these aetiologies with an aortic diameter below 45 mm.

All these subjects were devoid of aneurysms in any other location. The Ethics committee of Ambroise Paré hospital approved the study (CCPPRB n° 05 04 32). Written, informed consent was obtained from all patients before inclusion.

Clinical evaluation and aorta imaging
Data from were collected during a complete clinical evaluation, including age, gender, the presence of cardiovascular risk factors, medical treatment. Marfan diagnosis was made according to international criteria³. Aortic diameters were usually measured using transthoracic echocardiography on a parasternal long axis view. Measurements were performed in accordance with European
Society of Cardiology (ESC) guidelines \(^1\), as well as description of location of maximal aortic dilatation (sinuses of Valsalva or ascending aorta). When echocardiography was impossible, TDM or NMR were used to evaluate the maximal diameter of the ascending aorta. Presence of aortic valve disease was also noted and quantified when present.

**Blood sampling**

Venous blood was sampled (24 hours before surgery for the groups concerned by an aortic replacement) on citrate, heparin and in dry tubes. Patients concerned by an aortic replacement and with a treatment by aspirin stopped the treatment 3 days before. Citrated whole blood was used for flow cytometry within 3 hours of collection. Platelet free plasma (PFP) was obtained by centrifugation at 2200 \(g\), at 20°C, for 15 minutes, and stored at -80°C until analysis. Serum was obtained after blood clotting (dry tubes) and centrifugation at 1300 \(g\) at 4°C for 10 minutes, and was stored at -80°C until analysis. Strictly similar conditions were applied for the control group.

**Aortic tissue sampling**

Samples of ascending aortic tissue from TAAA patients were collected during surgery for aortic replacement. Control tissues were fragments of normal aorta sampled from donors during organ transplantation (authorization from the French Biomedicine Agency). Only non-atherosclerotic aortas were accepted as controls. For ethical reasons, clinical data concerning anonymous controls (transplantation donors) were not accessible.

Since the adventitia of samples removed at surgery may be contaminated by coagulated blood, adventicectomy was gently performed, and aortic media samples were washed 3 times in RPMI before use. Samples from TAAA and control aortas were weighed and incubated in serum free culture medium as previously described in order to collect *conditioned media* into which several mediators had diffused from the aortic media \(^4\). Conditioned media were then collected, centrifuged and frozen.

**Histological and immunohistochemical analysis**

Samples of aortic tissue were fixed in 3.7% paraformaldehyde and embedded in paraffin. Aortic wall specimens were oriented to obtain cross-sections, perpendicular to the axis of blood flow *in vivo*. Since mucoid degeneration is a common feature in TAAA whatever the aetiology \(^5\), paraffin sections were stained with Alcian blue (Nuclear fast red counterstaining) and serial sections were used for immunodetection of prothrombin and thrombin (with
antibody from rabbit (IgG), Hyphen BioMed), antigen retrieval was performed, endogenous peroxidase was blocked with 3% H₂O₂ in aqueous solution, and non-specific binding was blocked with non-fat dry milk. Slides were incubated overnight with the primary antibody, followed by secondary antibody (Kit LSAB, Dako). The binding reaction was detected using Histogreen (AbCys), producing a green/blue reaction. Sections were then counterstained with nuclear fast red (5 minutes at room temperature).

**Immunoblot**
Conditioned media were used for immunoblotting. Electrophoresis was performed in non-reducing conditions. The antibody (rabbit IgG) used for prothrombin/thrombin was the same as for immunohistochemistry (Hyphen BioMed).

**Microparticles**
Microparticles, isolated from PFP as described by Biro et al. ⁶, were analyzed by flow cytometry (Coulter Epics XL™ with Expo 32 software, Beckman Coulter) using annexin-V-FITC (fluoroisothiocyanate) labeling of surface-expressed phosphatidylserine. CD-41 (GPIIb) phycoerythrin-conjugated monoclonal antibody (Immunotech) was used to quantify microparticles of platelet origin. Background values were set using annexin-V in the absence of calcium or isotype irrelevant antibodies. Known amounts of fluorescent beads were added to the plasma samples before analysis for quantification of microparticles.

**Platelet analysis by Flow cytometry**
Platelet activation markers were measured on whole blood, by flow cytometry, using an anti-CD62P-FITC antibody (Beckman-Coulters) for P-selectin and an anti-Fibrinogen-FITC antibody (Biocytex) for fibrinogen. Background values were determined using an irrelevant antibody (isotype matched, Beckman-Coulters) for P-selectin and an excess of non-fluorescent antibody for fibrinogen (Biocytex).

Analysis of P-selectin and fibrinogen expression was made using 2 methods: the classical method which evaluates the percentage of positive events when compared to the negative control, and by the automated calculation of the percentage of positive shift of immunofluorescence between relevant (anti-P-selectin and anti-fibrinogen) and irrelevant immunoglobulin histograms as described by Overton ⁷.
**Enzyme-Linked Immunosorbent Assay (ELISA)**

ELISAs were performed on plasma samples and conditioned media (for TAT complexes) of TAAAs and controls, according to the manufacturers’ instructions, to quantify:

- **Platelet activation markers**: Soluble glycoprotein V (sGPV) (Diagnostica Stago) and CD40 ligand (sCD40L) (R&D systems).
- **Coagulation markers**: Tissue Factor (TF) (American Diagnostica) and Thrombin-antithrombin complex III (TAT) (Dade-Behring).
- **Endothelial markers**: Soluble CD146 (Biocytex), and VCAM-1 (R&D systems).

**Inflammatory markers**

Highly sensitive C Reactive Protein (hsCRP) was measured by immunonephelemetry in serum. Citrated plasma was used for quantification of fibrinogen by the same method.

**Procoagulant Activity**

Plasma samples of TAAA patients and controls were screened for activation of coagulation using a one-step recalcification assay of citrated platelet-poor plasma (PPP). The prolongation of the clotting times induced by an anti-TF antibody (10 µg/mL, American Diagnostica) was measured.

**Thrombogram**

Thrombin generation was measured in citrated PPP using the Calibrated Automated Thrombogram (CAT, fluorskan ascent, Biodis, France). The assay was carried out according to the manufacturer’s instructions and, as described by Hemker et al. The PPP reagent was diluted twice in Hepes buffer pH=7.35 (20 mM Hepes, 140 mM NaCl, bovine serum albumin 5 mg/mL) in order to unmask potential modifications of the initiation phase of thrombin generation. Total thrombin generation was evaluated by the ‘Endogenous Thrombin Potential’ (ETP) which represents the level of thrombin generated during 50 min. The maximal concentration of thrombin generated was also evaluated (peak height). For each plasma sample, measurement was performed in triplicate.

**Statistical Analysis**

Results are expressed as mean values ± SEM. Pair-wise comparisons were made using the Mann-Whitney U test for continuous variables and the Chi-square test for proportions. In order to assess for confounding variables, comparisons for biological markers between TAAA
and control groups, and between the different aneurysmal aetiologies were adjusted for the presence of cardiovascular risk factors and use of treatment by using non-parametric analysis of variance (multivariate analysis). Spearman’s rank correlation test was used for intra-group correlations. Statistical testing was performed at the 2-tailed alpha level of 0.05. Data were analysed using Statview® software.

References


Fig I: Absence of thrombus.
A: Macroscopic view of a normal aorta
B: Macroscopic view of an aortic aneurysm of the ascending aorta. Note that the luminal surface is smooth and devoid of any thrombus (arrow).
C: External view of the same arterial dilation (arrow).

Fig II: Fibrin and thrombin generation.
A: One-step recalcification clotting times were measured on citrated PPP from patients and controls (n=20). Clotting time was significantly shorter in patients with large aneurysm compared to controls (*: p<0.05).
B: Endogenous thrombin potential was measured on citrated PPP as described in methods.
B1: The calculated mean curve of thrombograms obtained with PPP from TAAA (red, n=17) and control (blue, n=15) groups is shown. Lag-times, times to peak and inhibition times were not significantly different between patients with large aneurysms and controls.
B2: The endogenous Thrombin Potential (ETP) corresponding to the area under the curve and the maximal thrombin generated was significantly higher (α<0.05) in TAAA patients compared to controls.
Detailed Figure Legends

Fig 1: Microparticles in relation to aortic diameter.
Number of circulating microparticles, in plasma from Marfan patients with various degrees of dilation and matched healthy individuals, was increased only when the dilation was superior to 45mm.

Fig 2: Platelet activation markers
Platelet expression of P-selectin (CD62P) determined by flow cytometry using Overton statistical analysis. For details please see www.ahajournals.org
In A, representative histograms are shown. In black: non-specific fixation of a non-relevant isotype matched FITC- IgG. Specific fixation of the FITC-coupled anti-CD62P IgG on platelets from a control subject (Blue) and from one patient with large aneurysm (Red). In green: quantitative integration of the shift produced by the specific antibody (Overton statistical analysis).
In B, the levels of platelet CD62P expression and platelet-linked fibrinogen measured as above in large aneurysms (n=20) and in controls (n=17) were compared (p<0.05: *; p<0.01: **).

Fig 3: Prothrombin/thrombin in the aortic wall
A: Immunohistochemistry of prothrombin/thrombin on control and pathological aortic media. We can observe prothrombin/thrombin (1, green) inside the aorta of large aneurysm (inset), which is colocalized with the mucoid degeneration area (2, Alcian blue, arrow) whereas normal aortic media is strictly negative (3) (magnification: X20). B: Immunoblot of prothrombin/thrombin of conditioned media from control and pathological aorta, and a positive control of active thrombin (IIa). C: Level of thrombin-antithrombin III (TAT) complexes in conditioned media from control and large aneurysms (** p<0.01)