Size regulation of von Willebrand factor-mediated platelet thrombi
by ADAMTS-13 in flowing blood

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ABSTRACT

The metalloproteinase, ADAMTS-13, regulates the size of released von Willebrand factor (VWF) multimers bound to endothelial cells, but it is unknown whether it can cleave plasma VWF during thrombogenesis. To address this issue, we perfused blood over immobilized VWF and used videomicroscopy to visualize an activation-independent platelet aggregation process mediated by soluble VWF at shear rates $>10,000$ s$^{-1}$. At normal Ca$^{2+}$ concentration, platelets formed rolling as well as surface-attached clusters that grew larger during the first 5 min but then lost $>70\%$ of their mass by 10 min. In contrast, platelet clusters were stable in size when metal ions were chelated, anti-ADAMTS-13 IgG were added, or washed blood cells were perfused with purified VWF but no plasma. In the latter case, addition of recombinant ADAMTS-13 reduced platelet cluster size by $>70\%$. Incubating ADAMTS-13 with VWF before perfusion did not prevent the initial platelet clustering, indicating that the enzyme may act on platelet-bound VWF under shear stress. At the concentrations tested, ADAMTS-13 had no effect on platelet aggregates formed upon blood perfusion over collagen fibrils. ADAMTS-13, therefore, may regulate thrombus size preferentially when the cohesion between platelets depends on VWF binding induced by pathologically elevated shear stress.
INTRODUCTION

The cleavage of von Willebrand factor (VWF) multimers by ADAMTS-13, a metalloproteinase, may prevent abnormal platelet aggregation and thrombus formation. This concept is based on the evidence that genetic defects or autoantibodies that alter ADAMTS-13 function are associated with thrombotic microangiopathies in which platelet-rich thrombi occlude the microcirculation causing severe organ damage. In many patients with thrombotic thrombocytopenic purpura (TTP), unusually large VWF multimers can be detected in plasma and may become a concurrent cause of acute thrombotic episodes triggered by still unknown factors. “Unusually large” refers to the size of VWF multimers that are present within endothelial cell Weibel-Palade bodies but normally not in circulating plasma, where they appear after release induced by agonists.

Unusually large VWF multimers are also contained in platelet α-granules, from which they are released upon platelet activation. ADAMTS-13 is thought to cleave VWF still bound to the endothelial cell membrane at the time of release, and a similar process may occur during thrombogenesis when stimulated platelets release VWF, thus explaining why the largest multimers are not circulating under normal resting conditions. That VWF undergoes a physiologic proteolytic processing is shown by the fact that subunit fragments are found in the plasma of all normal individuals. The isolation and sequence characterization of these fragments revealed their origin from a single peptide bond cleavage at Tyr842-Met843, later identified as the specific ADAMTS-13 cleavage site.

The information on how ADAMTS-13 cleaves newly released VWF is paralleled by a lack of knowledge as to whether the protease may further process circulating VWF. Platelet thrombus formation requires VWF function in areas of the circulation with rapid blood flow and shear rate above a threshold limit of approximately 1,000 s⁻¹, conditions that occur in the
Plasma VWF multimers support platelet adhesion at sites of vascular injury after becoming immobilized onto extracellular matrix components, and contribute to platelet aggregation after binding to membrane receptors. The largest multimers, such as those newly released from endothelial cells and platelets, may be particularly efficient in performing these functions owing to their ability to form strong adhesive bonds with cell receptors. Normal circulating multimers, however, display adequate thrombogenic function in ex vivo systems and may contribute significantly to platelet adhesion and aggregation during hemostasis and thrombosis in vivo. It should also be noted that plasma VWF multimers may enhance their adhesive properties by self-aggregating onto surfaces, such that their cleavage could represent an additional mechanism for the control of platelet aggregate size by ADAMTS-13 during thrombogenesis. To investigate this possibility, we have established a model of activation-independent platelet cohesion mediated by plasma VWF under conditions of extremely elevated shear stress, and evaluated whether ADAMTS-13 had any influence on the size of aggregates formed. Our findings delineate a mechanism for the modulation of plasma VWF-mediated platelet responses to thrombogenic stimuli.
MATERIALS AND METHODS

Patients and control samples. Blood for perfusion studies was drawn from an antecubital vein of healthy and medication-free human volunteers and collected into syringes containing D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone dihydrochloride (PPACK, final concentration 93 μM; Bachem Bioscience Inc., King of Prussia, PA) to prevent clotting. Plasma for the preparation of anti-ADAMTS-13 IgG was obtained from patients with acquired TTP during therapeutic exchange procedures. Platelet-poor plasma was prepared by centrifuging blood, containing 0.011 M trisodium citrate as the anticoagulant, for 20 min at 3,000 g and approximately 22 °C (room temperature). All studies involving human subjects were conducted in accordance with the Declaration of Helsinki and were approved by institutional review boards of The Scripps Research Institute and Mario Negri Institute. Informed consent to participate in the studies was obtained from all the subjects.

Construction of a recombinant plasmid expressing ADAMTS-13. Two μg total RNA from human liver (Stratagene, La Jolla CA) was heated for 5 min at 65 °C and reverse transcribed for 50 min at 42 °C with the SuperScript First-Strand synthesis system (Invitrogen, Carlsbad, CA) using random primers according to the manufacturer’s instructions. The resulting cDNA was subjected to polymerase chain reaction (PCR) using a 5’ end BglII tail primer (5’-CTAGCCAGATCTGCTGCAGGCAGGGCATCCTACACCTGGAG) and a 3’ end AgeI tail primer (5’-TATGCCACCGGTGCGGTTCCTTCCTTTCCCTTCC). The amplified ADAMTS-13 coding region started with the codon for the first Ala residue known to be the amino terminus of the plasma enzyme. PCR was performed using a high fidelity DNA polymerase (Platinum Pfx, Invitrogen) with the following cycle profile: 15 s at 94 °C, 30 s at 55 °C and 280 s at 68 °C (1 min extension for approximately 1 Kb), repeated for 30 cycles. The resulting amplified product
was blunt-end ligated into an intermediate cloning vector, PCR-Blunt (Invitrogen). Individual clones were then screened to verify that no spontaneous mutations had arisen during the PCR. The ADAMTS-13 cDNA was subsequently removed from the PCR-Blunt construct by digestion with Bgl II and AgeI, and cloned into the corresponding restriction sites of the expression vector pMTBip/V5-HisA (Invitrogen) in frame with a sequence coding for six His residues. The final expression vector, which contained the coding sequence for the BiP secretion signal, directed the synthesis of a soluble carboxyl terminal His-tagged ADAMTS-13 fusion protein under the control of a copper sulfate-inducible metallothionein promoter.

Expression of recombinant ADAMTS-13. Plasmid DNA was purified through a CsCl₂ gradient prior to transfection into Drosophila melanogaster S2 cells (Invitrogen). The cells were maintained at 24°C in Schneider's Drosophila medium supplemented with 10% heat-inactivated fetal bovine serum and transfected (2-3 x 10⁶/ml, >95% viability, 3 ml/well in 6 well plates) using the Calcium Phosphate Transfection Kit. To obtain stable cell lines expressing human ADAMTS-13, 19 μg of the purified pMT/Bip/ADAMTS-13 vector was co-transfected with 1 μg of pCoHYGRO, a vector carrying a hygromycin B resistance gene. Cells resistant to hygromycin (used at a final concentration of 300 μg/ml) were selected after 4 weeks as a stable polyclonal population and induced with 0.5 mM copper sulfate in serum-free expression medium. Cell-free conditioned medium was collected 3 days post-induction, clarified by centrifugation, and examined for ADAMTS-13 expression.

ADAMTS-13 Western blot analysis. Proteins in solution were separated by 6% polyacrylamide gel electrophoresis (PAGE) in the presence of 2% SDS and after disulfide bond reduction with 5 mM dithiothreitol, and then transferred to nitrocellulose membranes (Invitrogen). Membranes were blocked for 1 h with TBS-T (20mM Tris, pH 7.6, 150 mM NaCl,
0.1% Tween 20) containing 5% nonfat dry milk (Blotto solution). Expression of ADAMTS-13 was monitored by detection with an anti-hexahistididine monoclonal antibody diluted 1:2,500 in Blotto solution. After incubation with the antibody, the membranes were washed three times with TBS-T and then incubated with a polyclonal horseradish peroxidase-conjugated rabbit anti-mouse IgG antiserum (1:2,000 dilution). Reacting protein bands were identified using an enhanced chemiluminescence detection system (Pierce Super Signal; Pierce Biotechnology, Rockford, IL).

**Purification of ADAMTS-13 from culture medium.** The first step in the purification of rADAMTS-13 was performed using immobilized-metal-ion-affinity chromatography (IMAC) for the direct capture of the His-tagged protein from conditioned medium. Filtered conditioned medium, 1.8 L, was incubated with 120 ml of 75% slurry of Chelating Sepharose Fast Flow (Pfizer Inc. New York, NY). The mixture was kept in agitation overnight at 4 °C, and then washed three times with 5 volumes of a buffer composed of 20 mM HEPES, 300 mM NaCl, 5 mM imidazole, pH 7.4. Elution was carried out with two gel volumes of elution buffer (20 mM HEPES, 300 mM NaCl, 300 mM imidazole, pH 7.4), and this step was repeated a total of three times. The three eluted fractions were subsequently pooled and dialyzed against 20 mM HEPES, pH 8, for further purification on a heparin-Sepharose (Pfizer) column prepared with 45 ml of gel and equilibrated with the same HEPES buffer. The IMAC-eluted sample was loaded onto the column at a flow rate of 3 ml/min, and the column was then washed with 20 mM HEPES, pH 7.4, until the absorbance at 280 nm was at baseline. Heparin-bound proteins were eluted with a stepwise NaCl gradient from 0-150, 150-300, 300-450 and 450-600 mM. Absorbance was monitored at 280 nm, and peak fractions obtained with 0-150 and 150-300 mM NaCl were collected as two pools; they displayed the same functional activity, as evaluated by cleavage of a
recombinant VWF A1-A2-A3 fragment, and were eventually mixed. The protein concentration was measured with the micro-BCA assay (Pierce) according to the manufacturer’s instructions, and the degree of purification was evaluated by densitometric analysis of the bands corresponding to the proteins in solution separated by SDS-PAGE (see above) and stained with Coomassie blue. The VWF-cleaving activity of purified ADAMTS-13 was assessed by measuring the residual collagen binding activity of VWF in a plasma substrate incubated with the test sample under conditions previously described.24 A pool of normal plasma samples obtained from 25 healthy donors was used as a reference for the assays and arbitrarily defined to contain 100% of the protease activity. The values of ADAMTS-13 activity were calculated from a dose-response curve obtained by testing serial dilutions (from 1:5 to 1:320) of the reference plasma.

**Cleavage of a recombinant VWF A1-A2-A3 fragment by rADAMTS-13.** A fragment of VWF spanning residues 508-1111 of the mature subunit and comprising the three type A domains in the molecule was expressed in Drosophila melanogaster S2 cells, using a combination of the methods described above for ADAMTS-13 and previously for obtaining domain A1 in Chinese hamster ovary cells.25 The expressed fragment was purified by IMAC and heparin-Sepharose chromatography followed by gel-permeation chromatography. A mixture of VWF fragment (1 μg/ml) and rADAMTS-13 (12% of normal plasma activity) was incubated for 2 h at 37 °C in 5 mM Tris buffer, pH 8, containing 1.5 M urea and 3 mM BaCl2. The proteins in solution were then separated by SDS-PAGE (10% polyacrylamide) followed by blotting onto a nitrocellulose membrane. Fragments of the VWF subunit were detected by reaction with a mouse monoclonal antibody directed against an epitope located within the A3 domain, thus carboxyl terminal with respect to the target 842-843 bond.12;13;26;27 Under these conditions, the intact
rVWF fragment of ~80 kDa and the carboxyl terminal fragment of ~30 kDa derived from cleavage at 842-843 could be easily differentiated. Proteins that reacted with the antibody were revealed by incubation with a rabbit anti-mouse IgG coupled to horseradish peroxidase followed by a chemiluminescence reaction. For inhibition of ADAMTS-13 activity, rADAMTS-13 was incubated for 10 minutes at 37°C with purified IgG antibodies (2 mg/ml) isolated by protein A chromatography from the plasma of a patient with acquired TTP.

**Isolation of plasma IgG from a patient with acquired TTP.** Plasma from a patient with high titer anti-ADAMTS-13 antibodies causing acquired TTP was obtained from therapeutic plasma exchange and collected in standard citrate-phosphate-dextrose (CPD) anticoagulant. After recalcification, the resulting serum was applied to a Sepharose-Protein A column (Sigma) and IgG was eluted with 0.1 M citric acid at pH 6, 4.5 and 3. All fractions were tested with respect to the ability of inhibiting the ADAMTS-13-dependent cleavage of VWF multimers reflected in a collagen-binding test (see above). The IgG fraction eluted at pH 3 displayed the highest inhibitory activity and, at the concentration of 2 mg/ml, completely blocked ADAMTS-13 function. The same concentration of commercially available IgG (Sigma) served as a control.

**Measurement of VWF-mediated and activation-independent platelet cohesion.** The detailed description of this assay will be reported elsewhere (Z.M. Ruggeri et al, manuscript in preparation). In brief, we have established a videomicroscopy method to demonstrate that platelets adhere to one another in an activation-independent manner when exposed to a surface presenting immobilized VWF and in the presence of soluble VWF multimers, but only when the wall shear rate is above a threshold of ~10,000 s⁻¹. The assay was performed with PPACK-containing human blood. When indicated, prostaglandin (PG) E₁ (10 μM, Sigma Chemical Co., St. Louis, MO) was added to inhibit platelet activation and EDTA (5 mM, Sigma) to chelate
divalent cations, respectively. To remove plasma VWF, in some cases blood cells were washed in modified Tyrode buffer, pH 7.4, as reported previously. The platelet count and hematocrit were adjusted to the original values in whole blood. Perfusion experiments were conducted at 37 °C using as adhesive substrate human multimeric VWF, purified from plasma as previously described, or fibrillar type I collagen from bovine tendon (acid insoluble; Sigma) immobilized onto glass coverslips that were subsequently assembled into a parallel plate rectangular flow chamber. The perfusion chamber was mounted on the stage of an inverted microscope (Axiovert 135M; Carl Zeiss, Thornwood, NY) for real-time visualization of platelet interactions with the immobilized substrates and with one another. Platelets were rendered fluorescent by the addition of 10 μM mepacrine (quinacrine dihydrochloride, Sigma). Blood flow through the chamber was maintained with a peristaltic pump and adjusted to obtain selected wall shear rates. All experiments were recorded on S-VHS videotape using a silicon-intensified high sensitivity camera (Hamamatsu, Japan) and a VCR (SVO-9500MD; Sony Corp., Tokyo, Japan) at the acquisition rate of 30 frames/s. Image analysis was performed off-line using the Metamorph software package (Universal Imaging, West Chester, PA). The number and size (surface area and length) of each individual object detected on the surface and interacting with it, as defined by a slow translocation or longer-lasting adhesion, was determined on images obtained at different positions in the flow path of the chamber. Platelet clusters were identified by the computerized program as continuous single objects larger that individual platelets and moving or stationary over a 5 s time period. Quantitative information was typically measured in 5 different positions for each experimental point. The size of these clusters was taken to indicate the cohesive function of larger VWF multimers. A threshold was applied to the images to distinguish platelets and platelet clusters from the background, and the images were binarized before object
measurement. The supporting movies available online were prepared by digitizing and editing
the recorded analogue tapes with Adobe Premiere (Adobe Systems, San Jose, CA).

**Measurement of platelet thrombus formation on immobilized collagen fibrils.** These
experiments were performed essentially as previously described\textsuperscript{20} by perfusing blood containing
93\(\mu\)M PPAK as anticoagulant over immobilized acid-insoluble type I collagen fibrils from
bovine tendon (Sigma). The volume of the platelet thrombi formed onto the collagen fibrils was
measured from confocal sections as previously described.\textsuperscript{20,21}

**Statistical analyses.** The statistical significance of differences between measured
parameters was evaluated with the Student’s \(t\)-test for unpaired groups of values. \(P<0.05\) was
considered significant.
RESULTS

In relation to the terminology used here, note that traditionally “aggregation” refers to platelet-platelet cohesion dependent on activation and mediated by adhesive ligands bound to the integrin αIIbβ3, while “agglutination” indicates an activation-independent process of platelet cohesion that in most instances is induced by exogenous modulators, such as ristocetin in the case of the typical VWF/GP Ibα-mediated platelet agglutination. In this report, the term “aggregation” is used in a broad sense to describe the formation of platelet clusters even independently of activation or αIIbβ3 function, as seen in blood perfused at high shear rate over immobilized VWF without addition of exogenous components.

![Fig. 1. Activation-independent and shear rate-dependent aggregation and disaggregation of platelets perfused over immobilized VWF. Whole blood containing 93 μM PPACK as the anticoagulant, 10 μM PG E1 to block platelet activation, and 10 μM mepacrine to render platelets fluorescent for visualization, was perfused over a surface coated with immobilized VWF. The images shown are single frames from a real time recording (see movie 1 available online). A timeline (black horizontal bar) indicates at what moment during the perfusion experiment a given image was taken, as shown by the position of the corresponding letter (A, 55 s; B, 130 s; C, 380 s). A vertical white bar separates the timeline in segments corresponding to periods of perfusion at different wall shear rates (5,500 or 13,000 s⁻¹). The images are representative of 3 separate experiments performed with blood from different donors.](image)

The perfusion of PG E1-containing blood over immobilized VWF exposed to a wall shear rate of 5,500 s⁻¹ resulted in the adhesion of single platelets exhibiting continuous translocation in the direction of flow (Fig. 1, panel A; movie 1, available online). PG E1, as previously demonstrated,²⁹ has no effect on the initial VWF interaction with GP Ibα but, by inhibiting
platelet activation, impairs the subsequent stable adhesion and aggregation mediated by ligand binding to activated αIIbβ3. In contrast, a new modality of platelet adhesion and aggregation emerged when the shear rate was increased to 13,000 s⁻¹. After ~1 min of perfusion at this higher shear rate, the number of individual platelets interacting with immobilized VWF decreased and clusters of several platelets linked to one another appeared, either rolling on the surface with variable shape or forming chain-like structures aligned in the direction of flow and stationary for variable periods of time (Fig. 1, panel B; movie 1). Translocating platelet clusters tended to arrest transiently onto the stationary ones, often detaching them from immobilized VWF and incorporating them into rolling aggregates that increased in size as they progressed along the surface. The number and size of the aggregates formed under these experimental conditions initially increased up to a maximum at ~3 min, but then started to decrease progressively and, after ~5 min of perfusion, mostly single platelets were seen interacting with the surface (Fig. 1, panel C; movie 1).

To evaluate the hypothesis that the progressive disappearance of activation-independent platelet aggregates formed under high shear stress resulted from changes in VWF, possibly caused by the metal ion-dependent protease ADAMTS-13, we performed a set of experiments using blood containing the metal ion chelator EDTA (5 mM) from the beginning of flow. The wall shear rate was kept at 3,000 s⁻¹ for ~2 min to allow the adhesion of single translocating platelets (Fig. 2A), then between ~2 and 6 min it was increased progressively to 17,000 s⁻¹ to induce activation-independent aggregation (Fig. 2B, C, D), and finally it was decreased to 3,000 s⁻¹ to monitor aggregate stability over a total period of ~15 min (Fig. 2E; see also movie 2). At this point, the EDTA-containing blood was replaced with the same blood but without EDTA and the perfusion continued for ~5 min. Platelet clusters that formed under high shear stress in the
presence of EDTA were stable for over 10 min, while they typically lasted only 2-3 min when forming in the absence of EDTA (compare Figs. 1 and 2 and movies 1 and 2).

Accordingly, platelet aggregates that had been stable for several minutes in the presence of EDTA were markedly and rapidly reduced in number when the latter was removed from the perfusing blood (+EDTA: 104 ± 6.70; -EDTA: 12 ± 1.43; Fig. 2). The few aggregates that remained after 3 min of perfusion without EDTA were significantly reduced in size, both with respect to their average length and surface area (Fig. 2). The effect of removing EDTA was even more apparent when the dimensions of the single largest aggregate rather than the average

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**Fig. 2. Effect of EDTA on the activation-independent and shear rate-dependent aggregation and disaggregation of platelets perfused over immobilized VWF.** Whole blood containing 93 μM PPACK, 10 μM PG E1, 10 μM mepacrine, and with the addition of EDTA (5 mM) from the beginning of flow, was perfused over immobilized VWF at varying wall shear rates. The images shown are single frames from a real time recording (see movie 2 available online). The timeline (black horizontal bar) shows at what moment during the experiment a given image was taken as well as the wall shear rate during different periods of perfusion, as explained in the legend to Fig. 1 (A, 50 s, 3,000 s⁻¹; B, 180 s, 13,000 s⁻¹; C, 240 s, 15,000 s⁻¹; D, 340 s, 17,000 s⁻¹; E, 540 s, 3,000 s⁻¹; F, 1020 s, 3,000 s⁻¹). An arrow at 14 min on the timeline indicates when blood without EDTA replaced the blood with EDTA (from the same donor) perfused from the beginning of the experiment. The bar graph shows the average count, length and area of all the platelet aggregates present in five different positions on the surface after 540 s (black bars) and 1020 s (grey bars) of perfusion under the conditions described above. All values are reported as mean ± SEM and differences are statistically significant (count: p<0.0001; length: p<0.001; area: p<0.006).

Accordingly, platelet aggregates that had been stable for several minutes in the presence of EDTA were markedly and rapidly reduced in number when the latter was removed from the perfusing blood (+EDTA: 104 ± 6.70; -EDTA: 12 ± 1.43; Fig. 2). The few aggregates that remained after 3 min of perfusion without EDTA were significantly reduced in size, both with respect to their average length and surface area (Fig. 2). The effect of removing EDTA was even more apparent when the dimensions of the single largest aggregate rather than the average
dimensions of all the aggregates present in a field of view were evaluated (Table 1). Note that
activation-independent platelet clustering required a shear rate above 10,000 s^{-1} to occur
effectively, but afterwards persisted even at the lower shear rate of 3,000 s^{-1} (movie 2).
ADAMTS-13 could efficiently reduce aggregate size even at the lower shear rate, suggesting that
the protease does not require extremely high shear stress to cleave VWF.

| Experimental details are given in the legend to Fig. 2. Blood containing PPACK and PG E1 was perfused over
immobilized VWF at varying shear rates. The blood contained EDTA for the initial 14 min, and then blood
from the same donor but without EDTA was perfused for an additional 6 min. The values (mean ± SEM of five
separate fields of view; see Fig. 2) represent the area of the single smallest and largest aggregate and the length
of the single shortest and longest aggregate detected on the surface at 9 min (blood + EDTA) or 17 min (blood -
EDTA) from the beginning of perfusion. The differences in maximal area (p<0.0007) and length (p<0.001) are
significant.

Table 1. Change in activation-independent platelet aggregate size in the presence or absence of EDTA.

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<th>+EDTA</th>
<th>-EDTA</th>
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<tr>
<td>Area min</td>
<td>23.39 ± 0.60</td>
<td>23.73 ± 0.65</td>
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<tr>
<td>Area max</td>
<td>1187.29 ± 187.58</td>
<td>329.00 ± 43.92</td>
</tr>
<tr>
<td>Length min</td>
<td>6.06 ± 0.32</td>
<td>8.64 ± 1.00</td>
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<tr>
<td>Length max</td>
<td>210.35 ± 36.81</td>
<td>61.31 ± 10.55</td>
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To confirm the identity of the plasma component responsible for the disappearance of
activation-independent platelet aggregates formed under high shear stress, we carried out
perfusion experiments with blood cells washed free of plasma and resuspended in a buffer
containing 20 μg/ml purified VWF multimers. Preliminary experiments (not shown)
demonstrated that no platelet clusters formed in the absence of added soluble VWF even above
the threshold shear rate of 10,000 s^{-1}. In the presence of soluble VWF, large platelet aggregates
could be seen attached to the immobilized VWF surface over an observation period of ~ 6 min,
but their stability was greatly reduced when recombinant ADAMTS-13 was added to the blood
cell suspension together with the VWF multimers before the beginning of perfusion (Fig. 3;
movie 3). The protease used in these experiments could cleave a recombinant VWF fragment
comprising domains A1-A2-A3 under denaturing conditions and in the absence of flow (not shown).

By densitometric analysis of Coomassie blue stained protein bands separated by SDS-PAGE, we estimated that ADAMTS-13 represented 25% of the protein present in the preparation. The corresponding functional activity, measured as cleavage of the larger VWF

Fig. 3. Effect of recombinant ADAMTS-13 of VWF-mediated and activation-independent platelet thrombus formation under flow. Plasma-free washed blood cells were suspended in a Hepes/Tyrode buffer (10 mM Hepes, 135 mM NaCl, 2.7 mM KCl, 0.4 mM NaH₂PO₄, 2.8 mM dextrose), pH 7.4, containing 10 μM PG E₁, 10 μM mepacrine, 20 μg/ml purified human VWF multimers, 2 mM Ca⁡⁺⁺ and 0.5 mM Mg⁡⁺⁺. The blood cell suspension (platelet count: 180,000-390,000/μl; hematocrit: 38-43%) was perfused over a surface coated with immobilized VWF without or with the addition of recombinant ADAMTS-13, as indicated. The protease activity added corresponded to 17.5% of that present in normal blood. The images shown are single frames from a real time recording (see movie 3 available online). The timeline (black horizontal bar) shows at what moment during the experiment a given image was taken as well as the wall shear rate during different periods of perfusion, as explained in the legend to Fig. 1 (A, 30 s, 5,500 s⁻¹; B, 170 s, 13,000 s⁻¹; C, 370 s, 13,000 s⁻¹). The bar graph shows the average count, length and area (mean ± SEM) of all the platelet aggregates measured in five separate fields of view after 10 min of perfusion in the absence or presence of recombinant ADAMTS-13 during each of three experiments performed with blood cells from different donors. Differences are statistically significant for count (p<0.009) and area (p<0.04), but not for length (p>0.05).

By densitometric analysis of Coomassie blue stained protein bands separated by SDS-PAGE, we estimated that ADAMTS-13 represented 25% of the protein present in the preparation. The corresponding functional activity, measured as cleavage of the larger VWF.
multimers resulting in reduced binding to collagen, was 231% of that present in a reference normal plasma. Thus, based on the amounts added, the ADAMTS-13 activity in the washed blood cell suspension used for the experiment shown in Fig. 3 was 17.5% of that in normal blood. Yet, at least in the absence of other plasma proteins, this level of activity was sufficient to regulate VWF-mediated platelet cohesion. In agreement with the results obtained in the presence or absence of EDTA (see above), the effect of ADAMTS-13 on activation-independent platelet aggregates was particularly evident in the reduction of their number (-ADAMTS-13: 86.3 ± 17.6; +ADAMTS-13: 4.0 ± 1.5; Fig. 3) as well as of the maximal size and length that they could attain (Table 2). Of note, the addition of ADAMTS-13 did not prevent the initial formation of activation-independent platelet aggregates, which in fact were as numerous and large as in the absence of the protease within ~3 min, but caused their disappearance as opposed to persistence over the subsequent 3 min (Fig. 3; movie 3). Preincubation of ADAMTS-13 with the blood cell/VWF suspension for over 1 hour before perfusion did not affect the initial formation of activation-independent platelet aggregates nor did it accelerate their disappearance, indicating that the enzyme likely acts under flow conditions after VWF multimers are bound to platelet GP Ibα and exposed to shear stress.

Table 2. Change in activation-independent platelet aggregate size in the presence or absence of recombinant ADAMTS-13.

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<th>No ADAMTS-13</th>
<th>+ ADAMTS-13</th>
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<tr>
<td>Area min</td>
<td>91.96 ± 0.59</td>
<td>84.25 ± 25.41</td>
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<tr>
<td>Area max</td>
<td>2400.00 ± 10.41</td>
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<tr>
<td>Length min</td>
<td>13.53 ± 0.91</td>
<td>20.00 ± 2.57</td>
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<tr>
<td>Length max</td>
<td>238.82 ± 22.36</td>
<td>53.83 ± 14.24</td>
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Experimental details are given in the legend to Fig. 3. A suspension of washed blood cells with added soluble VWF and without or with added ADAMTS-13 (protease activity added before perfusion = 17.5% of that present in normal blood) was perfused over immobilized VWF at varying shear rates. The values (mean ± SEM of five different fields of view in three separate experiments; see Fig. 3) represent the area of the single smallest and largest aggregate and the length of the single shortest and longest aggregate detected on the surface. The differences in maximal area (p<0.0004) and length (p<0.002) are significant.
Additional experiments confirmed the role of ADAMTS-13 in controlling the number and size of activation independent platelet aggregates formed under high shear stress. First, we used the IgG fraction derived from the plasma of a patient with recurrent TTP who had developed function-blocking anti-ADAMTS-13 antibodies. When this IgG was added to normal blood, the subsequent perfusion over immobilized VWF resulted in the formation of activation-independent aggregates that persisted more numerous and were larger in size than after addition of control IgG prepared from the plasma of a normal volunteer (Fig. 4; Table 3).

**Fig. 4.** Effect of IgG from a TTP patient on VWF-mediated and activation-independent platelet thrombus formation under flow. Whole blood containing 93 μM PPACK as anticoagulant and 10 μM PG E₁ to inhibit platelet activation was supplemented with 2 mg/ml of IgG purified from the plasma of a normal individual (control IgG) or 2 mg/ml of IgG purified from the plasma of a patient with function-blocking antibodies against ADAMTS-13 (patient IgG) and then perfused over immobilized VWF. The images shown are single frames from a real time recording. The timeline (black horizontal bar) shows at what moment during the experiment a given image was taken as well as the wall shear rate during different periods of perfusion, as explained in the legend to Fig. 1 (A, 120 s, 13,000 s⁻¹; B, 540 s, 13,000 s⁻¹). The bar graph shows the average count, length and area of all the platelet aggregates present in a field of view after 2 and 9 min of perfusion in the presence of control or patient IgG, as indicated.
Table 3. Change in activation-independent platelet aggregate size in the presence or absence of IgG from a patient with neutralizing antibodies against ADAMTS-13.

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<th>Control IgG</th>
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<th>Patient IgG</th>
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<tr>
<td>Area max</td>
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Experimental details are given in the legend to Fig. 4. Normal blood with added IgG isolated from normal plasma or the plasma of a patient with neutralizing antibodies against ADAMTS-13 was perfused over immobilized VWF at varying shear rates. The values represent the average area of the single smallest and largest aggregate and the average length of the single shortest and longest aggregate detected in a field of view of the surface at different times of perfusion, as indicated.

In another experiment, we prepared washed normal blood cells and resuspended them either in normal plasma or in the plasma of a TTP patient with a congenital deficiency of ADAMTS-13. In the latter case, the activation-independent platelet aggregates formed upon perfusion over immobilized VWF were more numerous and larger in size after 10 min of perfusion than seen with normal plasma (Fig. 5; Table 4).

Fig. 5. Effect of plasma from a congenital TTP patient on VWF-mediated and activation-independent platelet thrombus formation under flow. Normal washed blood cells were resuspended either in the plasma of a normal individual (control plasma) or that of a patient with TTP obtained from therapeutic plasmapheresis (patient plasma). The bar graph shows the average count, length and area (mean ± SEM) of all the platelet aggregates present in five separate fields of view measured after a 10 min perfusion. Only the difference in aggregate count was significant (p<0.0001).
Table 4. Change in activation-independent platelet aggregate size in the presence of normal plasma or plasma from a patient with undetectable ADAMTS-13 activity.

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Experimental details are given in the legend to Fig. 5. Washed blood cells were resuspended in plasma either from a normal individual or from a patient with unmeasurable ADAMTS-13 activity and then perfused over immobilized VWF at varying shear rates. The values represent the area of the single smallest and largest aggregate and the length of the single shortest and longest aggregate detected on the surface (mean ± SEM of five separate fields of view; see Fig. 5). The differences in maximal area (p<0.007) and length (p<0.005) are significant.

For comparison, we then evaluated whether the addition of ADAMTS-13 could influence the stability of platelet aggregates formed upon perfusion of blood over immobilized collagen type I fibrils. In this case, inhibitors of platelet activation were not included in the perfusion. Recombinant ADAMTS-13, added to enhance the endogenous activity already present in normal blood, had no significant effect on the size of platelet thrombi formed at shear rates as high as 6,000 s$^{-1}$ (Fig. 6).

Fig. 6. Effect of recombinant ADAMTS-13 on platelet formation on fibrillar collagen type I under flow conditions. The bar graph shows the volume of thrombi formed after perfusion of whole blood containing 93 µM PPACK over a surface of 62,500 µm$^2$ coated with collagen type I fibrils. The blood was supplemented or not with recombinant ADAMTS-13, as indicated, added at least 15 min before perfusion. The protease activity added corresponded to 17.5% of that present in normal blood. The wall shear rate was 1,500 s$^{-1}$ for 7 min or 6,000 s$^{-1}$ for 4.5 min, as indicated. The values represent the mean ± SEM of 2 experiments performed with blood from different donors. The results obtained with or without addition of ADAMTS-13 were not significantly different.
The supplementary protease activity was 17.5% of that present in normal blood, the same concentration that by itself was sufficient to reduce considerably the size of activation-independent and VWF-mediated platelet clusters (see above, Fig. 3). Whether higher concentrations of ADAMTS-13 could yield an effect on collagen-induced platelet thrombi remains to be established, but it is clear that, in any case, this would require concentrations of the protease well above physiological levels.
DISCUSSION

Our findings indicate that ADAMTS-13 can cleave plasma VWF multimers bound to platelets, as judged from the reduction in the number and size of platelet clusters formed under conditions of elevated shear stress and in the absence of activation. This unique form of platelet cohesion depends on a mechanism that we will report in detail elsewhere (ZM. Ruggeri et al., manuscript in preparation) and is briefly described below. It is known that individual platelets interact with and roll onto immobilized VWF when exposed to the shear rates found in the normal arteriolar circulation. As shown here, however, clustered rather than individual platelets roll onto immobilized VWF when the shear rate is above a critical threshold of approximately 10,000 s\(^{-1}\). This phenomenon is strictly dependent on the presence of immobilized VWF to tether platelets onto the surface as well as soluble VWF to mediate interplatelet cohesion, and occurs in whole blood or washed blood cells suspended in a buffer with purified VWF multimers. The resulting clumps, which may consist of hundreds of platelets, attach more firmly to immobilized VWF and acquire an elongated string-like shape when the shear rate is above 20,000 s\(^{-1}\). In the latter situation, the length of such strings can exceed 200 \(\mu\)m. Platelet clusters do not form if soluble VWF is not present or is preincubated with a monoclonal antibody blocking A1 domain function. Formation of these VWF-dependent clusters does not require platelet activation or integrin function, as it may occur in the presence of PG E\(_1\) and EDTA. In this respect, therefore, the process of platelet cohesion visualized onto an immobilized VWF surface in the presence of soluble VWF is different from the previously described shear-induced aggregation detected with platelets in suspension in the absence of surface interactions. Common to both processes is the dependence on soluble VWF multimers and elevated shear stress, but the clusters visualized here onto immobilized VWF are clearly distinct in that they do not require platelet activation or
αIIbβ3 function. The activation-independent and VWF/GP Ibα-mediated platelet cohesion shown here may represent in an amplified manner the step preceding activation during the previously described shear-induced aggregation, and the ability to detect it may depend exclusively on the real time visualization of the interaction with immobilized VWF. Of note, activation-independent and VWF/GP Ibα-mediated platelet cohesion involves only physiologic blood components and, thus, has the potential to occur in vivo under conditions of pathologically elevated shear stress. This process may become relevant in thrombotic microangiopathies when induced in the context of an altered vessel wall that promotes platelet aggregate stability.

The function of ADAMTS-13 has been demonstrated previously on nascent ultralarge VWF multimers newly released from and still bound to the surface of endothelial cells, but several unique characteristics distinguish the process described here in the presence of plasma VWF. The platelets appearing as beads-on-a-string, as reported by Dong and colleagues, clearly represent single platelets each adhering individually to long VWF multimers, and could only assemble on the surface of stimulated endothelial cells but not on unstimulated cells in the presence of plasma VWF. Moreover, this type of adhesion to ultralarge multimers occurred in a range of shear stress typical of both venous and arterial blood flow, reportedly between 2.5 and 50 dyn/cm² (with washed platelets resuspended in buffer, these values correspond to shear rates between 250 and 5,000 s⁻¹). Even the highest value is well below the threshold we found to be necessary for inducing VWF-mediated and activation-independent platelet cohesion (with platelets in whole blood, the threshold of 10,000 s⁻¹ corresponds to a shear stress of 400 dyn/cm²). It is noteworthy that platelet adhesion to ultralarge endothelial VWF multimers was reported to decrease progressively with increasing shear stress, a behavior opposite to the enhanced VWF-mediated platelet cohesion reported here with increasing shear rates. These
differences are likely to reflect distinct mechanisms involved in the two processes. Adhesion to endothelial VWF appears to be a special case of platelet interaction with immobilized VWF, which is known to occur independently of elevated shear stress, and is characterized by the much greater stability of the bonds formed. Shear-induced platelet cohesion, on the other hand, may require the induction of conformational changes in soluble plasma VWF multimers to allow the initiation of the interaction with platelet GP Ibα. In either case, the conclusion that endothelial or plasma VWF multimers are cleaved by ADAMTS-13 was based on the demonstrated breakdown of platelet adhesion or cohesion clearly dependent exclusively on VWF. Albeit indirect, the evidence presented here was obtained with different types of experiments in which the prevention or acceleration of the breakdown of platelet cohesion was correlated to the inhibition or enhancement, respectively, of ADAMTS-13 function. Our findings, therefore, add to the concept of ADAMTS-13-mediated regulation of VWF multimer size at the time of secretion from stimulated endothelial cells, as it is now apparent that plasma VWF multimers can be further cleaved as they mediate platelet-to-platelet cohesion under elevated shear stress. This observation does not exclude the possibility that ADAMTS-13 may cleave plasma VWF exposed to shear stress even when not bound to platelets. In any case, cleavage of VWF multimers is a time-dependent process, as suggested by the observation that even supplementation of normal blood, which already contains ADAMTS-13, with additional amounts of recombinant protease could not prevent the initial formation of VWF-mediated platelet aggregates.

It is difficult to ascribe a direct pathophysiological significance to the observations presented here beyond the evidence that ADAMTS-13 can effectively regulate the size and persistence in time of plasma VWF-mediated platelet aggregates. In contrast, there was
apparently no similar effect on the size of thrombi formed upon blood perfusion over collagen type I fibrils, a situation in which platelets are fully activated and platelet-to-platelet cohesion is mediated by αIIbβ3 through the binding of different ligands.\textsuperscript{20,21} It is possible that under the latter conditions the role of VWF interacting with GP Ibα is temporally limited to supporting the very initial platelet-surface and platelet-platelet contacts,\textsuperscript{20,21} while stable bonds are then created by other ligands, such as fibrinogen and fibronectin,\textsuperscript{35,36} which cannot be cleaved by ADAMTS-13. In agreement with these observations, the functional absence of ADAMTS-13 is a known risk factor for the development of platelet-rich thrombi in the microarteriolar circulation, where shear rates are the highest, but not for the thrombotic occlusion of larger arteries. Our results suggest that VWF may be the main adhesive protein bridging platelets to one another and precipitating microarteriolar thrombosis in patients with acute episodes of TTP. The presence of an abnormal endothelial cell surface altered by inflammatory processes and the local release of unusually large VWF multimers may be necessary to initiate such episodes, but it is apparent from our findings that plasma VWF may also contribute to platelet aggregation if not regulated by ADAMTS-13.

**Acknowledgments:** We thank Dr. Miriam Galbusera for her help in the study of TTP patients, and Mr. Rolf Haberman for help in preparing the supplementary movies posted on line. Cristina Capoferrì is a recipient of a grant from Fondazione ARMR through a donation in memory of Maria Zanetti.

**References**


15. Tsai HM: Physiologic cleavage of von Willebrand factor by a plasma protease is dependent on its conformation and requires calcium ion. Blood 87: 4235-4244, 1996


FIGURE LEGENDS

**Fig. 1. Activation-independent and shear rate-dependent aggregation and disaggregation of platelets perfused over immobilized VWF.** Whole blood containing 93 μM PPACK as the anticoagulant, 10 μM PG E₁ to block platelet activation, and 10 μM mepacrine to render platelets fluorescent for visualization, was perfused over a surface coated with immobilized VWF. The images shown are single frames from a real time recording (see movie 1 available online). A timeline (black horizontal bar) indicates at what moment during the perfusion experiment a given image was taken, as shown by the position of the corresponding letter (A, 55 s; B, 130 s; C, 380 s). A vertical white bar separates the timeline in segments corresponding to periods of perfusion at different wall shear rates (5,500 or 13,000 s⁻¹). The images are representative of 3 separate experiments performed with blood from different donors.

**Fig. 2. Effect of EDTA on the activation-independent and shear rate-dependent aggregation and disaggregation of platelets perfused over immobilized VWF.** Whole blood containing 93 μM PPACK, 10 μM PG E₁, 10 μM mepacrine, and with the addition of EDTA (5 mM) from the beginning of flow, was perfused over immobilized VWF at varying wall shear rates. The images shown are single frames from a real time recording (see movie 2 available online). The timeline (black horizontal bar) shows at what moment during the experiment a given image was taken as well as the wall shear rate during different periods of perfusion, as explained in the legend to Fig. 1 (A, 50 s, 3,000 s⁻¹; B, 180 s, 13,000 s⁻¹; C, 240 s, 15,000 s⁻¹; D, 340 s, 17,000 s⁻¹; E, 540 s, 3,000 s⁻¹; F, 1020 s, 3,000 s⁻¹). An arrow at 14 min on the timeline indicates when blood without EDTA replaced the blood with EDTA (from the same donor) perfused from the beginning of the experiment. The bar graph shows the average count, length and area of all the platelet aggregates present in five different positions on the surface after 540 s
(black bars) and 1020 s (grey bars) of perfusion under the conditions described above. All values are reported as mean ± SEM and differences are statistically significant (count: p<0.0001; length: p<0.001; area: p<0.006).

**Fig. 3. Effect of recombinant ADAMTS-13 of VWF-mediated and activation-independent platelet thrombus formation under flow.** Plasma-free washed blood cells were suspended in a Hepes/Tyrode buffer (10 mM Hepes, 135 mM NaCl, 2.7 mM KCl, 0.4 mM NaH2PO4, 2.8 mM dextrose), pH 7.4, containing 10 μM PG E1, 10 μM mepacrine, 20 μg/ml purified human VWF multimers, 2 mM Ca++ and 0.5 mM Mg++. The blood cell suspension (platelet count: 180,000-390,000/μl; hematocrit: 38-43%) was perfused over a surface coated with immobilized VWF without or with the addition of recombinant ADAMTS-13, as indicated. The protease activity added corresponded to 17.5% of that present in normal blood. The images shown are single frames from a real time recording (see movie 3 available online). The timeline (black horizontal bar) shows at what moment during the experiment a given image was taken as well as the wall shear rate during different periods of perfusion, as explained in the legend to Fig. 1 (A, 30 s, 5,500 s⁻¹; B, 170 s, 13,000 s⁻¹; C, 370 s, 13,000 s⁻¹). The bar graph shows the average count, length and area (mean ± SEM) of all the platelet aggregates measured in five separate fields of view after 10 min of perfusion in the absence or presence of recombinant ADAMTS-13 during each of three experiments performed with blood cells from different donors. Differences are statistically significant for count (p<0.009) and area (p<0.04), but not for length (p>0.05).

**Fig. 4. Effect of IgG from a TTP patient on VWF-mediated and activation-independent platelet thrombus formation under flow.** Whole blood containing 93 μM PPACK as anticoagulant and 10 μM PG E1 to inhibit platelet activation was supplemented with 2 mg/ml of IgG purified from the plasma of a normal individual (control IgG) or 2 mg/ml of IgG purified...
from the plasma of a patient with function-blocking antibodies against ADAMTS-13 (patient IgG) and then perfused over immobilized VWF. The images shown are single frames from a real time recording. The timeline (black horizontal bar) shows at what moment during the experiment a given image was taken as well as the wall shear rate during different periods of perfusion, as explained in the legend to Fig. 1 (A, 120 s, 13,000 s\(^{-1}\); B, 540 s, 13,000 s\(^{-1}\)). The bar graph shows the average count, length and area of all the platelet aggregates present in a field of view after 2 and 9 min of perfusion in the presence of control or patient IgG, as indicated.

**Fig. 5. Effect of plasma from a congenital TTP patient on VWF-mediated and activation-independent platelet thrombus formation under flow.** Normal washed blood cells were resuspended either in the plasma of a normal individual (control plasma) or that of a patient with TTP obtained from therapeutic plasmapheresis (patient plasma). The bar graph shows the average count, length and area (mean ± SEM) of all the platelet aggregates present in five separate fields of view measured after a 10 min perfusion. Only the difference in aggregate count was significant (p<0.0001).

**Fig. 6. Effect of recombinant ADAMTS-13 on platelet formation on fibrillar collagen type I under flow conditions.** The bar graph shows the volume of thrombi formed after perfusion of whole blood containing 93 \(\mu\)M PPACK over a surface of 62,500 \(\mu\)m\(^2\) coated with collagen type I fibrils. The blood was supplemented or not with recombinant ADAMTS-13, as indicated, added at least 15 min before perfusion. The protease activity added corresponded to 17.5% of that present in normal blood. The wall shear rate was 1,500 s\(^{-1}\) for 7 min or 6,000 s\(^{-1}\) for 4.5 min, as indicated. The values represent the mean ± SEM of 2 experiments performed with blood from different donors. The results obtained with or without addition of ADAMTS-13 were not significantly different.
Table 1. Change in activation-independent platelet aggregate size in the presence or absence of EDTA.

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<td>Length max</td>
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Experimental details are given in the legend to Fig. 2. Blood containing PPACK and PG E₁ was perfused over immobilized VWF at varying shear rates. The blood contained EDTA for the initial 14 min, and then blood from the same donor but without EDTA was perfused for an additional 6 min. The values (mean ± SEM of five separate fields of view; see Fig. 2) represent the area of the single smallest and largest aggregate and the length of the single shortest and longest aggregate detected on the surface at 9 min (blood + EDTA) or 17 min (blood - EDTA) from the beginning of perfusion. The differences in maximal area (p<0.0007) and length (p<0.001) are significant.
Table 2. Change in activation-independent platelet aggregate size in the presence or absence of recombinant ADAMTS-13.

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<td>Length max</td>
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Experimental details are given in the legend to Fig. 3. A suspension of washed blood cells with added soluble VWF and without or with added ADAMTS-13 (protease activity added before perfusion = 17.5% of that present in normal blood) was perfused over immobilized VWF at varying shear rates. The values (mean ± SEM of five different fields of view in three separate experiments; see Fig. 3) represent the area of the single smallest and largest aggregate and the length of the single shortest and longest aggregate detected on the surface. The differences in maximal area (p<0.0004) and length (p<0.002) are significant.
Table 3. Change in activation-independent platelet aggregate size in the presence or absence of IgG from a patient with neutralizing antibodies against ADAMTS-13.

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Size regulation of von Willebrand factor-mediated platelet thrombi by ADAMTS-13 in flowing blood

Roberta Donadelli, Jennifer N Orje, Cristina Capoferrì, Giuseppe Remuzzi and Zaverio M Ruggeri