Complement activation: the missing link between ADAMTS-13 deficiency and microvascular thrombosis of thrombotic microangiopathies

Maria Piedad Ruiz-Torres¹,², Federica Casiraghi¹, Miriam Galbusera¹, Daniela Macconi¹, Sara Gastoldi¹, Marta Todeschini¹, Francesca Porrati¹, Daniela Belotti³, Enrico Maria Pogliani³, Marina Noris¹, Giuseppe Remuzzi¹,⁴

¹Mario Negri Institute for Pharmacological Research, Center for Research on Organ Transplantation “Chiara Cucchi de Alessandri e Gilberto Crespi”, Villa Camozzi–Ranica, Italy
²Department of Physiology, Alcala University, Alcala de Henares, Madrid, Spain
³Division of Haematology, “San Gerardo” Hospital, Monza, Italy and ⁴Unit of Nephrology and Dialysis, Azienda Ospedaliera, Ospedali Riuniti di Bergamo, Italy

Summary
Endothelial injury is the central factor in the events leading to thrombotic microangiopathy (TMA); however, the mechanisms involved are not fully understood. Here we investigate the role of neutrophils (PMNs) and of complement activation in inducing microvascular damage and loss of thromboresistance in TMA associated with ADAMTS-13 deficiency. PMNs isolated during the acute phase of the disease released excessive amounts of reactive-oxygen species (ROS), N-derived oxidants and proteinases and induced damage and thromboresistance loss in human microvascular endothelial cell line (HMEC-1) ex vivo. Endothelial cytotoxicity and thromboresistance loss was also induced by TMA serum. Complement-derived products were responsible for the above effects: in fact, TMA serum caused C3 and Membrane Attack Complex (MAC) deposition on HMEC-1 and its cytotoxic effect was abolished by complement inhibition. TMA serum caused surface expression of P-selectin on HMEC-1 which may promote PMN adhesion and resulted in increased PMN cytotoxicity, indicating that complement may have a role in PMN activation. In addition, TMA serum stimulated control PMNs to release ROS and proteinases, and to cause endothelial cell cytotoxicity. All of the above effects were abrogated by complement inactivation. These data document for the first time that complement-initiated PMN activation and endothelial injury may have a crucial role in microvascular thrombosis of TMA associated with ADAMTS-13 deficiency.

Keywords
Thrombotic microangiopathy, ADAMTS-13, reactive oxygen species, neutrophils, complement

Introduction
The term thrombotic microangiopathy (TMA) defines a lesion of vessel wall thickening (mainly arterioles and capillaries) with intraluminal platelet thrombosis (1). Depending on whether renal or brain lesions prevail, two different entities have been described: the haemolytic uraemic syndrome (HUS) and the thrombotic thrombocytopenic purpura (TTP). However, clinical overlapping makes the differential diagnosis difficult. Conversely to the most common form of TMA in children, the so-called D+HUS associated with Escherichia coli infection that has an excellent prognosis, forms of TMA that occur in older children and adults have a much poorer prognosis and are often relapsing. Death and end-stage renal failure or neurological sequelae are the final outcome in the majority of cases. Evidence is now emerging that some of these atypical forms are associated with abnormalities of the complement system due to genetic deficiency of factor H or MCP – two regulatory proteins that inhibit the activation of the alternative pathway of complement (1–3). Other forms may be triggered by a deficiency of ADAMTS-13, a plasma metalloprotease that cleaves ultralarge (UL) multimers of von Willebrand factor (VWF) soon after their secretion by en-
endothelial cells (4). The deficiency of ADAMTS-13 activity may be constitutive, as in patients carrying mutations in the gene encoding for the protease (4–7), or acquired due to the presence of a circulating autoantibody (6–8).

Injury to endothelial cells is the central and likely inciting factor in the sequence leading to all forms of TMA (1). Loss of physiological thromboresistance, leukocyte adhesion to damaged endothelium, and increased vascular shear stress sustain and amplify the microangiopathic process (1). Evidence is available that neutrophil (PMN)-derived toxic compounds contribute to microvascular endothelial damage. PMNs from patients with D+HUS were hyper-adhesive to human endothelial cells and induced endothelial injury by degrading cell fibronectin (9). Release of reactive oxygen species (ROS) in vivo was higher than normal in PMNs from patients with recurrent TMA taken during the acute phase and normalized at remission (10). In HUS secondary to quinine, drug-dependent circulating antibodies induced PMN activation and increased adhesion to endothelial cells (11). However, other authors documented that blood humoral factors may also have a pathogenic role in TMA. Plasma from patients with acute TTP or HUS induced apoptosis in human microvascular endothelial cells of renal, cerebral and dermal origin, but not of pulmonary or hepatic lineage (12), which reflected the distribution of lesions in both disorders. In other studies, plasma from patients with TTP caused monocyte and PMN activation, as measured by ROS production and CD11b expression (13) and induced the formation of platelet-leukocyte aggregates (14), suggesting that humoral substances, not yet identified, are present in TTP circulation triggering PMN activation. ADAMTS-13 activity was not evaluated in any of the above studies, thus whether the same pathogenetic mechanisms could also apply to TMA associated with ADAMTS-13 deficiency is still not understood.

The present study was designed to clarify the biochemical mechanisms leading to microvascular thrombosis in TMA associated with ADAMTS-13 deficiency. We first evaluated whether patients’ PMNs are activated during the acute phase and investigated the capability of PMNs and serum to induce endothelial cell damage and thromboresistance loss; second we evaluated the mechanisms responsible for PMN activation and finally we searched for possible biochemical mediators of the above effects.

### Patients, materials and methods

#### Patients

Eight patients with TMA were recruited among those referred to the International Registry of HUS and TTP, an International network of more than 60 Haematology and Nephrology Units established under the coordination of the Clinical Research Center for Rare Diseases “Aldo e Celso Daccò”. Patients were selected on the basis of severe deficiency of ADAMTS-13 (activity <10%) during the acute phase of the disease. In 4 patients ADAMTS-13 deficiency was associated with a congenital defect, all of whom had a chronic relapsing form of the disease with onset in childhood. Diagnosis of congenital ADAMTS-13 deficiency rests on the following evidence: 1) undetectable activity found in different occasions; 2) absence of anti-ADAMTS-13 autoantibodies and 3) subjects with half normal levels of the protease consistent with the heterozygous carrier state in the family tree. Mutations in ADAMTS-13 gene have been found in 3 patients (manuscript in preparation). In 3 patients with several recurrences, the defect was acquired due to a circulating inhibitor. The eighth patient had a single TMA episode following ticlopidine administration, ADAMTS-13 was undetectable during the acute phase, however, no inhibitor was detected. Patients were studied twice, during the acute phase (before any treatment, n=8) and at remission (n=7, one patient was lost at follow-up). Acute episodes of TMA were diagnosed on the basis of haematocrit less than 30%, haemoglobin less than 10 mg/dL, LDH greater than 460 IU/L, undetect-

#### Table 1: Clinical and laboratory values of TMA and control patients.

<table>
<thead>
<tr>
<th>Cod #</th>
<th>Diagnosis</th>
<th>ADAMTS-13 activity (%)</th>
<th>PLTS (x10^3/L)</th>
<th>LDH (IU/L)</th>
<th>HGB (mg/dl)</th>
<th>S Cr (mg/dl)</th>
<th>C3 (mg/dl)</th>
<th>C4 (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMA Patients (sex)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R.020 (F)</td>
<td>Recurrent TMA</td>
<td>0 (inh)</td>
<td>20%</td>
<td>68</td>
<td>187</td>
<td>330</td>
<td>308</td>
<td>13.3</td>
</tr>
<tr>
<td>R012 (M)</td>
<td>Recurrent TMA</td>
<td>0 (con)</td>
<td>0 (con)</td>
<td>62</td>
<td>117</td>
<td>418</td>
<td>290</td>
<td>11.1</td>
</tr>
<tr>
<td>R.014 (M)</td>
<td>Recurrent TMA</td>
<td>0 (inh)</td>
<td>0 (inh)</td>
<td>60</td>
<td>197</td>
<td>556</td>
<td>417</td>
<td>9.5</td>
</tr>
<tr>
<td>R.002 (F)</td>
<td>Recurrent TMA</td>
<td>0 (con)</td>
<td>0 (con)</td>
<td>36</td>
<td>398</td>
<td>2190</td>
<td>392</td>
<td>10.7</td>
</tr>
<tr>
<td>R.006 (M)</td>
<td>Recurrent TMA</td>
<td>0 (con)</td>
<td>0 (con)</td>
<td>43</td>
<td>136</td>
<td>1576</td>
<td>290</td>
<td>9.7</td>
</tr>
<tr>
<td>R.019 (M)</td>
<td>Recurrent TMA</td>
<td>0 (con)</td>
<td>0 (con)</td>
<td>26</td>
<td>166</td>
<td>600</td>
<td>258</td>
<td>14.0</td>
</tr>
<tr>
<td>S.025 (M)</td>
<td>Ticlopidine</td>
<td>0 (no inh)</td>
<td>13%</td>
<td>15</td>
<td>366</td>
<td>859</td>
<td>380</td>
<td>7.0</td>
</tr>
<tr>
<td>S.024 (M)</td>
<td>Ticlopidine</td>
<td>0 (no inh)</td>
<td>nd</td>
<td>9</td>
<td>507</td>
<td>1370</td>
<td>nd</td>
<td>14.9</td>
</tr>
<tr>
<td>Control Patients (sex)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C.001 (M)</td>
<td>Csa microangiopathy</td>
<td>45</td>
<td>129</td>
<td>557</td>
<td>10.3</td>
<td>10.1</td>
<td>80</td>
<td>26</td>
</tr>
<tr>
<td>C.002 (F)</td>
<td>Sclerodema</td>
<td>68</td>
<td>325</td>
<td>505</td>
<td>9.0</td>
<td>5.5</td>
<td>101</td>
<td>23</td>
</tr>
<tr>
<td>C.003 (M)</td>
<td>Malignant hypertension</td>
<td>70</td>
<td>276</td>
<td>391</td>
<td>12.8</td>
<td>3.1</td>
<td>174</td>
<td>56</td>
</tr>
<tr>
<td>Healthy Subjects</td>
<td></td>
<td>50-150</td>
<td>150-450</td>
<td>230-460</td>
<td>M: 14-18; F: 12-16</td>
<td>0.5-1.3</td>
<td>83-177</td>
<td>15-45</td>
</tr>
</tbody>
</table>

LDH: lactate dehydrogenase; HGB: hemoglobin; S Cr: serum creatinine; inh: inhibitors; con: congenital; a: acute; r: remission; nd: not done.
able haptoglobin, fragmented erythrocytes in the peripheral blood smear and platelet count less than 150,000/μL. Remission was defined as normalisation of the above parameters, as reported (6). Eight age and sex-matched healthy subjects were studied simultaneously as controls. In selected experiments three patients with microvascular diseases and normal plasma ADAMTS-13 activity, one with microangiopathy following cyclosporine (CsA) administration, one with scleroderma and one with malignant hypertension, were also studied as additional controls. Patients and controls received detailed information on the purpose and design of the study and provided informed consent according to the guidelines of the Declaration of Helsinki. The protocol was approved by the Institutional Review Board of the Mario Negri Institute. Patient laboratory values are shown in Table 1.

Study design

To evaluate the activation of PMNs, release of ROS, metalloproteinase-9 (MMP-9) and N-derived oxidants (NO2-Cl and NO3-), PMNs from TMA patients and healthy subjects were assessed ex vivo. Release of ROS by PMNs from control patients was also evaluated.

To investigate whether TMA PMNs induced endothelial damage, HMEC-1 were co-cultured with PMNs (PMNs/HMEC-1 ratio, 2:1, in 500 μl test medium: HBSS 0.5% BSA) from patients or controls for 4 hours, then endothelial cytotoxicity was evaluated as 51Cr release. To evaluate the effect of humoral substances present in patient circulation on endothelial cell viability, HMEC-1 were exposed to serum (500 μl, diluted 1:2 in test medium) from TMA patients, control patients or healthy subjects for 4 h. The role of complement activation products was assessed by repeating the experiments with heat-inactivated (58°C for 30 min) serum or by adding soluble complement receptor-1 (sCR1-TP10, 150 μg/ml, a gift from Avant Immunotherapeutics, Needham, MA). The effect of serum on C3, C4c, MAC deposition and P-selectin expression by HMEC-1 was evaluated by immunofluorescence.

To evaluate whether serum and PMNs synergize in inducing endothelial damage, HMEC-1 were exposed to serum from TMA patients or healthy subjects for 4 hours, then rinsed and incubated with PMNs from the same subjects for 4 h. To evaluate the relative role of ROS, NDO or proteinases in mediating endothelial damage by patients’ PMNs and/or serum, the above experiments were repeated in the presence of catalase (H2O2 scavenger, 1000 U/ml, Sigma Chemical Co., St Louis, MO), taurine (HOCI scavenger, 5 mM, Sigma), alpha-1-antitrypsin (α1-AT, elastase inhibitor, 1 mg/ml, Sigma), or BB-3103 (metalloproteinase inhibitor, 10 μM, a gift from British Biotech Pharmaceuticals Ltd, Oxon, UK). To further study whether substances in the circulation of patients with acute TMA induce PMN activation, PMN suspensions (5x10⁵ cells each) from healthy subjects (control PMNs) were exposed to serum from patients or controls for 4 hours, then washed and co-cultured with HMEC-1 as above.

Finally, to evaluate whether biochemical modifications induced on HMEC-1 by PMNs and serum may result in increased thrombus formation, platelet adhesion experiments were performed on HMEC-1 pre-exposed to serum (4 h) or to PMNs (5x10⁵/ml, 2 h) from TMA patients or healthy subjects and then perfused in a flow chamber with whole blood from healthy subjects. Incubation with PMNs was shortened to 2 h to limit the cytotoxic effect of PMNs, which could affect the endothelial monolayer integrity. The relative role of ROS, NDO and proteinases in thrombus formation was assessed by repeating experiments in the presence of specific inhibitors. The role of complement was investigated repeating the assay after serum decomposition by heat inactivation.

To identify platelet receptors involved in thrombus formation, HMEC-1 pretreated with serum or PMNs were perfused with whole blood preincubated with inhibitors of GPIb (polymeric aurin tricarboxylic acid (16), ATA trisodium salt, Aldrich Chemical, Milwaukee, WI, 100 μg/ml) or of αβ3 integrin receptor, using the chimeric 7E3 Fab anti-β1 integrin subunit, 50 μg/ml (Abciximab, Reopro; Eli Lilly, Indianapolis, IN) (16).

Polymorphonuclear cell isolation

PMNs were isolated from blood collected on heparin by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation, followed by treatment with Emagel, as reported (10).

Measurement of ADAMTS-13 activity

ADAMTS-13 activity was measured on citrated plasma as previously described (6) using the collagen binding assay. The presence of ADAMTS-13 inhibitory antibodies was assayed by testing ADAMTS-13 activity in mixtures of patient plasma and plasma pool at different dilutions (6).

Reactive oxygen species and N-derived oxidants

Superoxide generation was measured by superoxide dismutase-inhibitable reduction of ferricytochrome c, as reported (10). Data were expressed as nmol of O2-/10⁶ cells/30 minutes. To evaluate the formation of chlorinating and nitrating N-derived oxidants (NO2-Cl and NO3-), PMNs (4x10⁶ cells/well) were suspended in 1 ml HBSS with CaCl2 and MgSO4 in the presence of 1 mM 4-hydroxyphenylacetic (HPA, used as a chemical probe to monitor nitration and chlorination reactions, Sigma) and incubated for 1 h at 37°C in 5% CO2–95% air. Additional PMN aliquots were incubated in the presence of catalase, or uric acid (1 mM, peroxynitrite scavenger, Sigma), or sodium azide (100 μM, MPO inhibitor, Sigma). Aliquots of PMNs activated with PMA, 50 ng/ml, were used as positive controls. The separation and quantification of 3-chloro-HPA (Cl-HPA) and 3-nitro-HPA (NO2-HPA) in PMN supernatant was performed by reverse-phase HPLC (Reduced activity 3x8 C18 4.6x80 mm, Brownlee Columns, Perkin Elmer) using the elution buffer: KH2PO4, 1 mM, pH 3/acetoni trile (85/15, vol/vol) at the flow rate of 1.5 ml/min, over 15 min. Products were identified by UV detection (Detector 166 NM, Beckman) at 280 nm and quantified by extrapolation from a curve obtained by injection of standard Cl-HPA and NO2-HPA. Data were expressed as μM Cl-HPA and μM NO2-HPA.

Zymography

MMP-9 activity was evaluated by zymography. Briefly, PMNs were incubated for 30 min at 37°C in test medium. Supernatants were concentrated, resuspended in sample buffer in non reducing...
conditions without heating and electrophoresed on SDS-PAGE co-polymerized with 1 mg/mL gelatine type B (Sigma). After washing, gels were incubated overnight in collagenase buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM CaCl₂, 0.02% Brij-35) at 37°C and stained. Zones of lysis were visualized as distinct unstained bands. Supernatants of WM983A non activated or activated with p-aminophenylmercuric acetate were run in parallel and used as reference standards for pro- (92 kDa) and active (85 kDa) forms of MMP-9, respectively. Bands of 92 and 85 kDa were quantified by densitometry with Microscan 1.0.5 and NIH Image 1.61/ppcs softwares.

### Endothelial cell culture

HMEC-1 cells (SV-40 transfected human dermal microvascular endothelial cells line [16, 17]), were a gift from Dr. F.J. Candal (CDC Atlanta Center for Disease Control, USA). Cells were grown in MCDB 131 medium (GIBCO-Invitrogen, Milan, Italy) containing 10% FCS, hydrocortisone (1 μg/ml), bovine brain extract and antibiotics, at 37°C in 5% CO₂-95% air. These cells constitutively express PAI-1, tPA, VWF and thrombomodulin (17).

Since for patients studied during the acute phase we had only few hours notice to organize the co-culture experiments with freshly isolated PMNs we needed to have endothelial cell monolayers ready, and this could only be achieved with an endothelial cell line.

### Cytotoxicity assay

HMEC-1 were added with Na⁺⁺CrO₄ (5 μCi/well, Amersham, Milan, Italy) at the time of plating into wells of a 24-well culture plate (about 1.7x10⁶ cells per well), 18 hours in advance of experiments. Cells were washed with HBSS and endothelial damage was evaluated by measuring ⁵¹Cr in the supernatant in a gamma-counter. Maximum release was determined by incubation in 1.0% Triton. Control release was determined by incubation with serum, PMNs or their combination from healthy subjects (control), run in parallel. Results were expressed as % of specific ⁵¹Cr release over control: (cpm test – cpm control)/(cpm Triton – cpm control).

### Immunofluorescence

HMEC-1 grown on coverslips were incubated with serum from patients or controls and then fixed in 3% paraformaldehyde. After washing and blocking of aspecific binding sites, cells were treated with mouse anti-human P-selectin (50 μg/mL; R&D Systems Europe, Abingdon, UK) followed by FITC-conjugated F(ab')₂ goat anti-mouse IgG (1:50; Jackson Immunoresearch Labs Inc, West Grove, PA) or with FITC-conjugated rabbit anti-human C3c-complement or C4c-complement (1:300; DAKO, Glostrup, Denmark) or with a rabbit anti-human C5b-9 (MACH 1:200; Calbiochem-Novachem, La Jolla, CA) followed by FITC-conjugated goat anti-rabbit IgG (1:50; Caltag Labs, Burlingame, CA).

Negative controls were performed by incubation with secondary antibody alone or by incubation with isotype controls followed by secondary antibody. Coverslips were then mounted and examined under confocal inverted laser microscope (InSight plus; Meridian Instruments Inc, Okemos, MI) (16).

### Platelet adhesion assay under flow

Platelet adhesion assay was performed as described (16) perfusing whole blood from healthy subjects (pre-labelled with the fluorescent dye mepacrine) in a flow chamber, regulated at 37°C, in which one surface of the perfusion channel was a glass slide seeded with a monolayer of endothelial cells, at constant flow rate of 1500 sec⁻¹ (60 dynes/cm²). After 3 min, perfusion was stopped and the slide was dehydrated and fixed in acetone for 20 minutes.

Images of platelet thrombi on endothelial cell surface were acquired by a confocal inverted laser microscope (16). The area occupied by thrombi was evaluated by automatic edge detection using built-in specific functions of the software Image 1.61 (NIH, Bethesda, MD), and expressed as μm²/field analyzed. Before each perfusion endothelial integrity was evaluated at in-
verted microscopy. Endothelial integrity was also checked in HMEC-1 pre-exposed to patient serum or PMNs and perfused with blood without mepacrine, then fixed with 0.5% glutaraldehyde (Fluka, Milan, Italy), dehydrated with methyl alcohol, and stained with May-Grunwald Giemsa (Carlo Erba Reagents, Milan, Italy).

Statistical analysis
Repeated-Measures Analysis of Variance (ANOVA) was used for the analysis of the following response variables: superoxide, metalloproteinase-9 (MMP-9) and N-derived oxidants (NDO, NO\textsubscript{2} and NO\textsubscript{2}Cl) generation, % specific cytotoxicity and thrombi area. Subsequently, between-group comparisons (e.g. healthy controls vs TMA acute phase) were performed by means of unpaired t-test, while within-group comparisons (e.g. acute vs remission phase of TMA) were evaluated by means of paired t-test. Data are mean ± SE. Statistical significance was defined as \( P \) less than 0.05. Stat-View 4.01 software was used for all the statistical evaluation.

Results
Increased production of ROS and NDO and metalloproteinase release by PMNs from TMA patients
As shown in Figure 1, panel A, \textit{ex vivo} release of superoxide anion (O\textsubscript{2}\textsuperscript{-}) by PMNs was significantly (\( P < 0.05 \)) higher in patients with acute TMA than in healthy subjects, but decreased at remission. \textit{Ex vivo} release of O\textsubscript{2}\textsuperscript{-} by PMNs from control patients was comparable to that of healthy subjects (2.16±0.67 vs. healthy subjects: 2.56±0.65 nmoles/1x10\textsuperscript{6} PMNs/30 min) and was significantly (\( P < 0.05 \)) lower than O\textsubscript{2}\textsuperscript{-} release by PMNs from TMA patients (acute and remission). Similarly, in the acute phase the levels of nitrating and chlorinating NDO compounds (NO\textsubscript{2} and NO\textsubscript{2}Cl) released by TMA PMNs were higher than normal and comparable to the ones by control PMNs maximally stimulated with PMA (Fig. 1, panels C and E). The amounts of NO\textsubscript{2}-HPA and of Cl-HPA normalized at remission (Fig. 1, panels C and E).

Release of NO\textsubscript{2}-HPA and of Cl-HPA by TMA PMNs was inhibited by catalase or sodium azide (Fig. 1, panels D and F) revealing a critical role for H\textsubscript{2}O\textsubscript{2} and for MPO in NDO formation (15). Densitometric analysis of MMP-9 activity in supernatants of PMNs from patients with acute TMA showed a significant increase of 92– (\( P < 0.05 \)) and 85-kDa (\( P < 0.001 \)) gelatinolytic bands, consistent with pro-MMP-9 and activated MMP-9.
respectively, in comparison with controls (Fig. 1, panel B). Both latent and active forms of the enzyme decreased at remission.

Altogether these data indicate that circulating PMNs are highly activated during the acute phase of TMA associated with ADAMTS-13 deficiency.

**Cytotoxic effect of PMNs from patients with acute TMA on HMEC-1: role of ROS, NDO and proteinases**

Exposure of HMEC-1 to PMNs from patients with active TMA led to a mild but specific cytotoxicity (calculated as percentage of $^{31}$Cr release over controls: HMEC-1 exposed to PMNs from healthy subjects), which was significantly higher ($P<0.05$) than cytotoxicity induced by PMNs isolated at remission (Fig. 2, panel A). The cytotoxic effect of patients’ PMNs was potentiated by pre-exposure of HMEC-1 to patients’ serum (acute TMA, $P<0.05$ vs remission; Fig. 2, panel B). Cytotoxicity induced by PMNs from patients with acute TMA, alone or after pre-exposure to patients’ serum, was significantly reduced by the ROS scavengers catalase and taurine (the latter by scavenging HOCl prevents MPO-mediated formation of NDO (15)) (Fig. 2, panels D and E), but not by the specific inhibitor of peroxynitrite, uric acid (not shown). The proteinase inhibitors, α1-AT and BB-3103 were also effective in reducing the cytotoxic effect of patients’ PMNs alone or in combination with serum (Fig. 2, panels D and E). These results indicate that ROS and MPO-derived NDO as well as proteinases released by PMNs may have a role in mediating endothelial damage in acute TMA. On the other hand lack of inhibitory effect of uric acid would exclude a role of peroxynitrite.

**Effect of serum from patients with acute TMA on HMEC-1: role of complement**

Serum from patients with acute TMA caused a mild cytotoxicity to HMEC-1 that was significantly higher ($P<0.05$) than that at remission (Fig. 2, panel C). The cytotoxicity observed when HMEC-1 were exposed to serum from control patients was very low and statistically different from that found after exposure to acute TMA serum (1.05 ±0.31 vs. 3.98±1.17 % specific cytotoxicity, $P<0.001$). Exposure to serum from patients with acute TMA also caused P-selectin expression on endothelial cell surface (Fig. 3, panel A) as evidenced by a granular staining distributed on the apical side. Neither serum taken at remission (Fig. 3, panel B) nor control serum (Fig. 3, panel D) induced P-selectin expression. Expression of P-selectin may have favoured PMNs-endothelial cell interaction, which may explain the higher cytotoxic effect of patients’ PMNs on HMEC-1 pre-exposed to patients’ serum.

**Figure 4: Representative micrographs of HMEC-1 exposed for 4 hours to serum from patients with TMA associated with ADAMTS-13 deficiency, studied during the acute phase (panels A, B and G) and at remission (panels C and D) or to serum from healthy subjects (controls, panels E and F) or from control patients (panel H) and stained for C3 (panels A, C and E) and for CS-b9 (MAC, panels B, D, F, G and H). Panel G: MAC deposition on HMEC-1 incubated with TMA serum taken during the acute phase and added with soluble complement receptor-1 (sCR1, 150 μg/ml). Panel H: MAC deposition on HMEC-1 incubated with serum from control patients (patients with microvascular diseases and normal ADAMTS-13 activity). HMEC-1 exposed to serum from acute TMA showed increased C3 and MAC deposition. MAC deposition induced by acute TMA serum was prevented by sCR1. Original magnification 600X.**
The effect of patients’ serum in inducing endothelial cell cytotoxicity (Fig. 2, panel C) and P-selectin expression (not shown) was not affected by addition of ROS (catalase: 4.66 ± 0.20 % cytotoxicity) and NDO (taurine: 4.31 ± 0.32 % cytotoxicity) inhibitors or by addition of the protease inhibitors α1-AT (3.87 ± 0.28 % cytotoxicity) and BB-3103 (4.18 ± 0.18 % cytotoxicity). These results indicate that substances other than PMN-derived toxic products are present in patients’ circulation that may induce endothelial damage. In search for other candidate mediators we took advantage of the observation that half of our patients with ADAMTS-13 deficient TMA had reduced serum levels of complement C3 and normal C4 serum levels during the acute phase (Table 1), which may be regarded as an index of activation of alternative pathway of complement. By immunofluorescence, we found strong deposition of C3 (Fig. 4, panel A) and of MAC (Fig. 4, panel B) on endothelial cells exposed to serum from patients with acute TMA, while little or no staining was found on cells exposed to serum taken at remission (Fig. 4, panels C and D), from healthy subjects (Fig. 4, panels E and F) and from control patients (Fig. 4, panel H). By contrast, C4 deposition on HMEC-1 exposed to serum from patients with acute TMA was very comparable to that induced by serum taken at remission and by healthy subject serum (data not shown), excluding the activation of the classical pathway of complement. MAC deposition was prevented by addition to TMA serum of the complement inhibitor, sCR1 (Fig. 4, panel G). sCR1 also completely abolished both cytotoxicity (Fig. 2, panel F) and P-selectin expression (Fig. 3, panel C) induced by TMA serum. Altogether these results indicate that breakdown products derived from complement activation via the alternative pathway mediate the biological effects of TMA serum on microvascular endothelial cells.

Serum from patients with acute TMA induces activation of PMNs
To clarify the mechanism(s) responsible for PMN activation in acute TMA, PMNs from healthy controls were pre-exposed to patients’ serum collected during the acute phase. As shown in Figure 5, right panel, control PMNs pre-exposed to TMA serum had a higher cytotoxic effect (P<0.05) on HMEC-1 than control PMNs pre-exposed to control serum. The cytotoxic effect of PMNs pre-exposed to TMA serum was significantly reduced by treating the cells with catalase, α1-AT or BB3103, indicating that TMA serum caused activation of PMN oxidative burst and degranulation. Complement inactivation by heating significantly prevented (P<0.05) the effect of patients’ serum on control PMNs (Fig. 5) indicating that complement activation products may represent crucial mediators of PMN activation during active TMA.

Figure 5: Cytotoxicity to HMEC-1 of PMNs isolated from healthy subjects and pre-exposed to serum from controls or from TMA patients studied during the acute phase (right panel). PMNs were isolated from control subjects and incubated for 4 hours in serum diluted 1:2 with test medium, rinsed and exposed to 5Cr pre-labelled HMEC-1 for 4 additional hours at 2:1 PMNs:HMEC-1 ratio (left panel). Control release was determined in HMEC-1 incubated with test medium, run in parallel. In additional samples catalase (cat, 1000 U/ml) or taurine (5 mM), or BB-3103 (BB, 10 μM) or α1-antitrypsin (α1-AT, 1 mg/ml) were added to control PMNs, previously exposed to serum from patients with acute TMA, before incubation with HMEC-1. None: no inhibitor added. In other samples TMA serum was heat inactivated (dec) before incubation with control PMNs. Data are mean ± SE. § P < 0.05 vs control PMNs + control serum; # P < 0.05 vs control PMNs + acute TMA serum none.

Effect of PMNs and serum from patients with acute TMA on thrombus formation: role of ROS, proteases and complement
As shown in Figure 6 (panels A and C), only limited platelet deposition was observed after blood perfusion on HMEC-1 pre-exposed to control PMNs. Pre-exposure of HMEC-1 to PMNs from patients with acute TMA significantly (P<0.05) increased platelet adhesion (Fig. 6, panels A and D), while the effect on thrombus formation of PMNs at remission (Fig. 6, panels A and E) was comparable to that of control PMNs. The prothrombogenic effect of TMA PMNs was inhibited by either catalase or BB-3103 (Fig. 6, panel B). Complete inhibition of platelet adhesion by ATA or 7E3 Fab (Fig. 6, panel B) indicates vWF as the major blood protein involved in platelet deposition. In these conditions the integrity of HMEC-1 treated with PMNs from patients with acute TMA and then perfused with blood was preserved as indicated by staining with May-Grunwald Giemsa (data not shown).

Limited platelet deposits were observed on HMEC-1 pre-exposed to control serum and then perfused with blood (Fig. 6, panel F). By contrast, pre-exposure to serum from patients with acute TMA caused a significant (P<0.05) increase of the endothelial surface covered by thrombi, while the effect of serum taken at remission was comparable to that of control serum (Fig. 6, panel F). Endothelial integrity as assessed by May-Grunwald Giemsa staining was preserved (data not shown). The prothrombogenic effect of TMA serum was completely abrogated by previous complement inactivation by heating (Fig. 6, panel G) but not by catalase or BB-3103 (Fig. 6, panel G). ATA or 7E3 Fab completely prevented thrombus formation (data not shown).

Discussion
Here we show that complement-initiated PMN activation and endothelial injury may have a crucial role in triggering the microvascular thrombosis in TMA associated with ADAMTS-13 deficiency. This was demonstrated by the following: 1- PMNs isolated during the acute phase released excessive amounts of ROS, NDO and proteases and induced microvascular endothelial cell damage; 2- patients’ serum caused C3 and MAC deposition and P-selectin expression on endothelial cells and potentiated the cytotoxic effect of PMNs; 3- control PMNs exposed to TMA serum released ROS and proteases and became cytotoxic to-
ward microvascular endothelium; 4- all the effects of TMA serum on endothelial cells and control PMNs were abrogated by complement inhibition; 5- PMNs and serum from patients caused endothelial thromboreistance loss, as documented by thrombus formation; 6- all the above abnormalities correlated with disease activity: they manifested during the acute phase and disappeared at remission.

That PMN may be implicated in pathogenesis of TMA rests on the evidence that PMN activation as indicated by increased oxidative burst (10, 18) and degranulation (19, 20) favour PMN adhesion to the endothelial cells leading to endothelium injury (9). Here we confirm the same activation mechanism in patients with TMA associated with ADAMTS-13 deficiency, as shown by an excessive production of ROS and NDO and by an excessive release of MMP-9 by PMNs isolated during the acute phase. In addition those cells caused a mild but significant cytotoxicity to microvascular endothelial cells, which was prevented by either antioxidants or proteinase inhibitors, suggesting a causal link between PMN products and endothelial damage. Pre-exposure to PMNs isolated during the acute phase caused a massive thrombus formation on endothelial cells perfused with blood, which was inhibited by the antioxidant catalase. This finding is consistent with data that PMN-derived ROS induce the release of vWF and P-selectin from Weibel-Palade bodies (21) and favour platelet adhesion on endothelial cell surface.

Our findings that patients’ serum induced cytotoxicity on endothelial cells are in line with previous studies showing that plasma from patients with TTP or HUS induced apoptosis in microvascular endothelial cells (12). Neither antioxidants or proteinase inhibitors blocked this effect indicating that PMN-derived products are likely scavenged by antioxidants and by proteinase inhibitors naturally present in human blood (20) and that PMNs exert their cytotoxic potential only locally at the sites of PMN–endothelial cell contact. On the other hand, soluble toxic mediators in TMA serum could also derive from the activation of alternative pathway of complement cascade as indicated by endothelial cytotoxicity associated with increased deposition of C3 and MAC, effects that were abolished by heat-inactivation or by sCR1.

Our findings that sCR1 also prevented endothelial P-selectin expression induced by TMA serum are consistent with previous in vitro data that either MAC assembly (21) or interaction of C5a with its receptors (22) induced P-selectin expression in human endothelial cells. Functional consequence of complement-mediated P-selectin expression in TMA is double: on one hand it may promote PMN-adhesion to endothelial cells resulting in increased PMN-mediated cytotoxicity, as we documented in endothelial cells pre-incubated with TMA serum and then exposed to patients’ PMNs. On the other hand, P-selectin expression may favour platelet adhesion (23) and thrombus formation as supported by our results showing a significant increase in platelet deposition on endothelial cells pre-exposed to serum from patients with acute TMA. Again, this prothrombogenic effect was completely abrogated by complement inactivation.

Beside a direct effect on endothelial cell adhesive properties, complement activation also affects PMN functions. Human PMNs constitutively express specific receptors for C3a and C5a generated by C3 and C5a cleavage (24), and engagement of either receptor evokes activation of PMN respiratory burst (25), that in turn may cause ROS-mediated injury to aortic endothelial cells (26). A similar pattern of PMN activation may occur in TMA. Indeed incubation with patients’ serum stimulated control PMNs to release ROS and proteinases and to cause endothelial cytotoxicity. All these effects were completely abrogated by complement inactivation. Consistent with present data are previous reports showing that plasma from patients with TTP induced ROS production, upregulation of CD11b expression (13), and promoted the formation of platelet-PMNs aggregates (14).
Activation of complement through the alternative pathway is a rather common finding in several forms of TMA. Reduced levels of C3 and C3 deposition in glomeruli have been reported in
D+HUS and in HUS associated with genetic deficiencies of fac-
tor H (1, 2). In a large series of patients with familial TMA, more
than 70% of cases had lower than normal C3 levels, irrespective
on whether they were classified as HUS or TTP (27); depressed
C3 levels did not parallel similar changes in C4 values, which in-
dicated a selective activation of the alternative pathway. In an-
other series of patients with TTP, 60% of cases were found to
have platelet-associated C3 (28) and in a young woman with
TTP, C3 deposition was demonstrated in capillary and small ar-
terioles (29).

However, no evaluation of ADAMTS-13 activity was done in
the above studies. Results of the present paper, for the first time,
provide the evidence that activation of complement may also play
a pathogenetic role in TMA associated with ADAMTS-13 deficien-
cy. ADAMTS-13 is a plasma metalloprotease that norm-
ally cleaves UL multimers of VWF into smaller forms soon after
their secretion (4). According to the “two hit model”, deficiency
of ADAMTS-13 predisposes to microvascular thrombosis and
TMA supervenes after a triggering event (infections, certain
drugs, even pregnancy) that activates microvascular endothelial
cells and causes the secretion of UL VWF multimers and P-se-
lectin expression. Platelets in flowing blood adhere transiently
to secreted VWF anchored on endothelial P-selectin (30). In nor-
mal conditions ADAMTS-13 cleaves VWF multimers, releases
the platelets, and limits the thrombus growth (30). Instead, in
the absence of ADAMTS-13, long strings of secreted UL VWF and
platelets remain bound to endothelial cells initiating the
formation of microthrombi (30).

But how our finding of complement activation in
ADAMTS-13 deficient TMA integrates into the above model? It
has been known since the late seventies, that activation of pla-
telets may cause complement activation (31). Human platelet
stimulated with thrombin formed a C3 convertase that activated
the terminal complement components (31). In a model of ex vivo
thrombosis induced by human whole blood recirculated through
cellulose acetate hallow fibers, plasma levels of C3 and C5 acti-
vation products increased, which did not occur when thrombosis
was prevented by heparin (32). In addition, C3 is phosphorylated
by a casein kinase released by activated human platelets (33), re-
sulting in increased binding of C3 to target surfaces, reduced
binding of C3b to factor H and reduced factor I dependent inac-
tivation of C3b into iC3b (32–35). In patients with systemic
lupus erythematosus, the phosphate content of plasma C3 in-
creased in parallel to platelet activation (36). These observations
document that thrombus formation may trigger activation of
complement and that C3 is the main target of this activation cas-
cade.

Based on previous reports and present data, we hypothesize
that in patients with ADAMTS-13 deficiency platelet micro-
thrombi cause the activation of the alternative pathway of com-
plement thus initiating a cascade of events which includes com-
plement deposition on microvascular endothelium, activation of
PMN oxidative burst and PMN adhesion to endothelial cells.
This cascade culminates in microvascular endothelial damage
and loss of thromboresistance, so that microvascular thrombi
continue to grow, causing tissue ischemia and infarction. If this
hypothesis is correct, it could define the interactions among
thrombus formation and endothelial cell damage in TMA pa-
tients with ADAMTS-13 deficiency.

Results from the present study may open new therapeutic
perspectives for the treatment of acute episodes of TMA. Com-
plement inhibitors, such as the soluble forms of complement re-
ceptor-1 now clinically available (37), combined with plasma
therapy, could be of value in limiting endothelial damage and
microvascular thrombosis.

Acknowledgements
Dr. Maria Piedad Ruiz-Torres is a recipient of the postdoctoral fellowship
from Spanish Ministry of Education and Sciences. Francesca Porrati is a
recipient of a fellowship in memory of Libera Dosss Giana. The authors
thank Paola Cassis, Stefania Angioletti, Marina Marchetti and Chiara Rossi
for excellent technical assistance. Dr Anna Falanga and dr FJ Candal kindly
provided HM1C-1. We are grateful to the nursing staff of the Unit of Neph-
rology, from Ospedali Riuniti, for their kind collaboration in patient recruit-
ment and blood collection.

References
1. Ruggenenti P, Noris M, Remuzzi G. Thrombotic
microangiopathy, hemolytic uremic syndrome, and
thrombotic thrombocytopenic purpura. Kidney Int
2001; 60: 831–46.
2. Caprio J, Bettnaglio P, Zippel PF, et al. The mol-
ecular basis of familial hemolytic uremic syndrome:
mutation analysis of factor H gene reveals a hot spot in
297–307.
3. Noris M, Brissoschi S, Caprio J, et al. for the Inter-
national Registry of Recurrent and Familial HUS/TTP.
Familial haemolytic ureemic syndrome and MCP mu-
4. Levy GG, Nichols WC, Lian EC, et al. Mutations in
a member of the ADAMTS gene family cause throm-
bolic thrombocytopenic purpura. Nature 2001; 413:
475–6.
5. Kokame K, Matsumoto M, Soejima K, et al. Mu-
tations and common polymorphisms in ADAMTS13
gene responsible for von Willebrand factor-cleaving
protease activity. Proc Natl Acad Sci USA. 2002; 99:
11902–7.
Willebrand factor cleaving protease (ADAMTS13) is
deficient in recurrent and familial thrombotic thomb-
boocytopenic purpura and hemolytic uremic syndrome.
Blood 2002; 100: 778–85.
7. Furlan M, Robles R, Galbusera M, et al. von Wille-
brand factor cleaving protease in thrombotic thromb-
boocytopenic purpura and the hemolytic-uremic syn-
8. Tsai HM, Lian EC. Antibodies to von Willebrand
factor-cleaving protease in acute thrombotic thrombo-
1585–94.
Neutrophil-mediated endothelial injury in haemolytic
ncreased nitric oxide formation in recurrent thrombotic
micrangiopathies: a possible mediator of microvascu-
11. Stoneck DE, Vercellotti GM, Ammerscheidt DE, et
al. Characterization of multiple quinine-dependent
antibodies in a patient with episodic hemolytic uremic
syndrome and immune glomerulonephritis. Blood 1992;
thrombocytopenic purpura and sporadic hemolytic-
uremic syndrome plasmas induce apoptosis in re-
stricted lineages of human microvascular endothelial
ma from patients with thrombotic thrombocytopenic
purpura induces activation of human monocytes and
120: 129–34.
thrombocytopenic purpura plasma enhances platelet-
17. Ribeiro MJA, Philipps DJ, Benson JM, et al. Hemostatic properties of the SV-40 transfected human microvascular endothelial cell line (HMEC-1). A representative in vitro model for microvascular endotheli-
opathic haemolytic-uraemic syndrome. Pediatr Neph-
or 1991; 5: 387–92.
20. Fitzpatrick MM, Shah V, Trompeter RS, et al. Interleukin-8 and polymorphonuclear neutrophil leukocyte activ-
21. KIlgore KS, Ward PA, Warren JS. Neutrophil ad-
hesion to human endothelial cells is induced by the membrane attack complex: the roles of P-selectin and platelet activating factor. Inflammation 1998; 22: 583–98.
23. McEver RP. Adhesive interaction of leukocytes, platelets, and the vessel wall during hemostasis and in-
24. van Epps DE, Chenoweth DE. Analysis of the bind-
25. Elsner J, Opperman M, Czech W, et al. C3a acti-
vates the respiratory burst in human polymorphonu-
clear neutrophilic leukocytes via pertussis toxin sensi-
26. Hardy MM, Flickinger AG, Riley DP, et al. Super-
plementemia discloses genetic predisposition to he-
emolytic uremic syndrome and thrombotic thrombocy-
ization of platelet glycoproteins and platelet/endothe-

tlial cell antibodies in patients with thrombotic thrombocy-
30. Dong JF, Moake JL, Nolasco L, et al. ADAMTS-13 rapidly cleaves newly secreted ultralarge von Wille-
34. Ekudahl KN, Nilsson B. Phosphorilation of complement component C3 and C3 fragments by a human pla-
35. Ekudahl KN, Nilsson B. Alteration in C3 activation and binding caused by phosphorilation by a casein ki-
nase released from activated human platelets. J Immu-
nol 1999; 162: 7426–33.
creased phosphate content in complement component C3, fibrinogen, vitronectin, and other plasma proteins in systemic lupus erythematosus: covariation with pla-
37. Smith RA. Targeting anticomplement agents. Bio-