Acquired TTP: ADAMTS13 meets the immune system
Sorvillo, N.

Citation for published version (APA):
Sorvillo, N. (2013). Acquired TTP: ADAMTS13 meets the immune system

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Residues Arg568 and Phe592 contribute to an antigenic surface for anti-ADAMTS13 antibodies in the spacer domain

Wouter Pos, Nicoletta Sorvillo, Rob Fijnheer, Hendrik B. Feys, Paul H.P Kaijen, Gestur Vidarsson and Jan Voorberg

Haematologica. 2011 June;96(11):1670-167
Chapter 2

Abstract

**Background:** The majority of patients diagnosed with thrombotic thrombocytopenic purpura (TTP) have autoantibodies directed towards the spacer domain of ADAMTS13. **Design and methods:** In this study we explored the epitope specificity and Ig class and IgG subclass distribution of anti-ADAMTS13 antibodies. The epitope specificity of anti-spacer domain antibodies was examined using plasma of 48 patients with acute acquired TTP by means of immunoprecipitation of ADAMTS13 variants containing single or multiple alanine substitutions. Using similar methods, we also determined the presence of anti-TSP2-8 and CUB1-2 domain antibodies in this cohort of patients. **Results:** Antibody profiling revealed that anti-ADAMTS13 IgG \textsubscript{1} and IgG \textsubscript{4} predominate in plasma of patients with acquired TTP. Analysis of anti-spacer domain antibodies revealed that Arg568 and Phe592, in addition to residues Arg660, Tyr661, and Tyr665, also contribute to an antigenic surface in the spacer domain. The majority of patients (90%) lost the reactivity towards the spacer domain following introduction of multiple alanine substitutions at Arg568, Phe592, Arg660, Tyr661 and Tyr665. Anti-TSP2-8 and anti-CUB1-2 domain directed antibodies were present in 17% and 35% of patient samples analyzed, respectively. **Conclusions:** IgG directed towards a single antigenic surface comprising residues Arg568, Phe592, Arg660, Tyr661 and Tyr665 predominates in plasma of patients with acquired TTP.
**Introduction**

Acquired thrombotic thrombocytopenic purpura (TTP) is a rare and life-threatening autoimmune disease characterized by the presence of autoantibodies directed towards ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13). Most autoantibodies directed towards ADAMTS13 are IgG’s although IgM and IgA have also been detected. Subclass analysis revealed that IgG₄ and to a lesser extent IgG₁ dominate the immune response to ADAMTS13. ADAMTS13 regulates the accumulation of ultra-large or unusually-large von Willebrand factor (UL-VWF) multimers on the surface of endothelial cells. The persistence of UL-VWF multimers promotes platelet aggregation resulting in obstruction of the microvasculature. VWF multimers are rapidly cleaved by ADAMTS13 at the Tyr1605-Met1606 scissile bond in the A2 domain of VWF. Shear stress induces unfolding of VWF multimers, thereby exposing the scissile bond in the A2 domain for cleavage by ADAMTS13. It has been postulated that multiple exosites within the disintegrin-like/TSP1/cysteine-rich/spacer (DTCS) domains interact with unfolded A2 domain. For example, Arg349 within the disintegrin domain has been shown to interact with residue Asn1614 of VWF whereas spacer domain residues Arg660, Tyr661 and Tyr665 interact with residues Glu1660-Arg1668 in the carboxy-terminal alpha-6 helix within the VWF A2 domain. Previously, we and others showed that the spacer domain of ADAMTS13 contains a major binding site for antibodies in patients with acquired TTP. Anti-ADAMTS13 antibodies present in the plasma of acquired TTP patients target an antigenic surface including residues Arg660, Tyr661 and Tyr665. However in 3 out of 6 patients analyzed residual binding was observed to an MDTCS variant in which Arg660, Tyr661 and Tyr665 were replaced by an alanine. This observation suggested that additional residues present within the spacer domain participate in binding of anti-ADAMTS13 antibodies. Previously, Arg568 and Phe592 have been shown to contribute to the binding of ADAMTS13 to VWF A2 domain. Therefore, we explored whether residues Arg568 and Phe592 also contribute to the binding of anti-spacer domain antibodies using plasma samples of 48 patients with acquired TTP. Several studies have reported the presence of antibodies directed towards the carboxy-terminal thrombospondin type repeats 2 to 8 (TSP2-8) and the CUB1-2 domains in patients with acquired TTP. The availability of a large cohort of patients allowed us to simultaneously address whether antibodies binding to the TSP2-8 and CUB1-2 domains are present in our cohort of patients with acquired TTP.


**Chapter 2**

**Materials and methods**

**Patients**

Plasma samples from a panel of 48 patients with acquired TTP containing high titers of anti-ADAMTS13 antibodies were included in this study. The study protocol was approved by the Medical Ethical Committee of the University Medical Center Utrecht in accordance with the Declaration of Helsinki. ADAMTS13 activity levels in all plasma samples were 10% as measured using the fluorogenic FRETS-VWF73 substrate assay kit (Peptides International, Liousville, KY, USA). Inhibitor titers measured with the Technozym ADAMTS13 inhibitor ELISA (Technoclone, Vienna, Austria) or with an in-house developed ELISA. All patients included had a history or were at presentation with primary acquired TTP with hemolytic anemia with fragmented erythrocytes and thrombocytopenia. ADAMTS13 inhibitor levels of plasma samples included in this study were >50 U/ml as measured by Technozym ADAMTS13 inhibitor ELISA.

**Construction and expression of recombinant ADAMTS13 and truncated variants**

Construction and expression of wild-type pcDNA3.1-propeptide/metalloprotease/disintegrin-like/TSP1/cysteine-rich/spacer V5-HIS fragment (Invitrogen, Carlsbad, CA, USA) (PMDTCS13-V5-HIS, but termed MDTCS here); a TSP2-8-V5-HIS fragment (TSP2-8) and a CUB1-2-V5-HIS domain fragment (CUB1-2) were produced in stably transfected HEK293 cells as described previously. An Ig-kappa signal peptide was cloned in front of the coding region of the TSP2-8 and CUB1-2 constructs in order to increase expression levels of these variants. Primer sequences are available upon request. Cells were grown in Optimem supplemented with glutamax medium (Invitrogen). Medium was harvested after four days and expression of ADAMTS13 was confirmed by Western blotting or immunoprecipitation using a monoclonal (horseradish-labeled (HRP)) anti-V5 antibody (Invitrogen), a mouse anti-TSP5-8 monoclonal antibody (20A5), a mouse anti-CUB1-2 domain monoclonal antibody (20D2) and a rabbit anti-metalloprotease polyclonal antibody (Abcam, Cambridge, UK). Monoclonal antibody 20D2 was prepared essentially as described previously. A sheep-anti-mouse-HRP labeled antibody (in conjunction with 20A5 and 20D2) and a donkey anti-rabbit-HRP labeled (in conjunction with the rabbit anti-metalloprotease antibody) both obtained from GE Healthcare (Bio-Sciences AB, Uppsala, Sweden) were used for chemiluminescence detection (Roche Applied Science, Almere, the Netherlands).
Arg568 and Phe592 bind anti-ADAMTS13 antibodies

Single alanine (R568A and F592A) and double alanine mutants (R568A/F592A) were introduced into the wild-type vector (pcDNA3.1-PMDTCS13-V5-HIS) using Quick-Change PCR (Stratagene, Amsterdam, the Netherlands). The same mutants were introduced into the previously described plasmid pcDNA3.1-PMDTCS13-R660A/Y661A/Y665A-V5-HIS (termed MDTCS-RYY) resulting in a total of eight variants including wild-type. Sequences of the sense and antisense oligonucleotide primers used for construction of these variants are available upon request. All vectors were verified by sequencing. All ADAMTS13 variants were transiently expressed essentially as described previously.

Epitope mapping of anti-ADAMTS13 antibodies

Recombinant antibodies or antibodies in plasma of the patients were bound to protein G sepharose (GE Healthcare) in 50 mM Tris pH 7.6 (Invitrogen), 500 mM NaCl (Merck), 1% (w/v) bovine serum albumin (BSA; Merck), 1% (w/v) Triton X-100 (Merck) and 0.1% (v/v) Tween-20 (Sigma Aldrich, Zwijndrecht, the Netherlands). As a negative control an anti-pneumococcal monoclonal antibody was included. The two human monoclonal anti-spacer domain antibodies I-9 and II-1 have been described previously. Monoclonal anti-V5 antibody (Invitrogen) coupled to protein G sepharose (GE Healthcare) was used as a positive control. Normal human pooled plasma from 47 healthy donors (NHP) was included as negative control. Thirty microliters of patient plasma was incubated with protein G sepharose for 1 hour at room temperature. Cell culture medium containing 1 µg of recombinant ADAMTS13 fragments was added to antibody-loaded sepharose and incubated in 50 mM Tris pH 7.6, 150 mM NaCl, 1% BSA, 1% Triton X-100 and 0.1% Tween-20 for at least 14 hours. After washing two to three times with buffer of the same composition, and a third to fourth time with 20 mM Tris pH 7.6, bound proteins were eluted with 100 mM glycine pH 2.0 and applied to either 7.5% or 10% SDS-polyacrylamide gels depending on the size of the fragments. Immunoblots were prepared on Immobilon-P polyvinylidene fluoride membranes (Millipore, Bedford, USA) and stained with HRP-labeled anti-V5 monoclonal antibody (Invitrogen). For detection BM chemiluminescence substrate (Roche Applied Science) and CL-Xposure films (Perbio Science, Etten-Leur, the Netherlands) were used. The composition of the immunoprecipitation buffer was adjusted for the experiments performed with the TSP2-8 and CUB1-2 variants by lowering the amount of Triton X-100 from 1% to 0.5% and Tween-20 from 0.1% to 0.05% in order to facilitate antibody binding towards these carboxy-terminal fragments.
Results

Anti-ADAMTS13 immunoglobulins in plasma of patients with acquired thrombotic thrombocytopenic purpura

Plasma samples of a panel of 48 patients with acquired TTP were analyzed for the presence of IgG, IgM and IgA as outlined in the Supplementary Information. Anti-ADAMTS13 IgM was found in 5 of these 48 patients (Figure 1). Anti-ADAMTS13 IgA was detected in 9 out of 48 patients (Figure 1). IgA antibodies can be further subdivided into IgA1 and IgA2. In all IgA positive samples anti-ADAMTS13 IgA1 was found suggesting that IgA1 (and not IgA2) antibodies develop in patients with acquired TTP. IgG was present in all patient samples analyzed. Levels ranged from 0.25 to 19.5 µg/ml. Analysis of the subclass of anti-ADAMTS13 IgG revealed that IgG1 was present in 35 out of 48 samples and IgG4 in 33 out of 48 samples analyzed (Figure 1). Low levels of IgG2 and IgG3 were observed in 7 and 4 patients respectively (Figure 1). Anti-ADAMTS13 IgG1 and IgG4 co-existed in 15 out of 48 patients. These results show that anti-ADAMTS13 IgG1 and IgG4 predominate in plasma of patients with acquired TTP.

Arg568 and Phe592 contribute to the binding of human monoclonal anti-spacer domain antibodies

Inspection of the three-dimensional structure of the spacer domain reveals that exposed residues Arg568 and Phe592 are in close proximity of Arg660, Tyr661 and Tyr665 (Figure 2A and B). This raises the possibility that Arg568 and Phe592 also contribute to the binding of anti-spacer domain antibodies. We assessed the binding of two patient-derived monoclonal antibodies I-9 and II-1 to MDTCS-R568A, MDTCS-F592A and MDTCS-R568A/F592A variant (Figure 2C). As a control, we monitored the reactivity of these antibodies with the previously described R660A/Y661A/Y665A (MDTCS-RYY) variant. Binding to RYY variants containing R568A, F592A and R568A/F592A substitutions was also evaluated. All variants were produced in HEK293 cells; the levels of expression of all variants were similar to that of wild-type MDTCS (data not shown). Antibody II-1 did not react with MDTCS-R568A and MDTCS-F592A (Figure 2). Antibody I-9 retained its reactivity towards MDTCS-F592A whereas binding to both MDTCS-R568A and MDTCS-R568A/F592A was abrogated (Figure 2C). As described previously, antibodies II-1 and I-9 did not bind to MDTCS-RYY. As expected MDTCS-RYY variants containing R568A, F592A or R568A/F592A mutations also did not interact with antibodies II-1 and I-9 (Figure 2C).
Arg568 and Phe592 bind anti-ADAMTS13 antibodies

Figure 1. Characteristics of anti-ADAMTS13 antibodies. Samples from 48 patients with acquired TTP were included in this study. Residual ADAMTS13 activity and antibody levels as measured by Technozym inhibitor ELISA are displayed. Levels of anti-ADAMTS13 IgG, IgA1, IgA2, and IgM are also included. IgG subclasses were measured in a qualitative manner. High levels (above 100-500 ng/ml) of anti-ADAMTS13 IgG1, IgG2, IgG3, and IgG4 are indicated by (++), intermediate or low levels (up to 100-500 ng/ml) are indicated by (+). Absence of IgG's, IgM and IgA's is indicated as ND (not detectable). The limit of detection for IgG's was 0.01 µg/ml; IgG1 0.002 µg/ml; IgG2-4 0.004 µg/ml; IgA1 0.01 µg/ml and IgM 0.02 µg/ml. Normal human pooled plasma from 47 healthy donors (NHP) was used as a negative control. Experimental details on Ig class and subclass measurements are included in the supplementary information.
Figure 2. (A) Three-dimensional presentation of ADAMTS13 fragment consisting of the DTCS-domains is depicted with the modeled metalloprotease domain (disintegrin domain (Dis) in green; the thrombospondin type repeat 1 (TSP1) in blue; the cysteine-rich domain (Cys) in pink and the spacer domain (Spacer) in light blue). The crystal structure of the metalloprotease domain of ADAMTS1, 4 and 5 were used as a template to model the metalloprotease domain (Met in light red) and was modeled using the HHpred server as described previously. Loops that were lacking in the DTCS crystal structure (323TFAREHLD330 and 459RSSPGGA465) were modeled using the MODELLER 9v7 program. (B) Residues Arg568, Phe592, Arg660, Tyr661 and Tyr665 are highlighted in red; these residues provide an exposed surface in the spacer domain. (C) Immunoprecipitation of MDTCS alanine(-hybrid)-mutants with recombinant monoclonal anti-ADAMTS13 antibodies I-9 and II-1. The reactivity of patient-derived anti-ADAMTS13 monoclonal antibodies I-9 and II-1 was determined by immunoprecipitation. An IgG1 isotype control antibody (-) and anti-V5 (+) antibody were used as controls. The first four lanes show MDTCS, MDTCS-R568A, MDTCS-F592A and the MDTCS-R568A/F592A double mutant. The second four lanes show the triple mutant R660A/Y661A/Y665A (RYY), MDTCS-RYY-R568A, MDTCS-RYY-F592A and MDTCS-RYY-R568A/F592A.

Spacer domain residues Arg568, Phe592, Arg660, Tyr661 and Tyr665 provide a major target for antibodies in patients with acquired thrombotic thrombocytopenic purpura

We analyzed the contribution of Arg660, Tyr661 and Tyr665 to binding of spacer domain antibodies in a cohort of 48 patients with acquired TTP. In addition, we determined whether residues Arg568 and Phe592 contribute to the binding of patient-derived anti-ADAMTS13 antibodies. Binding of patient IgG to MDTCS, MDTCS-R568A, MDTCS-F592A, MDTCS-R568A/F592A, MDTCS-R660A/Y661A/Y665A (in short MDTCS-RYY), MDTCS-RYY-R568A, MDTCS-RYY-F592A and MDTCS-RYY-R568A/F592A was evaluated. Replacement of Arg568 by an alanine had little impact on binding of patient-derived IgG; all patient samples bound to this variant (Figure 3). Replacement of Phe592 resulted in a decline in reactivity with the MDTCS-fragment for 12 out of 48 patients (patient samples 1, 4, 7, 10, 12, 16, 22, 25, 28, 38, 43 and 47). Reactivity with MDTCS-R568A/F592A was absent or strongly reduced for 16 out
of 48 patient samples analyzed (patients 1, 3, 4, 5, 7, 9, 10, 12, 16, 22, 25, 28, 38, 39, 43 and 47). Together, these findings indicate that residues Arg568 and Phe592 contribute to the binding of antibodies to the MDTCS-fragment in a significant number of patients. Next, we determined the binding of patient-derived IgG to MDTCS-RYY (Figure 3). In 33 out of 48 samples analyzed, the binding was significantly reduced compared to that with “wild-type” MDTCS. The introduction of F592A into the RYY-variant resulted in a decrease in binding in all samples analyzed (see, for example, patient 11, 14, 34 and 41 in Figure 3). In contrast, substitution of Arg568 for Ala had little impact on binding of patient derived IgG to the MDTCS-RYY fragment (see, for example, patient 8, 19 and 41 in Figure 3). Binding of patient derived IgG to MDTCS-RYY was completely abrogated for 43 out of 48 patients analyzed when both Arg568 and Phe592 were replaced by an alanine residue (Figure 3). Residual binding to MDTCS-RYY-R568A/F592A was still observed for patient samples 6, 16 and 37.

<table>
<thead>
<tr>
<th>Patient</th>
<th>WT</th>
<th>RYY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 48</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3. Immunoprecipitation of MDTCS alanine(-hybrid)-mutants with plasma samples from patients with acquired TTP. The reactivity of anti-ADAMTS13 antibodies present in plasma of 48 patients with acquired TTP was determined by an immunoprecipitation experiment using the MDTCS-variants described in the Legend of Figure 2. Normal human pooled plasma (-) and anti-V5 (+) antibody were used as controls.
Antibodies directed towards the TSP2-8 and CUB1-2 domains of ADAMTS13 in patients with acquired thrombotic thrombocytopenic purpura

We determined whether antibodies directed towards TSP2-8 and CUB1-2 domains were present in plasma from the 48 patients with acquired TTP included in this study. Only a few patients had anti-TSP2-8 directed antibodies (Figure 4). IgG derived from one patient’s sample (patient 3) reacted very well with the TSP2-8 fragment; for 2 other samples (from patients 4 and 16) weak signals were observed whereas 5 additional patient samples (from patients 6, 13, 18, 26 and 28) showed weak but detectable binding to TSP2-8 (Figure 4). Thus, TSP2-8 directed antibodies were present in 17% of patient samples analyzed.

Considerable amounts of antibodies directed towards the CUB1-2 domains were detected in 5 samples (from patients 8, 9, 24, 26 and 28) (Figure 5). In 6 samples (from patients 3, 4, 10, 11, 34 and 43) lower levels of anti-CUB1-2 domain antibodies were present whereas very low levels were observed in samples from patients 1, 40, 41, 42, 44 and 45 (Figure 5). Anti-CUB1-2 domain antibodies were found in approximately 35% of the samples analyzed. Only patients 3, 4, 26 and 28 had both anti-TSP2-8 and anti-CUB1-2 domain directed antibodies, although levels of anti-CUB1-2 and anti-TSP2-8 antibodies varied considerably. For instance, patient 3 had high levels of anti-TSP2-8 directed antibodies whereas levels of CUB1-2 directed antibodies in these patients were very low (Figure 4 and 5). Conversely, patient 26 and 28 had relatively high levels of anti-CUB1-2 domain antibodies but low levels of anti-TSP2-8 antibodies.

Figure 4. Immunoprecipitation of TSP2-8 domains with TTP plasma. Reactivity of autoantibodies present in the plasma of 48 patients used in this study with a fragment corresponding to thrombospondin type repeats 2 to 8 (TSP2-8). Normal human pooled plasma (-) and anti-V5 (+) antibody were used as controls.

Figure 5. Immunoprecipitation of the CUB1-2 domains with TTP plasma. Immunoprecipitations performed with the CUB1-2 domains (CUB1-2) of ADAMTS13. Normal human pooled plasma (-) and anti-V5 (+) antibody were used as controls.
Discussion

IgG, IgM and IgA autoantibodies directed towards ADAMTS13 are found in plasma of patients with acquired TTP with IgG as the dominant isotype. In agreement with previous studies, we found that anti-ADAMTS13 IgM and IgA are present in a subset of patients with acquired TTP. IgA consists of two subclasses IgA1 and IgA2; we found that only anti-ADAMTS13 IgA1 is present in plasma of patients with acquired TTP. The possible significance of this observation is presently unclear (see also discussion in the supplementary information). Our findings confirm previous observations on the predominance of IgG1 and IgG4 but indicate that the contribution of IgG2 and IgG3 to the total level of anti-ADAMTS13 IgG is less pronounced than that suggested by previous studies.

It has been well-established that the spacer domain harbours a major binding site for the anti-ADAMTS13 antibodies that develop in patients with acquired TTP. We recently showed that residues Arg660, Tyr661 and Tyr665 contribute to an antigenic surface in the spacer domain. In this study we show that Arg568 and Phe592, which are in close proximity to Arg660, Tyr661 and Tyr665 also contribute to the binding of anti-spacer domain antibodies. The antigenic surface covered by the variable domains of an antibody usually covers around 1500 Å². The surface accessibility of an arginine is ~211 Å², a tyrosine can cover ~104 Å² and a phenylalanine ~123 Å². The potential surface area covered by Arg568, Phe592, Arg660, Tyr661 and Tyr665 is approximately 750 Å². This value was confirmed by using a surface accessibility calculator. Arg568, Phe592 and Arg660, Tyr661 and Tyr665 originate from three distinct surface loops. All patient samples with the exception of samples 6 and 37 display a strong reduction in binding to an MDTCS fragment in which Arg568, Phe592, Arg660, Tyr661 and Tyr665 have been replaced by Ala (Figure 3). This observation strongly suggests that Arg568, Phe592, Arg660, Tyr661 and Tyr665 comprise an immunodominant region that is targeted by antibodies that developed in the majority of patients with acquired TTP. It should be noted that the ADAMTS13 variants described in this study partially retain their VWF processing activity (Supplemental Figure 2). In view of the restricted epitope specificity of anti-ADAMTS13 antibodies this observation may provide a basis for the design of ADAMTS13 variants with reduced antigenicity. Binding of IgG derived from patients 6 and 37 (and to a lesser extent also 16, 27 and 31) to the MDTCS fragment was not completely abolished following replacement of Arg568, Phe592, Arg660, Tyr661 and Tyr665. We anticipate that these may bind to other exposed surfaces in the MDTCS-fragment. Antibodies binding to the protease domain and
TSP1-repeat have been identified previously.\textsuperscript{16} In view of the multiple contacts between ADAMTS13 and VWF A2 domain it can certainly not be excluded that these antibodies impair ADAMTS13 function.\textsuperscript{11,12} In this study we also explored whether antibodies directed towards the proximal TSP2-8 repeats and CUB1-2 domains were present in our cohort of patients. Anti-TSP2-8 antibodies were found in 17% of patients analyzed. Weak signals were observed when the TSP2-8 fragments were precipitated with patient-derived immunoglobulins (Figure 4). Anti-TSP2-8 antibodies were found in 37% and 28% of patient samples analyzed in two previous studies.\textsuperscript{16,19} Anti-CUB1-2 antibodies were observed in 35% of patients analyzed. This value is similar to the value of 31% reported by Zheng and co-workers.\textsuperscript{19} In an early study anti-CUB1-2 domain antibodies were found in 64% of the patients analyzed.\textsuperscript{16} The observed discrepancies are potentially explained by differences in patient’s characteristics. Alternatively, the weak signals of the precipitated TSP2-8 and CUB1-2 domains observed in our study may be due to the relatively stringent conditions of our immunoprecipitation assay. The functional significance of anti-TSP2-8 and anti-CUB1-2 antibodies is presently unclear. Several studies have shown that the carboxy-terminal TSP2-8 and CUB1-2 domains are important for processing of VWF by ADAMTS13.\textsuperscript{22,30,31} Zheng and co-workers showed that platelet counts on admission were lower in patients with anti-TSP2-8 and/or anti-CUB1-2 IgG.\textsuperscript{19} This observation suggests that antibodies directed towards the carboxy-terminal domains inhibit ADAMTS13 activity or alternatively enhance its clearance from the circulation. Isolation of human monoclonal antibodies directed towards the TSP2-8 and CUB1-2 domains from patients with acquired TTP will help to determine whether these antibodies also impair ADAMTS13 function.

**Acknowledgements**

We are grateful to the Department of Blood Coagulation and Diagnostics at Sanquin for determining ADAMTS13 activity levels in plasma of the 48 acquired TTP patients.

**Author contribution**

W.P., N.S., P.K., G.V. and H.B.F. performed experiments, W.P., N.S. and J.V. designed the study and analysed results. W.P., N.S., G.V. and J.V. wrote the manuscript. R.F. provided patient samples and coordinated clinical aspects of the study. G.V. and H.B.F. provided essential reagents and protocols.

**Disclosures of Conflict of Interest**

The authors declare no competing financial interests.
References


Supplemental information

I. Quantification of anti-ADAMTS13 IgG, IgM and IgA in plasma of patients with acquired thrombotic thrombocytopenic purpura

Most anti-ADAMTS13 antibodies are of immunoglobulin class G (IgG), although in a limited number of patients antibodies of immunoglobulin class M (IgM) and A (IgA) have also been detected. While anti-ADAMTS13 antibodies of the IgG subclass dominate the immune response to ADAMTS13, IgG1 and IgG2 have been found in approximately 50% of the patient samples analyzed. In this study we addressed the Ig class distribution in 48 patients with acquired thrombotic thrombocytopenic purpura (TTP) using human monoclonal antibody II-1 as an internal standard.

Materials and methods

Construction and expression of anti-ADAMTS13 IgG1-2-3-4/M/A1-2 antibody II-1

The variable heavy chain of antibody II-1 (IgG1) was subcloned into the pcDNA3.1 vector together with different constant regions of IgG2 (Cγ2)-IgG3 (Cγ3)-IgG4 (Cγ4), IgM (Cμ), IgA1 (Ca1) and IgA2 (Ca2) with the BamHI/NotI/KpnI restriction sites/ enzymes (NEB, Ipswich, United Kingdom). All variants were transiently expressed in the Freestyle system (Invitrogen) including the light chain of antibody II-1 and quantified using a human IgG or IgM or IgA quantitation kit from Bethyl Laboratories (Montgomery, TX, USA). IgM, IgA1 and IgA2 were co-expressed with the J-chain (present in the pcDNA3.1 vector) in order to let the antibodies multimerize into pentamers/hexamers for IgM or dimers for the IgA1 and IgA2 molecules. All antibody variants were analyzed by sodium dodecylsulfate polyacrylamide (SDS-PAGE) under reducing conditions. Western blots were stained using an anti-IgG-, anti-IgM- and anti-IgA-horseradish peroxidase (HRP)-labeled antibody (DAKO, Glostrup, Denmark).

Detection of anti-ADAMTS13 IgG/M/A1-2 antibodies from TTP plasma

The presence of anti-ADAMTS13 antibodies in plasma from patients with acquired TTP was determined by immobilizing an anti-V5 antibody (1 μg/ml) on Maxisorp microtiter plates (NUNC, Roskilde, Denmark) in 50 mM NaHCO3 pH 9.8 overnight at 4°C. Wells were subsequently blocked with PBS 2% BSA 0.1% Tween-20. After washing three times with PBS 0.1% Tween-20, purified recombinant V5-tagged ADAMTS13 (1 μg/ml) was incubated in blocking buffer for 2 hours at 37°C. The purification and analysis of ADAMTS13 has been described previously. Subsequently,
Arg568 and Phe592 bind anti-ADAMTS13 antibodies

unbound ADAMTS13 was washed away and plasma samples, diluted in PBS 2% BSA 0.1% Tween-20, were added to the wells and incubated for 2 hours at 37°C. After washing, immunoglobulins were detected with a mix of HRP-labeled monoclonal anti-human IgG1, IgG2, IgG3 and IgG4 (1:2000; Sanquin Reagents, Amsterdam, the Netherlands) for the detection of total IgG, HRP-labeled monoclonal anti-human IgM (1:750; Sanquin Reagents) and HRP-labeled monoclonal anti-human specific for IgA1 or IgA2 (1:1000; Southern Biotech, Birmingham, Alabama, USA). A pool of normal human plasma (NHP) derived from 47 donors and plasma from two congenital TTP patients were used as negative controls. An equimolar mixture of II-1 IgG1, 2, 3, and IgG4 was used as an internal standard for determining IgG levels in patients samples. II-1 IgM was used as an internal standard for determining IgM levels in patients samples. II-1 IgA1 and II-1 IgA2 were used as an internal standard for determining the level of anti-ADAMTS13 IgA1 and IgA2 in patients samples. Anti-ADAMTS13 IgG subclass antibodies in plasma from patients with acquired TTP were detected by directly coating ADAMTS13 (1 μg/ml) on Maxisorp microtiter plates (NUNC, Roskilde, Denmark) in 50 mM NaHCO3 pH 9.8 overnight at 4°C. After blocking for 1 hour at 37°C with PBS 2% BSA 0.1% Tween-20, plasma samples and recombinant anti-ADAMTS13 antibodies (standard; start dilution of 100 ng/ml for IgG1 and 500 ng/ml for IgG2, 3, 4) were added for 2 hours at 37°C, allowing antibodies to bind ADAMTS13 in solution. Immunoglobulins were detected with HRP-labeled monoclonal anti-human IgG1, IgG2, 3, and IgG4 (1:2000; Sanquin Reagents, Amsterdam, the Netherlands); detection of IgG2 required an additional step of incubation with a primary anti-human IgG2 antibody and then a secondary HRP-labeled antibody (1:2000; Sanquin Reagents, Amsterdam, the Netherlands). A pool of normal human plasma (NHP) derived from 47 donors was used as a negative control.

Results and Discussion

Plasma samples of a panel of 48 patients with acquired TTP were analyzed for the presence of IgG, IgM and IgA. In all patients ADAMTS13 activity levels were 10% or less as determined by FRETS-VWF73 assaya (Figure 1). The levels of the immunoglobulins were quantified using human monoclonal anti-ADAMTS13 antibody II-1 as an internal reference. The variable domains of antibody II-1 were fused to the constant regions of human IgG1, IgG2, IgG3, IgG4, IgM, IgA1, and IgA2. Analysis of supernatants of transfected 293 cells by SDS-PAGE revealed that all variants were expressed (Supplemental Figure 1). Anti-ADAMTS13 IgG was present in plasma samples of all 48 patients analyzed (Figure 1). Levels of anti-ADAMTS13
IgG ranged primarily from 0.50-1.50 μg/ml; higher values of anti-ADAMTS13 IgG were found in 11 plasma samples. The highest level of anti-ADAMTS13 IgG was detected in plasma of patient 7 (19.5 μg/ml).

Residual levels of ADAMTS13 activity of 10% were still present in plasma of this patient. Levels of anti-ADAMTS13 IgG did not correlate with residual levels of ADAMTS13 activity (data not shown). IgG subclass analysis revealed that IgG₁ was present in 35 out of 48 samples and IgG₄ in 33 out of 48 samples (Figure 1). Low levels of IgG₂ and IgG₃ were observed in seven and four samples, respectively. Patient’s plasma samples that were negative for the presence of anti-ADAMTS13 IgG₁,₂,₃ or IgG₄ were denoted as non-detected (ND). Due to limitations in availability of patient’s samples we only assessed the presence of anti-ADAMTS13 IgG₁ and IgG₄ in plasma diluted 10 times. We cannot, therefore, exclude that small amounts of IgG₂ and IgG₃ are present in the patient’s samples included in this study. Nevertheless, our findings suggest anti-ADAMTS13 IgG consists primarily of IgG₁ and IgG₄. Anti-ADAMTS13 IgM was found in 5 out of 48 patients. In a previous study the presence of anti-ADAMTS13 IgM was reported in 4 out of 58 patients.³ Levels of anti-IgM varied between 0.15-1.20 μg/ml. Anti-ADAMTS13 IgA was detected in 9 out of 48 patients. This value closely corresponds to results from a previous study in which 10 out of 47 patients had anti-ADAMTS13 IgA’s.³ Anti-ADAMTS13 IgA levels were low (0.02-0.60 μg/ml) when compared to anti-ADAMTS13 IgG in most patients with the exception of patient 3 (Figure 1). The percentage of patients positive for the combinations of IgG/IgA, IgG/IgM and IgG/IgM/IgA were similar to values reported in a previous study.² IgA antibodies can be further subdivided into IgA₁ and IgA₂, IgA₃.
Arg568 and Phe592 bind anti-ADAMTS13 antibodies is the major component of serum IgA and secretory IgA except in the large intestines and the female genital tract where IgA₂ constitutes the majority of IgA found. We show that patients only have subclass anti-ADAMTS13 IgA₁ in their plasma; no anti-ADAMTS13 IgA₂ was detected. Taken together, our data suggest that the majority of anti-ADAMTS13 antibodies consist of IgG, although significant amounts of IgM and IgA₁ antibodies can also be found in plasma of patients with acquired TTP (Figure 1). Our findings are in agreement with previous studies on the presence of anti-ADAMTS13 IgA and IgM in patients with acquired TTP. IgA consists of two subclasses IgA₁ and IgA₂; IgA₁ has a unique hinge region lacking in IgA₂ containing O-linked glycans that can mediate binding to various lectins (galectin-1, Fcα1/γR on T cells). Serum IgA is exclusively monomeric, and is mostly composed of IgA₁ (~80%). In contrast, IgA produced at the mucosa is mostly dimeric or polymeric and associated with the J-chain, allowing for interaction with the polymeric Ig-Receptor (pIgR) expressed on mucosal epithelial cells. The pIgR transports the IgA mucosal surfaces of the gastro-intestinal, respiratory and urinogenital tracts where it prevents pathogen penetration, interacts with dietary antigens and controls commensal microbes through immune exclusion. Here, IgA₁ is also more abundant than IgA₂ (except in the large intestine and female genital tract), and both subclasses remain covalently attached to the extracellular part of the pIgR (secretory component). The monomeric IgA in serum, on the other hand, has been suggested to comprise a second line of defense for incoming pathogens, which are eliminated by FcαRI expressed on monocytes and neutrophils and Kupffer cells of the liver. FcαRI interacts exclusively with serum IgA, but not secretory IgA due to steric hindrance by the secretory component, and can mediate a strong degranulation of neutrophils and release of inflammatory mediators upon receptor crosslinking. Deposits of systemic IgA immune complexes can give rise to purpura in the skin and kidney failure in patients with Henoch-Schönlein purpura and patients with IgA nephropathy, respectively. High titers of anti-ADAMTS13 IgA and IgG₁ have previously been linked to clinical outcome following a first TTP event. Here we show that anti-ADAMTS13 IgA is exclusively composed of subclass IgA₁. Presently, it is not known whether immune complexes consisting of anti-ADAMTS13 IgA₁ contribute to the pathogenesis of acquired TTP.

II. Quantification of binding of spacer domain variants to patient’s IgG

In order to quantitatively determine differences in reactivity of the spacer domain variants for anti-ADAMTS13 IgG present in the patient samples we performed densitometric scanning of the western blots shown in Figure 2 (Supplemental Figure 2)
Supplemental Figure 2. Quantification of binding of spacer domain variants to patient’s IgG. Signals obtained for binding of patient derived IgG to the different spacer domain variants were plotted as percentage of binding to wild-type MDTCS. The spacer domain variants used in this study are displayed on the x-axis. Each data point presents the reactivity of IgG present in a single patients sample to the variant listed on the x-axis. Means and standard error of the means of the reactivity of the 48 patient samples with the different variants is indicated by horizontal bars. A progressive decline in reactivity of patient IgG in the order WT, WT-R568A, WT-F592A, WT-R568A/F592A, RYY, RYY-R568A, RYY-F592A and RYY-R568A/F592A is observed.

III. VWF processing of ADAMTS13 spacer domain variants

The activity of the MDTCS variants included in this study was determined using the fluorogenic FRETS-VWF73 assay (Peptides International, Liousville, KY, USA). Unexpectedly, VWF processing activity of MDTCS-F568A and MDCTS-F592A was increased when compared to wild-type MDCTS. Introduction of both amino acid substitutions did not further enhance the VWF processing activity of MDTCS (see results obtained for MDTCS-R568A/F592A; Supplemental Figure 3). In accordance with previous findings the VWF processing activity of MDTCS-RYY was strongly reduced when compared to wild type MDCTS. Introduction of the F592A, R568A or both the F592A and R568A substitutions results in an increased activity when compared to MDTCS-RYY (Supplemental Figure 3). Overall, our findings suggest that introduction of the R568A and/or F592A substitutions does not induce major structural alterations. The strongly reduced binding of patient IgG to MDTCS-RYY-R568A/F592A and the relatively modest effect of these amino acid substitutions on ADAMTS13 activity may provide a basis for the design of spacer domain variants with reduced antigenicity which at least partially retain their ability to process VWF.
Arg568 and Phe592 bind anti-ADAMTS13 antibodies

Supplemental Figure 3. VWF processing activity of spacer domain variants. Spacer domain variants (5 nM) were incubated with 2 μM of FRETS-VWF73 substrate for 60 minutes at 30°C. Fluorescence signal was measured using a Wallac 1420 ARVO multilabel counter. The VWF processing activity of the different spacer domain variants is expressed as percentage of that observed for wild type MDTCS.
References


