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A suit of armor around ADAMTS13

Exploring paths towards resistance against autoantibodies of immune TTP patients

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Chapter 3

Immunogenic hotspots in the spacer domain of ADAMTS13 in immune-mediated thrombotic thrombocytopenic purpura

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Summary

Background: Immune-mediated thrombotic thrombocytopenic purpura (iTTP) is caused by anti-ADAMTS13 autoantibodies inducing a severe deficiency of ADAMTS13. Epitope mapping studies on samples obtained during acute iTTP episodes have shown that the iTTP immune response is polyclonal, with almost all patients having autoantibodies targeting the spacer domain of ADAMTS13.

Objectives: To identify the immunogenic hotspots in the spacer domain of ADAMTS13.

Patients/Methods: A library of 11 full-length ADAMTS13 spacer hybrids was created in which amino acids regions of the spacer domain of ADAMTS13 were exchanged by the corresponding region of the spacer domain of ADAMTS1. Next, the full-length ADAMTS13 spacer hybrids were used in ELISA to epitope map anti-spacer autoantibodies in 138 samples from acute and remission iTTP patients.

Results: Sixteen different anti-spacer autoantibody profiles were identified with a similar distribution in acute and remission patients. There was no association between the anti-spacer autoantibody profiles and disease severity. Almost all iTTP samples contained anti-spacer autoantibodies against the following three regions: amino acid residues 588-592, 602-610 and 657-666 (hybrids E, G and M). Between 31 and 57% of the samples had anti-spacer autoantibodies against amino acid regions 572-579, 629-638, 667-676 (hybrids C, J and N). In contrast, none of the samples had anti-spacer autoantibodies against amino acid regions 556-563, 564-571, 649-656 and 677-685 (hybrids A, B, L and O).

Conclusion: We identified 3 hotspot regions (amino acid regions 588-592, 602-610, and 657-666) in the spacer domain of ADAMTS13 that are targeted by anti-spacer autoantibodies found in a large cohort of iTTP patients.

Essentials

- The immune response in immune-mediated Thrombotic Thrombocytopenic Purpura (iTTP) is polyclonal.
- The majority of iTTP patients have autoantibodies that target the spacer domain of ADAMTS13.
- We fine mapped anti-spacer autoantibodies present in plasma from acute and remission iTTP patients.
- Amino acid regions 588-592, 602-610, 657-666 are anti-spacer autoantibody hotspots.

Keywords: ADAMTS13, autoantibodies, epitope mapping, immunophenotyping, Thrombotic Thrombocytopenic Purpura

Introduction

Immune-mediated thrombotic thrombocytopenic purpura (iTTP) is a rare and life-threatening disorder, caused by autoantibodies that inhibit or clear the plasma protease ADAMTS13 (A Disintegrin and Metalloprotease with ThromboSpondin Type 1 repeats, member 13) [1-7]. ADAMTS13 consists of 14 domains: a metalloprotease (MP), a disintegrin-like (Dis), a first thrombospondin type-1 repeat (T1), a cysteine-rich (Cys), a spacer (S), seven additional T1 repeats (T2–8), and two CUB domains [8]. ADAMTS13 is responsible for the cleavage of ultra-large von Willebrand factor (UL-VWF) multimers. Upon secretion from the endothelial cells, UL-VWF multimers unfold due to shear stress in the flowing blood and are cleaved by ADAMTS13 into smaller multimers to avoid spontaneous platelet binding [9, 10]. Absence of functional ADAMTS13 leads to the accumulation of UL-VWF which induces the formation of platelet-rich microthrombi. When these microthrombi block arterioles and capillaries, organ failure and even death can occur when left untreated [3]. Current treatment options for iTTP include plasma exchange, to supply functional ADAMTS13 and to remove anti-ADAMTS13 autoantibodies; caplacizumab, to block the interaction between VWF and platelets; and corticosteroids with often rituximab, to reduce anti-ADAMTS13 autoantibody production [2, 11-13]. As an alternative for the invasive and cumbersome plasma exchange procedures, recombinant ADAMTS13 is being tested as a novel therapy in a clinical trial (NCT03922308), to investigate whether recombinant ADAMTS13 can overcome the anti-ADAMTS13 autoantibodies and increase the ADAMTS13 activity in iTTP patients [11, 14]. To further refine this approach, active recombinant ADAMTS13 variants resistant to anti-ADAMTS13 autoantibodies are under development [15]. However, to estimate the success of this approach, a thorough knowledge about the epitopes of anti-ADAMTS13 autoantibodies is crucial [11, 15, 16].

To date, epitope mapping studies of anti-ADAMTS13 autoantibodies from iTTP patients using ADAMTS13 variants have shown that the immune response in iTTP patients is polyclonal with different studies reporting 62–100% of the iTTP patients having anti-ADAMTS13 autoantibodies that target the spacer domain of ADAMTS13 [16-19]. In small cohort studies, the anti-ADAMTS13 autoantibodies targeting the spacer domain were screened in more detail and it was shown that the amino acid regions 572-579 and 657-666 are mainly targeted in acute iTTP patients [16, 19-24]. Additionally, by introducing alanine mutations in ADAMTS13, it was possible to identify that the amino acid residues $R^{568}F^{592}R^{660}Y^{661}Y^{665}$ form the main immunogenic region in the spacer domain. Based on this finding, ADAMTS13 variants were developed with a reduced anti-ADAMTS13 autoantibody binding and an increased ADAMTS13 activity [15, 16]. However, it was shown that additional residues with varying importance also participate in the binding of anti-spacer autoantibodies [19-22]. Therefore, large scale

epitope mapping studies are needed, using both acute and remission samples, to gain more insight into the immunogenic regions of the spacer domain. Additionally, the individual patient's anti-spacer autoantibody profile will identify which patients could benefit from therapies using ADAMTS13 spacer variants. Finally, investigating whether anti-ADAMTS13 autoantibody (spacer) profiles are linked with disease severity [19-22], could also be of prognostic value and help to treat iTTP. A link between autoantibody profiles and disease severity has been demonstrated in other autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus (SLE) [25, 26].

In this study, we developed a high-throughput ELISA using full-length ADAMTS13 spacer hybrids to identify the autoantibody hotspots in the spacer domain of ADAMTS13. We also used these data to identify anti-spacer autoantibody profiles in both acute and remission phase samples from a large multicenter cohort of iTTP patients and studied possible links with disease severity.

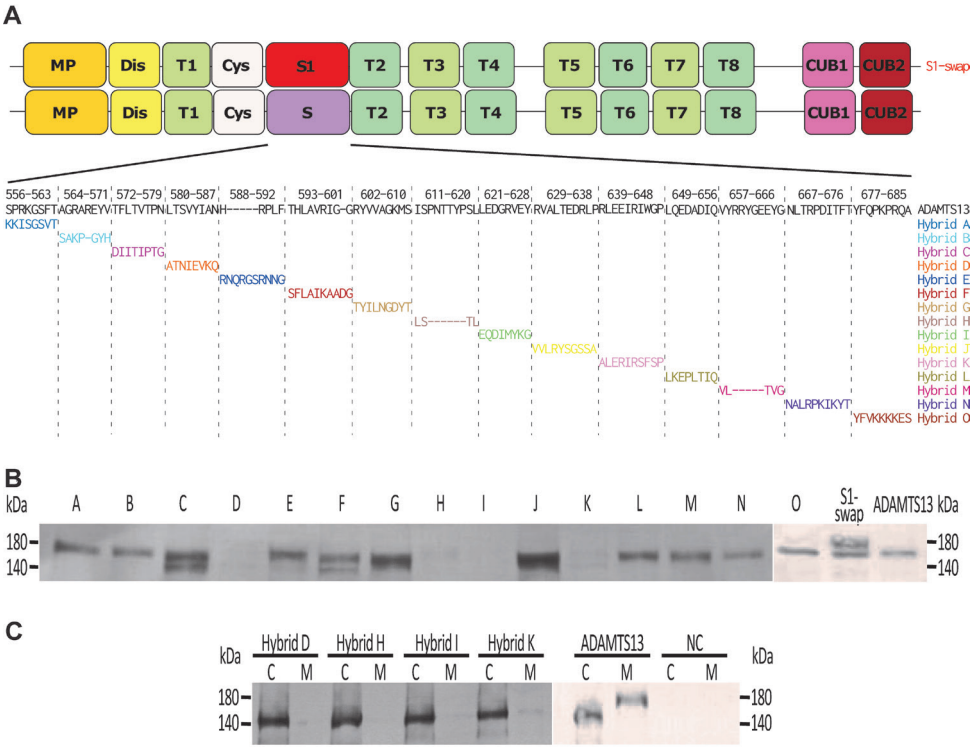

Figure 1 - Expression of full-length WT ADAMTS13 and spacer hybrids.

A. Schematic representation of full-length ADAMTS13, consisting of a metalloprotease (MP), a disintegrin-like (Dis), a first thrombospondin type-1 repeat (T1), a cysteine-rich (Cys), a spacer (S), seven additional T1 repeats (T2–8), and two CUB domains; the spacer domain of ADAMTS1 that was used to create the hybrid S1-swap is shown in red. Under the domain structure of ADAMTS13, the amino acid sequence of the

spacer domain is shown. The name of each hybrid and the amino acid regions exchanged between the spacer domain of ADAMTS13 and ADAMTS1 are labelled using the same color. B. Western blot of expression media from the 16 full-length spacer hybrids and WT ADAMTS13. Expression media of cells transfected with each one of the hybrids (A-O, S1-swap) and of WT ADAMTS13 were loaded onto an 8% SDS-polyacrylamide gel. After blotting, the membrane was incubated with a mouse monoclonal anti-His tag antibody, followed by HRP labelled goat anti-mouse Immunoglobulin G (IgG). Colorimetric development was performed using a TMB solution III. C. Western blot of cell lysates (C) and expression media (M) of cells transfected with the hybrids D, H, I and K constructs (constructs that were not well secreted in the expression medium). Cells transfected with the WT ADAMTS13 construct were used as a positive control and of non-transfected cells (NC) as a negative control. Detection was done as described in **B.**

Materials and methods

Monoclonal anti-ADAMTS13 antibodies

We used monoclonal anti-ADAMTS13 antibodies: 3H9 (anti-metalloprotease domain) [27-31], 19H4 (anti-thrombospondin type-1 repeat 8 domain) [27-30], 17G2 (anti-CUB1 domain) [27-30], and 3E4 [32], 15D1 [32], I-9 [33] and II-1 (all anti-spacer domain) [33]. See **Supporting methods** for details.

Generation of full-length wild type (WT) ADAMTS13 and spacer hybrids

Construction of expression vectors: To epitope map anti-spacer autoantibodies in iTTP patient plasma, we generated full-length WT ADAMTS13 and a library of 16 spacer hybrids where specific amino acid regions of the spacer domain of ADAMTS13 were exchanged by the corresponding regions of the spacer domain of ADAMTS1 [21]. (**Fig. 1A).** See **Supporting methods** for details.

Expression and concentration determination: Full-length WT ADAMTS13 and spacer hybrids were expressed in CHOEBNALT85 cells (European Patent EP1851319B1, Icosagen Cell factory OÜ, Kambja, Estonia), analyzed by Western blot and the concentration quantified in cell culture medium using our in-house developed ADAMTS13 antigen ELISA [27, 29, 30]. See **Supporting methods** for details.

Binding of monoclonal antibodies to full-length WT ADAMTS13 and the spacer hybrids.

General folding of full-length WT ADAMTS13 and the spacer hybrids was analyzed using monoclonal anti-spacer antibodies. See **Supporting methods** for details.

Plasma samples

In this study, 384 plasma samples from 244 iTTP patients in acute and/or remission phase obtained from the TTP reference centers in Marseille, Budapest, Mainz, and Paris,

as well as 418 samples from healthy donors from the Blood Service of the Belgian Red Cross-Flanders were used. Informed consent was obtained according to the Declaration of Helsinki. See **Supporting methods** for details.

Epitope mapping of anti-ADAMTS13 spacer autoantibodies in the plasma of iTTP patients

To identify which spacer domain regions were targeted by anti-ADAMTS13 autoantibodies (see **Supporting methods** for anti-ADAMTS13 autoantibody determination) of iTTP patients, an ELISA was developed. Anti-ADAMS13 antibody 3H9 (5 µg/mL) was coated on a 96-well plate to capture full-length WT ADAMTS13 and spacer hybrids (100 ng/mL) in culture medium. Next, plasma (diluted 1:40) or human monoclonal antibody II-1 (0.05 µg/mL, used as a reference) were added. Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-human immunoglobulin G (Fc specific; 1: 80,000; Sigma-Aldrich, St Louis, MO). *O*-phenylenediamine dihydrochloride and H_2O_2 compromised the substrate for colorimetric development. The reaction was stopped with 4 M $H_2SO_{4,}$ and the absorbance was measured at 492 nm. Optical density (OD) value for the binding of antibody II-1 (0.05 µg/mL) to WT ADAMTS13 was set as 1 on each plate. All other OD values were first normalized to this value, to compare data between different ELISA plates. Afterwards, the ratio of the OD corresponding to the binding of patient autoantibodies to the hybrids, to the OD corresponding to the binding of patient autoantibodies to WT ADAMTS13 was made (hybrid/ADAMTS13 ratio). Fifteen iTTP patient samples (Marseille cohort) were used in quadruplicates and a statistical analysis was performed to calculate the value that determines the presence of anti-spacer autoantibodies recognizing a specific hybrid.

Statistics

Statistical analysis was performed using GraphPad Prism v5 (GraphPad Software, San Diego, CA) and IBM SPSS statistics, v19 (IBM Corp., Armonk, NY). See **Supporting methods** for details.

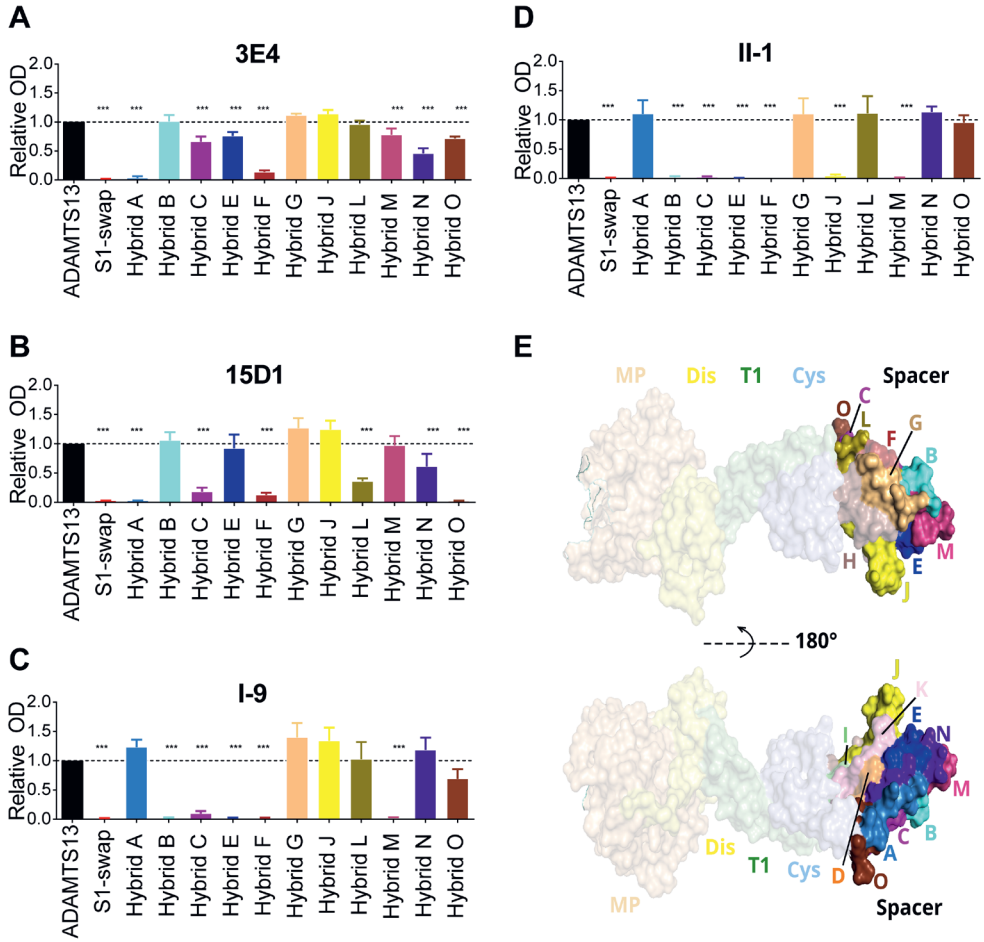

Figure 2 – Binding of monoclonal anti-ADAMTS13 antibodies recognizing conformational epitopes to secreted full-length spacer hybrids.

A-D. Binding of the murine monoclonal anti-spacer antibodies, A. 3E4, B. 15D1 and human monoclonal anti-spacer antibodies C. I-9 and D. II-1 to full-length WT ADAMTS13 and spacer hybrids A, B, C, E, F, G, J, L, M, N, O, S1-swap. The mouse monoclonal anti- metalloprotease domain antibody 3H9 was used to capture full-length WT ADAMTS13 or the spacer hybrids. Next, biotinylated mouse monoclonal anti-spacer antibodies (3E4 and 15D1) or human monoclonal anti-spacer antibodies (I-9 and II-1) were added. Bound murine or human anti-ADAMTS13 antibodies were detected using HRP conjugated streptavidin or HRP conjugated goat anti-human IgG Fc specific antibody, respectively. Data are expressed as relative OD to the binding of WT ADAMTS13. Differences were statistically analyzed using one-way ANOVA with Tukey's post-hoc test (***p-value ≤0.001, n=3). Data are expressed as mean ± SD. **E.** Structure of MDTCS in which the regions that were exchanged in the full-length spacer hybrid are highlighted. The crystal structure of MDTCS available at the RCSB Protein Data Bank (PDB) under code 6QIG was used as a template to depict the regions exchanged per full-length spacer hybrid using PyMOL Molecular Graphics System, Version 2.0 (Schrödinger, LLC). The MDTC domains are depicted in pale colors; metalloprotease (MP) domain in wheat, disintegrin-like (Dis) domain in yellow, thrombospondin type-1 repeat (T1) domain in green, and cysteine-rich (Cys) domain in light blue. The regions corresponding to the hybrids

used for epitope mapping are depicted in bright colors: hybrid A in sky-blue (556–563), hybrid B in cyan (564–571), hybrid C in magenta (572–579), hybrid E in blue (588-592), hybrid G in light orange (602–610), hybrid J in yellow (629–638), hybrid L in olive (649-656), hybrid M in warm pink (657–666), Hybrid N in purple-blue (667-676), and hybrid O in chocolate (677-685). The regions corresponding to the hybrids not used for epitope mapping are depicted in pale colors: hybrid D in orange (580-587), hybrid F in firebrick (593-601), hybrid H in dark salmon (611-620), hybrid I in lime (621-628), and hybrid K in pink (639-648). The structure is shown in front and back view.

Results

Construction and expression of full-length WT ADAMTS13 and spacer hybrids

To allow in-depth anti-spacer autoantibody profiling in iTTP patients, we constructed full-length WT ADAMTS13 and 16 spacer hybrids and expressed them in CHOEBNALT85 cells (**Fig. 1A**). The presence of the hybrid protein in the expression media was analyzed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (**Fig. 1B**). An immunoreactive band of WT ADAMTS13 and 12 of the 16 hybrids (hybrid A, B, C, E, F, G, J, L, M, N, O, S1-swap) was observed in the expression media (**Fig. 1B**), showing that those constructs were expressed and secreted into the medium. In contrast, for 4 of the 16 hybrids (hybrid D, H, I and K) almost no protein was detected in the expression media (**Fig. 1B**). Analysis of the cell lysates of hybrids D, H, I and K showed that the hybrids were expressed (**Fig. 1C**), but were not efficiently secreted into expression medium, possibly due to aberrant protein folding. Therefore, only the 12 secreted full-length spacer hybrids (hybrid A, B, C, E, F, G, J, L, M, N, O, S1-swap) could be used for epitope mapping of the anti-ADAMTS13 autoantibodies in iTTP patient plasma (see below).

General folding of the secreted full-length spacer hybrids

Swapping regions of the ADAMTS13 spacer domain by a region of the ADAMTS1 spacer domain could influence the overall folding of the spacer domain. Therefore, we studied the binding of 4 selected monoclonal antibodies to full-length WT ADAMTS13 and the 12 secreted spacer hybrids. The 4 selected monoclonal antibodies were 2 mouse, 3E4 and 15D1, and 2 human, I-9 and II-1, anti-spacer antibodies that all recognize a conformational epitope in the spacer domain, the epitopes of 3E4, 15D1 and I-9 do not overlap, while the epitope of I-9 and II-1 do overlap. Hence, we considered the epitopes of 3E4, 15D1 and I-9/II-1 to be sufficiently spread over the spacer domain to study the folding of the hybrids. If at least 1 of the 4 antibodies bound well to the hybrid, the hybrid was considered well folded. If no antibodies bound, the hybrid was considered misfolded.

ADAMTS13 spacer hybrids were captured using the mouse monoclonal anti-

metalloprotease domain antibody 3H9 and binding of all 4 monoclonal antibodies (3E4, 15D1, I-9 and II-1) to the 12 secreted full-length spacer hybrids was assessed. As shown in **Fig. 2 A-D**, hybrids A, B, C, E, G, J, L, M, N and O, were properly folded as at least one monoclonal antibody bound well to these hybrids. In contrast, hybrid F was not correctly folded as it was not recognized by any of the monoclonal antibodies (**Fig. 2 A-D**). Therefore, hybrids A, B, C, E, G, J, L, M, N and O were used for epitope mapping of anti-spacer autoantibodies in iTTP plasma (see below). The location of the full-length spacer hybrids A, B, C, E, G, J, L, M, N and O in the spacer domain on the three-dimensional structure of the MDTCS (metalloprotease through the spacer domain of ADAMTS13) is given in **Fig. 2E.**

Identification of anti-spacer autoantibody profiles in iTTP patients

The anti-spacer autoantibody profile was determined using an ELISA where the full-length spacer hybrids A, B, C, E, G, J, L, M, N or O were captured on the mouse monoclonal anti-metalloprotease domain antibody 3H9. Of note, capturing ADAMTS13 on 3H9 ensures an open ADAMTS13 conformation with the cryptic domains in the spacer domain exposed [34]. In this way, the conformation of ADAMTS13 captured on 3H9 is similar to the conformation of directly coated ADAMTS13. ELISA's using directly coated ADAMTS13 are used in clinical laboratories to study the presence of anti-ADAMTS13 autoantibodies in iTTP patients [35]. The availability of open ADAMTS13 in both assays is crucial as it is known that patient autoantibodies recognize cryptic residues in the spacer domain [29, 34].

The binding of autoantibodies in patient plasma to spacer hybrids A, B, C, E, G, J, L, M, N or O was tested and compared to binding to WT ADAMTS13. When the hybrid/ADAMTS13 ratio was <0.75, the plasma samples contained autoantibodies against the original spacer sequence that was swapped in the full-length spacer hybrid (see **Supporting data** for validation of the ELISA and determination of this threshold and **Supporting Fig. 1**). Hence, we analyzed the anti-spacer autoantibody profile in a large cohort of iTTP patient plasma samples selected to have anti-spacer autoantibodies (n=138, see **Supporting data** for the selection of the iTTP plasma samples used in the epitope mapping study and **Supporting Fig. 2, Supporting Table 2**). Data analysis of the anti-spacer autoantibody profiles in the 138 selected plasma samples (102 in the acute phase, 36 in remission) (**Supporting Table 2**), showed that the plasma samples could be grouped in 16 different anti-spacer autoantibody profiles (in 6 plasmas of the 138 samples, no anti-spacer autoantibody profile could be determined because the overall anti-spacer autoantibody titer was too low) (**Fig. 3A**, **Supporting Table 2**). Of these 16 profiles, seven profiles were represented well in our cohort being present in 89% (118/132) of the samples (**Fig. 3A**, **Supporting Table 2**). **Fig. 3B** shows the 16 different anti-spacer autoantibody profiles in the crystal structure of MDTCS highlighting the different regions in the spacer domain that are recognized by anti-spacer autoantibodies.

A

PROFILE	iTTP		Hybrid									
	Number of acute phase patients (number of samples)	Number of remission phase patients (number of samples)	A	B	C	E	G	J	L	M	N	O
1	19 (19)	8 (9)										
2	20 (21)	5 (7)										
3	13 (13)	4 (5)										
4	13 (13)	4 (4)										
5	6 (6)	4 (4)										
6	7 (7)	2 (2)										
7	8 (8)											
8	2 (2)	1 (1)										
9	1 (1)	1 (2)										
10	1 (1)	1 (1)										
11	1 (1)											
12	1 (1)											
13	1 (1)											
14	1 (1)											
15	1 (1)											
16	1 (1)											
undefined	5 (5)	1 (1)										

B

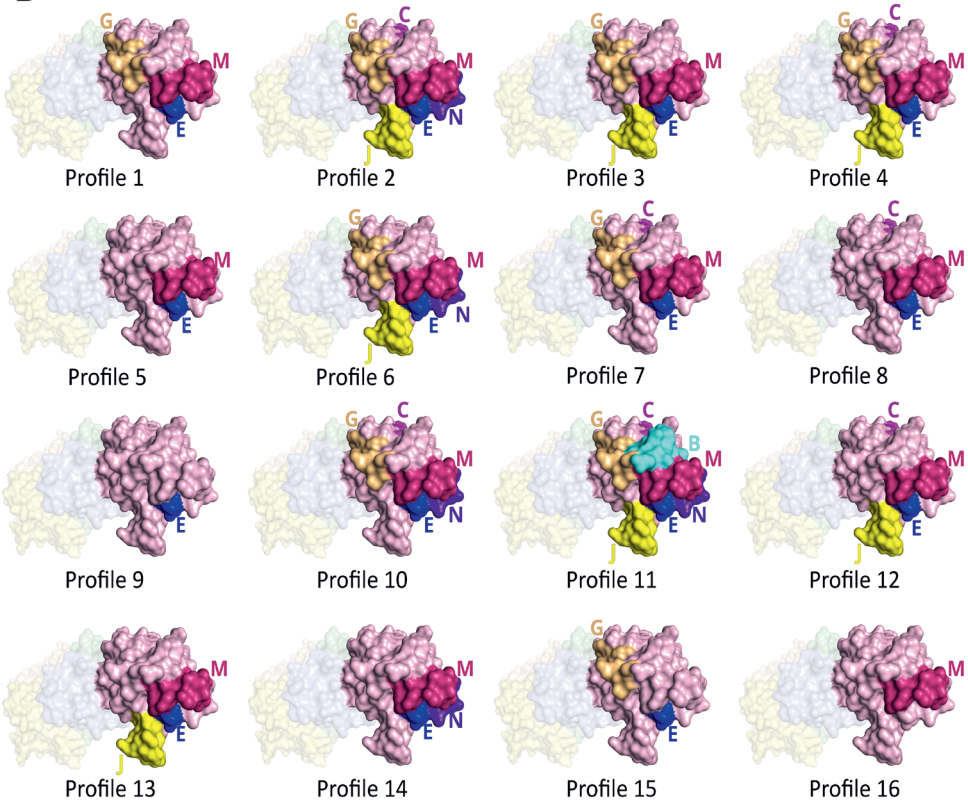

Figure 3 – Anti-spacer autoantibody profiles in iTTP patients.

A. Schematic overview of the anti-spacer autoantibody profiles identified in iTTP patients in the acute and remission phase. Since some patients have samples taken during follow up visits, the number of samples with a certain profile is shown in brackets. Profiles were determined by epitope mapping of anti-spacer ADAMTS13 autoantibodies using an ELISA where full-length WT ADAMTS13 spacer hybrids were captured by mouse monoclonal anti-metalloprotease domain antibody 3H9. Next, plasma samples were added. Bound human anti-ADAMTS13 autoantibodies were detected using HRP conjugated goat anti-human IgG Fc specific antibody. The binding of the anti-ADAMTS13 autoantibodies to the full-length spacer hybrids was calculated relative to the binding to WT ADAMTS13 (hybrid/ADAMTS13 ratio). The amino acid regions which are involved in the anti-spacer autoantibody profile are depicted in green (hybrid/ADAMTS13 ratio <0.75). In contrast, the regions which are not involved are represented in red (hybrid/ADAMTS13 ratio ≥0.75). **B.** The structure MDTCS (PDB ID: 6QIG) showing the 16 identified anti-spacer ADAMTS13 autoantibody profiles found in the screened iTTP plasma samples. The MDTC domains are depicted in pale colors; metalloprotease (MP) domain in wheat, disintegrin-like (Dis) domain in yellow, thrombospondin type-1 repeat (T1) domain in green, and cysteine-rich (Cys) domain in light blue. Bright colors are used to show the spacer domain in light pink as well as the full-length hybrids containing regions part of the epitope of anti-ADAMTS13 spacer antibodies: hybrid B in cyan (564–571), hybrid C in magenta (572–579), hybrid E in blue (588-592), hybrid G in light orange (602–610), hybrid J in yellow (629–638), hybrid M in warm pink (657–666) and hybrid N in purple-blue (667-676).

Finally, we could study if anti-spacer immunoprofiles changed during disease course in 5 iTTP patients as at least three follow-up samples were available in these patients (patient HUN1003, HUN113, HUN280, HUN31, and HUN198; **Supporting Table 3**). In general, these data showed that there were no major changes in anti-spacer immunoprofiles during the disease course.

Anti-spacer autoantibody profiles are not associated with disease severity

To investigate whether the identified anti-spacer autoantibody profiles (**Fig. 3**, **Supporting Table 2**) are associated with disease severity (**Supporting Fig. 3**), parameters of disease outcome and treatment were analyzed as previously described [36]. Disease outcome was measured considering the Benhamou score [37]. The Benhamou score is a risk score for early death in TTP and encompasses three factors related to clinical and biological presentation (age, high lactate dehydrogenase level (LDH; >2500 IU) and cerebral involvement) [37]. This score ranges between 1 and 4 in which a score ≥3 has a positive predictive value for mortality (patients at risk for 30-day mortality after treatment initiation), while a score <3 has a negative predictive value [37]. The association study between the Benhamou score, or its individual parameters (age, LDH levels and cerebral involvement), or treatment, and anti-spacer autoantibody profile was performed on 43 acute TTP patients from the TTP Reference Center in Paris since detailed information on these laboratory and clinical outcome parameters were available from this cohort. Information on age, cerebral involvement and treatment were available for 4 additional patients of the French cohort and hence they were included in the association study between age, LDH or treatment and the anti-spacer autoantibody profile (n=47). Finally, the anti-spacer autoantibody profiles 1 to 7 (**Fig. 3**) were included

in the analysis as each of those profiles were detected in more than 2 patients.

The statistical analysis showed no significant differences between anti-spacer autoantibody profile and Benhamou score (Fisher's exact test, *p-value*=0.59) (**Supporting Fig. 3A**), and no significant differences for the individual parameters included in the Benhamou score (age: One-way ANOVA, *p*-value=0.31; LDH level: Fisher's exact test, *p*-value=0.38; and cerebral involvement: Fisher's exact test, *p*-value=0.59) (**Supporting Fig. 3 B-D**), indicating that anti-spacer autoantibody profile is not associated with disease outcome. We also analyzed the possible association between anti-spacer autoantibody profile in patients treated with plasma exchange without rituximab or in combination with rituximab (with/without rituximab) and patients treated with plasma exchange without rituximab or in combination with rituximab together with additional treatment(s) (like steroids or other immunosuppressive drugs (like cyclophosphamide and bortezomib); and/or splenectomy; and/or caplacizumab). However, also here, no significant differences were observed (Fisher's exact test, *p*-value=0.64) (**Supporting Fig. 3E**). These results indicate that the anti-spacer autoantibody profile is not associated with disease severity.

The spacer domain contains three hotspots (on amino acid regions 588-592, 602-610, 657-666) targeted by anti-spacer autoantibodies

The 16 different anti-spacer autoantibody profiles showed that the majority of the plasma samples contained anti-spacer autoantibodies targeting three regions in the spacer domain. From the 132 epitope mapped samples, 131 showed a reduced binding towards hybrid E (99%), 112 towards hybrid G (85%), and 128 towards hybrid M (97%), in which amino acid regions 588-592, 602-610, and 657-666 were changed, respectively. In effect, those regions contain epitope hotspots for anti-ADAMTS13 autoantibodies in the spacer domain (**Fig. 4A**). Anti-spacer autoantibodies targeting the regions 572-579 (hybrid C), 629-638 (hybrid J) and 667-676 (hybrid N) were less dominant, since only 45% (60/132), 57% (75/132) and 31% (41/132) of the samples showed a reduced binding towards hybrids C, J and N, respectively (**Fig. 4A**). Interestingly, none of the samples showed a reduced binding towards hybrid A (556-563), hybrid L (649-656) and hybrid O (677-685) (**Fig. 4A**), while only 1 patient showed a reduced binding towards hybrid B (564-571), showing that those regions do not seem to play a role in anti-spacer autoantibody binding. The regions mainly (hybrids E, G and M), moderately (hybrids C, J and N) and not involved (hybrids A, B, L and O) in anti-spacer autoantibody binding were visualized in the MDTCS crystal structure (**Fig. 4 B-D**). **Fig. 4B** shows that the three hotspots are located closely together, while the other regions are distributed along the spacer domain.

In summary, anti-spacer autoantibody mapping in a multicenter iTTP patient cohort identified three hotspots (588-592, 602-610 and 657-666) in the spacer domain (exchanged in hybrids E, G and M), since almost all patients have anti-spacer

autoantibodies binding to those regions.

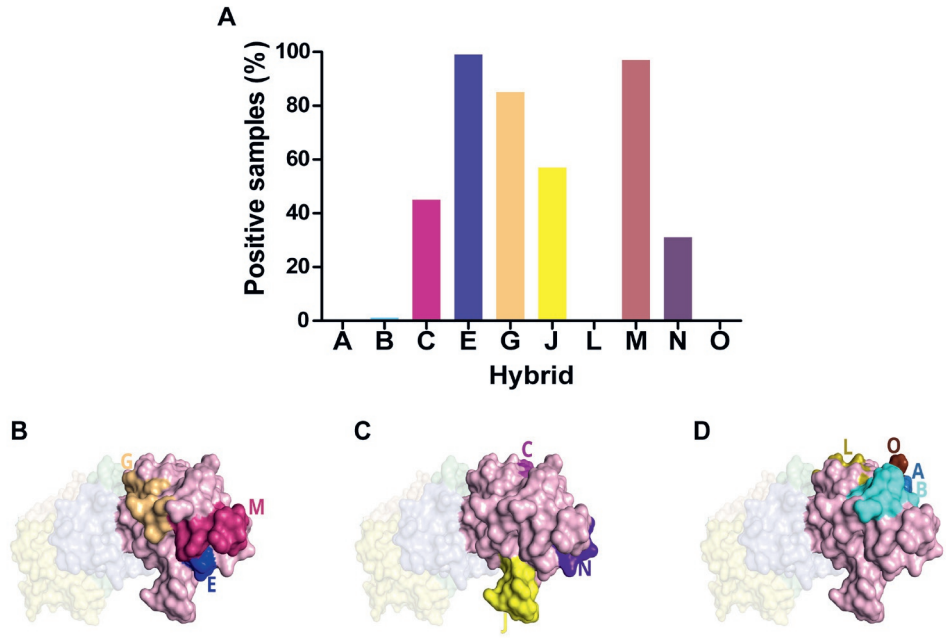

Figure 4 - Regions exchanged in hybrid E, G and M are hotspots for anti-spacer autoantibody binding.

A. Bar graph indicating the percentage of samples with anti-spacer autoantibodies targeting the exchanged regions per full-length spacer hybrid. **B-D.** Structure of MDTCS (PDB ID: 6QIG) showing the regions in the spacer domain targeted by autoantibodies. The MDTC domains are depicted in pale colors; metalloprotease (MP) domain in wheat, disintegrin-like (Dis) domain in yellow, thrombospondin type-1 repeat (T1) domain in green, and cysteine-rich (Cys) domain in light blue. The spacer domain is indicated in light pink with the hybrids: **B.** mainly involved: hybrid E in blue (588-592), hybrid G in light orange (602–610), and hybrid M in warm pink (657–666); C. moderately involved: hybrid C in magenta (572–579), hybrid J in yellow (629–638), and hybrid N in purple-blue (667-676); or **D.** not involved: hybrid A in sky-blue (556–563), hybrid B in cyan (564–563), hybrid L in olive (649-656), and hybrid O in chocolate (677-685) in anti-ADAMTS13 autoantibody binding.

Discussion

In this study, we performed a large multicenter in-depth epitope mapping of anti-spacer autoantibodies present in plasma samples from patients during acute iTTP and during remission using full-length ADAMTS13 spacer hybrids. By using those full-length ADAMTS13 spacer hybrids, we analyzed 132 plasma samples and could identify 16 different anti-spacer autoantibody profiles. Each profile represented a specific set of hybrids to which anti-spacer autoantibodies bound. Seven profiles were represented

well in our cohort as they were found in 89% of the samples (118/132). None of the seven profiles could be linked with disease severity. An overall analysis of the 16 different anti-spacer autoantibody profiles showed that regions 588-592 (hybrid E), 602-610 (hybrid G) and 657-666 (hybrid M) are hotspots in the spacer domain of ADAMTS13 since they are part of the epitopes of anti-spacer autoantibodies in almost all samples (85%-99%). Also, other regions 572-579, 629-638, 667-676 (hybrids C, J and N) were targeted by anti-spacer autoantibodies but not in all samples (31%-57%). In contrast, other regions 556-563, 564-571, 649-656, 677-685 (hybrid A, hybrid B, hybrid L and hybrid O) do not seem to be involved in anti-spacer autoantibody binding.

We constructed 16 full-length spacer hybrids in full-length ADAMTS13, similar to the 16 spacer hybrids that have previously been expressed in the DTCS fragment by Luken et al. [21] In both studies hybrids A, B, C, E, G, J, L, M, N, O and S1-swap were well secreted and recognized by anti-spacer antibodies. However, changing small fragments of the ADAMTS13 domains can affect the secretion and/or folding of ADAMTS13. In the current study hybrids D, H, I and K were not secreted and hybrid F was not properly folded as anti-spacer antibodies did not recognize hybrid F; consequently, hybrid D, F, H, I and K were excluded for further analysis. Expression of those hybrids was also affected in the study of Luken et al. [21].

Luken et al. [21] were the first to investigate the epitopes of anti-ADAMTS13 autoantibodies targeting the spacer domain in a small patient cohort (6 iTTP patients) using the ADAMTS13 fragment, DTCS (and not full-length ADAMTS13 like we used in our study) (**Supporting Fig. 4**). They found that all 6 patients had anti-ADAMTS13 autoantibodies against regions 572-579 (hybrid C) and 657-666 (hybrid M), while 5/6 (83%) patients had anti-spacer autoantibodies recognizing region 602-610 (hybrid G) and region 629-638 (hybrid J) (**Supporting Fig. 4**). In our large cohort study, the regions 602-610 (hybrid G) and 657-666 (hybrid M) seem to have a prominent role in anti-spacer autoantibody binding, while the regions 572-579 (hybrid C) and 629-638 (hybrid J) only seem to be moderately involved (**Supporting Fig. 4**). In both studies region 667-676 (hybrid N) had a moderate role in anti-spacer autoantibody binding (31-50%, respectively), while regions 556-563 (hybrid A), 564-571 (hybrid B), 649-656 (hybrid L) and 677-685 (hybrid O) were not important in anti-ADAMTS13 autoantibody binding since none or only a few samples showed a reduced binding towards these hybrids (**Supporting Fig. 4**) [21]. This shows that our findings are largely in agreement with previous reports [19-22]. Hence, our study revealed three hotspots (588-592, 602-610 and 657-666) in the spacer domain (exchanged in hybrids E, G and M) involved in anti-spacer autoantibody binding of iTTP patients (**Supporting Fig. 4**) [19, 21].

Studies searching for specific residues involved in anti-spacer autoantibody binding identified the residues $R^{568}F^{592}R^{660}Y^{661}Y^{665}$, located in hybrids B, E and M, as an immunogenic exosite in the spacer domain after testing at the most 48 patients [15, 16,

21, 23, 24, 38-40]. Since those residues seem to have a major role in anti-ADAMTS13 autoantibody binding, modifications of these residues are under investigation to create ADAMTS13 spacer variants which are active, but with reduced binding to anti-ADAMTS13 autoantibodies as a potential therapy for iTTP patients [15, 16, 39]. Although, this is a promising therapy, a thorough knowledge about the epitopes of anti-ADAMTS13 autoantibodies is crucial to know which patients are eligible for this therapy. This knowledge can now be provided by our assay. To be eligible for this novel therapy, patients should have anti-spacer autoantibodies, in particular against regions 564-571, 588-592, 657-666 (hybrids B, E and M). Therefore, we speculate that patients with anti-spacer autoantibody profile 5, 9 and 16 (**Fig. 3A**) could benefit from the administration of previously published gain of function ADAMTS13 variants [15-16]. It would be interesting to investigate whether patients with anti-spacer autoantibodies outside hybrid B, E and M would also efficiently benefit from this potential novel therapy. It should be mentioned that not all patients have only anti-spacer autoantibodies but also have antibodies against other domains in ADAMTS13 [6, 17, 19, 24, 41]. Also our study showed that 67% of the samples had anti-spacer autoantibodies, this finding was confirmed in another study in which the plasma samples were epitope mapped using ADAMTS13 fragments MDTCS, M, DT, CS, T2-C2, T2-T5 T6-T8 CUB1-2 (Kangro et al., manuscript in preparation).

Although a link between autoantibody profiles and disease severity has been demonstrated in other autoimmune diseases [25, 26], Thomas et al. could not find a link between disease severity and domain specificity of anti-ADAMTS13 autoantibodies in iTTP [6]. In line with that, focusing on only the anti-spacer autoantibodies, we could also not identify a link between the anti-spacer autoantibody profiles and disease severity in terms of disease outcome and treatment.

Two human monoclonal anti-ADAMTS13 autoantibodies II-1 and I-9, previously cloned from iTTP patients [33], have been analyzed for their binding towards our full-length spacer hybrids. Alanine scanning has previously been used to determine the epitope of those 2 antibodies to be $R^{568}F^{592}R^{660}Y^{661}Y^{665}$ and $R^{568}R^{660}Y^{661}Y^{665}$, respectively [24]. As expected a reduced binding towards hybrids B, E and M (regions 564-571, 588-592 and 657-666, respectively) was seen for II-1, while for I-9 no binding towards hybrid B and M (regions 564-571 and 657-666, respectively) was observed. However, in the current study binding was also abrogated towards hybrids C and J (regions 572-579 and 629-638, respectively) for antibody II-1 and towards hybrids C and E (regions 572-579 and 588-592, respectively) for antibody I-9, indicating that additional amino acids might also be involved in the epitope of those two cloned human monoclonal antibodies.

In summary, the current study provides an extensive investigation of antigenic regions in the spacer domain. We developed a robust ELISA for epitope mapping of anti-spacer autoantibodies. Using this assay, we have shown that 67% of the iTTP

samples from acute and remission phase with anti-ADAMTS13 autoantibodies have antibodies targeting the spacer domain. Additionally, we identified three hotspots for anti-ADAMTS13 autoantibody binding comprising the amino acid regions 588-592 (hybrid E), 602-610 (hybrid G) and 657-666 (hybrid M). However, depending on the patient, other regions in and outside the spacer domain can also be involved in anti-ADAMTS13 autoantibody binding.

Authorship Contributions

L.C. Velasquez Pereira designed and performed the experiments, analyzed and interpreted the data, and wrote the manuscript; E. Roose analyzed and interpreted the data and wrote the manuscript; N.A.G. Graça provided the pUC57-Mut1.1 and pQMCF3-ADAMTS13-V5-His; K. Kangro expressed further full-length spacer hybrids; G. Sinkovits, B.S. Joly, E. Tellier, G. Kaplanski, T. Falter, C. Von Auer, H. Rossmann, H.B. Feys, M. Reti, Z. Prohászka, B. Lämmle, P. Coppo, and A. Veyradier provided the plasma samples; J. Voorberg provided pMIB-DTCS plasmids containing the spacer hybrids; A. Männik designed the experiments, analyzed and interpreted the data, and critically reviewed the manuscript; N.A.G. Graça, G. Sinkovits, K. Kangro, B.S. Joly, E. Tellier, G. Kaplanski, T. Falter, C. Von Auer, H. Rossmann, H.B. Feys, M. Reti, Z. Prohászka, B. Lämmle, J. Voorberg, P. Coppo, A. Veyradier, and S.F. De Meyer critically reviewed the manuscript; K. Vanhoorelbeke designed the experiments, analyzed and interpreted the data, wrote the manuscript and provided funding.

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Disclosure of Conflict of Interests

L.C. Velasquez Pereira, E. Roose, N.A.G. Graça, G. Sinkovits, K. Kangro, B.S. Joly, E. Tellier, G. Kaplanski, T. Falter, C. Von Auer, H. Rossmann, H.B. Feys, M. Reti, Z. Prohászka, J. Voorberg, S.F. De Meyer, A. Männik
do not have any conflict of interest to declare. B. Lämmle is chairman of the Data Monitoring Board for studies of recombinant ADAMTS13 in congenital and acquired TTP (Shire-Takeda 281102; SHP655-201), is a member of the Advisory Board of Ablynx-Sanofi for caplacizumab and received congress travel support and/or lecture fees

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Supporting Information

Supporting methods

Monoclonal anti-ADAMTS13 antibodies

The following in-house developed mouse monoclonal anti-ADAMTS13 antibodies were used in this study: 3H9 [1-5], 19H4 [1-4], 17G2 [1-4], 3E4 [5], 15D1 [5]. The antibody 3H9 is directed against the metalloprotease domain [6] and is a good antibody to capture ADAMTS13 from expression medium or plasma in ELISA [1, 3, 4]. The antibodies 19H4 and 17G2 target the T8 and CUB1 domain and are used to detect ADAMTS13 [1, 3, 4]. The antibody 3E4 binds to the spacer domain of ADAMTS13 and increases ADAMTS13 activity, while antibody 15D1 also binds to the spacer domain but does not influence ADAMTS13 activity [5].

Human monoclonal anti-ADAMTS13 autoantibodies I-9 and II-1 were isolated from iTTP patients and inhibit ADAMTS13 activity [7]. They both bind to the spacer domain of ADAMTS13, recognizing the residues $R^{568}R^{660}Y^{661}Y^{665}$. Antibody II- 1 also targets F^{592} [8].

Generation of full-length wild type (WT) ADAMTS13 and spacer hybrids

Construction of expression vectors containing full-length WT ADAMTS13 and spacer hybrid sequences: The full-length WT ADAMTS13 was constructed as previously described [8-10]. In addition, a library of 16 full-length spacer hybrids was created to fine map the epitopes of anti-spacer autoantibodies (**Fig. 1A**): 15 hybrids (named A-O), in which 5-10 amino acids of the spacer domain of ADAMTS13 were exchanged by the corresponding regions of the spacer domain of ADAMTS1 and one hybrid in which the complete spacer domain of ADAMTS13 was exchanged by the one of ADAMTS1 (S1-swap). The exchanged regions are identical to the ones exchanged in the DTCS spacer hybrids of Luken et al. [9] The cloning strategy to introduce the spacer hybrid sequences into the pQMCF3 mammalian expression vector containing the full-length WT ADAMTS13 sequence was performed using a 3-step cloning process: i) amplification of the region containing the spacer hybrid (hybrid A-N) from the pMIB-DTCS [9] vector (ADAMTS13 fragment disintegrin-like through spacer domain) and ordering synthetic sequences encoding for hybrid O or S1-swap, ii) digestion and ligation of the region containing the spacer hybrids into an intermediate vector (pUC57-Mut1.1) [10], and iii) digestion and ligation of the region containing the spacer hybrids into the expression vector (pQMCF3-ADAMTS13-V5-His). The first step corresponding to the amplification of the region of interest from the pMIB-DTCS plasmids [9] for hybrids A to N was done through PCR using specific primers (**Supporting Table 1**). For hybrid O and S1-swap synthetic sequences were ordered (Genewiz, Essex, UK); hybrid O a sequence encompassing nucleotides 1731 to 2184 of WT ADAMTS13 coding for the

fragment of ADAMTS13 that range over the spacer to the thrombospondin type-1 repeat 2 domain (p.V583-P734; pUC57-DTCS-hybrid O) while for the S1-swap a sequence encompassing nucleotides 1522 to 2184 of WT ADAMTS13 coding for the fragment of ADAMTS13 from the cysteine-rich to the thrombospondin type-1 repeat 2 domain (p.R514-V734; pUC57-DTCS-S1-swap). Next, the PCR products from hybrids A, B, C and D and the pUC57-Mut1.1 vector [10] were digested with the restriction enzymes *AvaI* and *HindIII*, and were ligated together. While for hybrids E, F, G, H, I, J, K, L, M and N, PCR products were digested with *AccI* and *HindIII* and ligated in the pUC57-Mut1.1 vector. Hybrids O and the S1-swap synthetic fragments were digested with the restriction enzymes *AccI* and *MscI*, and *AvaI* and *MscI*, respectively and ligated in the pUC57-Mut1.1 vector. Finally, after ligation of all hybrid sequences in the pUC57-Mut1.1 vector, the vector of each hybrid was digested with *Esp3I* and *PagI*, which cleave in the cysteine-rich and thrombospondin type-1 repeat 2 domain of ADAMTS13, respectively. Additionally, also the pQMCF3-ADAMTS13-V5-His vector was digested with *Esp3I* and *PagI* to introduce the hybrid spacer regions through ligation. All spacer modifications in ADAMTS13 were confirmed by restriction analyses and sequencing of the spacer domain (Institute of Genomics Core Facility, University of Tartu, Tartu, EE).

Expression of full-length WT ADAMTS13 and spacer hybrids: To produce full-length WT ADAMTS13 and spacer hybrids, WT ADAMTS13 and all 16 constructs (10 µg) were transiently transfected into 10 mL of suspension culture of the Chinese hamster ovary-derived cell line CHOEBNALT85 (1.5 x 10^7 cells; clone 1E9, European Patent EP1851319B1, Icosagen Cell factory OÜ, Kambja, Estonia) using chemical transfection Reagent 007 (Icosagen Cell Factory OÜ) [11]. The transfected cells were grown in BalanCD® Transfectory™ CHO (Irvine Scientific, Santa Ana, CA) medium supplemented with 6 mM Gibco® GlutaMAX™ Supplement (Life Technologies, Carlsbad, CA), and 1% Penicillin-Streptomycin (Life Technologies) on an orbital shaker platform at 37°C in the presence of 8% CO_2. Three days after transfection, to increase protein expression, the temperature was shifted to 30°C, and BalanCD® CHO Feed 4 (Irvine Scientific) was added at regular intervals of 3, 6 and 8 days. After twelve days of transfection, the cell culture medium was harvested and centrifuged (30 minutes, 1000 g, 15°C). To avoid proteolytic degradation of full-length WT ADAMTS13 or spacer hybrids, phenylmethylsulfonyl fluoride (PMSF; Carl Roth, Karlsruhe, DE) was added at a final concentration of 0.5 mM.

To investigate the expression of full-length WT ADAMTS13 and spacer hybrid recombinant proteins, cell culture media and cell lysates were analyzed through Western blot. Therefore, cell culture media and cell lysates of cells expressing the full-length spacer hybrids (10 µL) were loaded on an 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel under reducing conditions and analyzed via SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Next, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Burlington, MA) and the membranes were blocked with 4%

milk in PBS (phosphate buffered saline) for 1 hour at room temperature. After blocking, the mouse monoclonal anti-His tag antibody (1:2,500; GenScript, Piscataway, NJ) was added, followed by a horseradish peroxidase (HRP) conjugated goat anti-mouse Immunoglobulin G (IgG) antibody (1:8,000; Abcam, Cambridge, UK). As negative controls, cell culture medium and cell lysate from non-transfected CHOEBNALT85 cells were run in parallel. The blots were developed with 3, 3', 5, 5'-Tetramethylbenzidine solution III (TMB solution III) (Biopanda Diagnostics, Belfast, UK).

Determination of full-length WT ADAMTS13 and spacer hybrid concentrations: The in-house developed ADAMTS13 antigen ELISA (ADAMTS13: Ag) [1, 3, 4] was used to quantify the concentration of full-length WT ADAMTS13 and spacer hybrids. In brief, a 96-well microtiter plate was coated with the mouse monoclonal anti-ADAMTS13 antibody 3H9 (5 μg/mL) in PBS. Next, normal human plasma (NHP; 1:25 starting dilution) or cell culture medium containing full-length WT ADAMTS13 or spacer hybrid (1:2 or 1:12.8 starting dilution depending on the hybrid) was added in a 1.5 over 2.5 dilution series. Bound ADAMTS13 was detected using two biotinylated mouse monoclonal anti-ADAMTS13 antibodies 17G2 and 19H4 (1.5 μg/mL), followed by HRP conjugated streptavidin (1:10,000; Thermo Fisher Scientific, Merelbeke, Belgium). O-phenylenediamine dihydrochloride (OPD) and H_2O_2 were used as a substrate for colorimetric development. The reaction was stopped with 4 M H_2SO_4, and the absorbance was measured at 492 nm. To calculate ADAMTS13 concentrations, NHP was used as a reference and set as 1 μg/mL ADAMTS13 [4].

Binding of monoclonal antibodies to full-length WT ADAMTS13 and spacer hybrids

To evaluate the general folding of full-length WT ADAMTS13 and the spacer hybrids, an ELISA was performed using monoclonal anti-spacer antibodies. A 96-well microtiter plate was coated with the mouse monoclonal anti-ADAMTS13 antibody 3H9 (5 μg/mL). Next, cell culture medium containing full-length WT ADAMTS13 or spacer hybrids (100 ng/mL) was added, followed by addition of the biotinylated mouse monoclonal anti-ADAMTS13 antibodies 3E4, 15D1 (1 μg/mL) or human monoclonal anti-ADAMTS13 antibodies I-9 or II-1 (1 μg/mL). To detect bound murine or human anti-ADAMTS13 antibodies, HRP conjugated streptavidin (1:10,000; Thermo Fisher Scientific), or HRP conjugated goat anti-human IgG Fc specific (1:80,000; Sigma-Aldrich, St Louis, MO) were added, respectively. Colorimetric development and absorbance measurement were performed as described above. Binding of the monoclonal antibodies to the hybrids was expressed as relative OD values compared to the binding of the monoclonal antibody to WT ADAMTS13 set as 1.

Plasma samples

A total of 384 plasma samples from 244 iTTP patients from acute and remission

phase were obtained from the TTP reference centers in Marseille (n=19), Budapest (n=90), Mainz (n=156 samples) and Paris (n=119). Eighty-five samples from the Paris cohort, twenty-four samples from the Budapest cohort, and the samples from Marseille and Mainz have been described previously [10, 12-15], but data related to the epitope mapping of anti-spacer antibodies are reported here for the first time. All plasma samples from acute phase were collected before plasma therapy. Acute iTTP was defined as a thrombotic microangiopathy (TMA) [16] linked with a severe ADAMTS13 deficiency (activity <10% measured with the FRETS-VWF73 assay) [17, 18]. A sustained normalization of platelet count and lactate dehydrogenase (LDH) level for more than 30 days after cessation of plasma exchange defined remission.

Additionally, plasma samples from 418 non-remunerated healthy donors (HD), which were available from the Blood Service of the Belgian Red Cross-Flanders, were used to establish the cut-off for positivity of the anti-ADAMTS13 autoantibody assay (see below).

The study was approved by the ethical committees: Project National de Recherche Clinique 2007 (#2007/23; Marseille, France), Ethik-kommission, Landesärztekammer Rheinland-Pfalz (837.506.15(10274); Mainz, Germany), Ethics Committee on Human Clinical Research (263/PI/2011 (8361-1/2011-ECU); Budapest, Hungary), Hospital Pitié-Salpêtrière and Hospital Saint-Antoine (NTC00426686; Paris, France), and the Ethics Committee of the KU Leuven (file number 260220; Leuven, Belgium).

Determining the presence of anti-ADAMTS13 autoantibodies in patient plasma

To identify which plasma samples were positive for anti-ADAMTS13 autoantibodies, antibody 3H9 (5 µg/mL) was coated on a 96-well plate and full-length WT ADAMTS13 (100 ng/mL) in culture medium was added. Next, plasma of 418 healthy donors (diluted 1:40) or human monoclonal antibody II-1 (0.05 µg/mL, used as a reference) were added. Bound antibodies were detected with HRP conjugated goat anti-human (IgG Fc specific) (1:80,000; Sigma-Aldrich). OPD and H_2O_2 were used as a substrate for colorimetric development. The reaction was stopped with 4 M $H_2SO_{4,}$ and the absorbance was measured at 492 nm. Binding of anti-ADAMTS13 autoantibodies was expressed as relative OD to allow comparison of data between different ELISA plates. The relative OD was obtained by recalculating the obtained OD to the OD value of II-1 binding (0.05 µg/mL) to WT ADAMTS13, which was set as 1. The 97.5th percentile of the II-1 relative OD values obtained in the 418 healthy donor samples was set as a threshold for anti-ADAMTS13 autoantibody positivity.

Statistics

GraphPad Prism v5 software (GraphPad Software, San Diego, CA, USA) and IBM SPSS statistics, v19 (IBM Corp., Armonk, NY, USA) were used for statistical analysis.

One-way ANOVA with Tukey's post-hoc test was used to determine if the exchanged region in a full-length spacer hybrid was involved in the epitope of anti-ADAMTS13 antibodies. The exchanged region in the hybrid was considered part of the epitope of the anti-spacer antibodies if there was a statistically significant reduction of the antibody binding in the hybrid/ADAMTS13 ratio compared to WT ADAMTS13. To test whether the anti-spacer autoantibody profiles were linked with age one-way ANOVA was used, while the Fisher's exact test was performed to test for an association of the anti-spacer autoantibody profiles with the Benhamou score, LDH level, cerebral involvement and treatment. A p-value <0.05 was considered statistically significant for all analyses.

Supporting data

Validation of the ELISA for epitope mapping of anti-ADAMTS13 spacer autoantibodies in the plasma of iTTP patients

To validate the ELISA for epitope mapping of anti-ADAMTS13 spacer autoantibodies, plasma samples from 18 acute iTTP patients (Marseille cohort) were used. Anti-ADAMTS13 autoantibodies were detected in 15 of these 18 samples considering a cut-off of II-1 relative OD of 0.34. This value was established by determining the 97.5th percentile of anti-ADAMTS13 autoantibody levels measured in healthy donors (n=418) (data not shown). Next, the presence of anti-spacer autoantibodies binding to either region A, B, C, E, G, J, L, M, N or O in the spacer domain was studied by adding patient plasma to S1-swap and hybrids region A, B, C, E, G, J, L, M, N and O. Data were expressed as the hybrid/ADAMTS13 ratio and a ratio below a value of 0.75 indicates the presence of anti-spacer autoantibodies against the region exchanged in this hybrid (**Supporting Fig. 1**). This value was based on a statistical analysis of the binding of anti-ADAMTS13 autoantibodies in plasma samples of the 15 iTTP patients to WT ADAMTS13, S1-swap and hybrids A, B, C, E, G, J, L, M, N and O which were studied in quadruplicate (**Supporting Fig. 1**). Thus, from the 15 patients with anti-ADAMTS13 autoantibodies, 9 had anti-spacer antibodies (**Supporting Fig. 1**), since a statistically significant reduction of the S1-swap/ADAMTS13 ratio compared to WT ADAMTS13 (One-way ANOVA with Tukey's post-hoc test, p-value <0.05) was observed. Analyzing the binding of the 9 samples with anti-spacer autoantibodies to hybrids A, B, C, E, G, J, L, M, N and O (**Supporting Fig. 1**) showed that for example iTTP patient MF-KB-TTP02-A had anti-spacer autoantibodies with epitopes in regions E, G, J and M (p-value <0.05, **Supporting Fig. 1**). When large cohorts of patients are screened, there is usually not enough plasma available to perform repeats, and hence data analysis cannot be done based on statistics. However, the statistical analysis of the data of all 9 patients consequently allowed us to set the cut-off value for autoantibody positivity against the spacer domain and regions A, B, C, E, G, J, L, M, N or O in the

spacer domain at a hybrid/ADAMTS13 ratio of 0.75 (**Supporting Fig. 1**).

Plasma samples used for analyzing the anti-spacer autoantibody profile in iTTP patients

For this multicenter study, a total of 384 plasma samples were received from 232 iTTP patients in acute and remission phase of the disease (**Supporting Fig. 2A**). During the processing of the samples, ten were excluded because of a low plasma volume (n=4) or a high background (n=6) (**Supporting Fig. 2A**). Thus, 374 plasma samples were included in the analysis. Of those, 176 samples were obtained from iTTP patients in an acute phase, while 198 samples were from remission (**Supporting Fig. 2A**). Patient samples in acute phase all exhibited ADAMTS13 activity <10% and 144 of the 176 samples (82%) had detectable anti-ADAMTS13 autoantibodies. Among patient samples in clinical remission, anti-ADAMTS13 autoantibodies could only be detected in 61 of the 198 (31%) samples in which 51 samples still had an ADAMTS13 activity <10% and detectable anti-ADAMTS13 autoantibodies and 10 samples had an ADAMTS13 activity >10% in spite of detectable anti-ADAMTS13 autoantibodies (II-1 relative OD >0.34; **Supporting Fig. 2 A-B**). Additionally, 101 samples from iTTP patients in clinical remission had ADAMTS13 activity >10% and were negative for anti-ADAMTS13 autoantibodies while 36 samples had ADAMTS13 activity <10% and were negative for anti-ADAMTS13 autoantibodies. Anti-spacer autoantibodies were found in 71% (102/144) of the acute and 59% (36/61) of the remission samples (**Supporting Fig. 2 A, C**) since a reduced binding of anti-ADAMTS13 autoantibodies towards the hybrid S1-swap (S1-swap/ADAMTS13 ratio <0.75) was observed.

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Supporting Tables

Supporting Table 1. Primers used in PCR to amplify the region containing the spacer hybrid (hybrid A-N) from the pMIB-DTCS plasmid.

Plasmid name	Primer sequences	cDNA position
pMIB-DTCShybridA		
pMIB-DTCShybridB	Forward: 5'-TTCCTCGATGGGACCCGGTGTATG-3'	c.1488T
pMIB-DTCShybridC	Reverse: 5'-CGTGAAGCTTGCCGTGGCTTAGGCTGGAAGTAG-3'	c.2012C
pMIB-DTCShybridD		
pMIB-DTCShybridE		
pMIB-DTCShybridF		
pMIB-DTCShybridG		
pMIB-DTCShybridH		
pMIB-DTCShybridI	Forward: 5'-CAGTTACTGCTGGCAGAGCGAGAG-3'	c.1666C
pMIB-DTCShybridJ	Reverse: 5'-CGTGAAGCTTGCCGTGGCTTAGGCTGGAAGTAG-3'	c.2012C
pMIB-DTCShybridK		
pMIB-DTCShybridL		
pMIB-DTCShybridM		
pMIB-DTCShybridN		

Supporting Table 2. Detailed representation of all iTTP patient samples used for anti-spacer autoantibody epitope mapping. Anti-spacer autoantibody profile, sample identity (ID), patient ID and disease phase are shown. The presence of anti-ADAMTS13 autoantibodies (II-1 relative OD >0.34, green boxes) and anti-spacer autoantibodies (S1-swap/ADAMTS13 ratio <0.75, green boxes) and the amino acid regions involved (hybrid/ADAMTS13 ratio <0.75, green boxes) and not involved (hybrid/ADAMTS13 ratio ≥0.75, red boxes), in the anti-spacer autoantibody profile are depicted.

Profile	Sample ID	Patient	Phase	Anti-ADAMTS13 IgG (II-1 relative OD)	Anti-spacer IgG (S1-swap/ ADAMTS13 ratio)	Hybrid (hybrid/ADAMTS13 ratio)									
						A	B	C	E	G	J	L	M	N	O
1	ID_072	HUN1063	Acute	0.38	0.72	1.01	0.90	0.78	0.65	0.73	0.83	1.00	0.68	0.81	1.06
1	ID_011	HUN31	Acute	0.37	0.55	1.10	1.08	0.91	0.59	0.63	0.86	1.09	0.60	0.96	1.07
1	ID_014	HUN53	Acute	0.43	0.63	1.07	1.05	0.83	0.52	0.58	0.78	1.04	0.52	0.87	1.08
1	ID_061	HUN920	Acute	0.36	0.61	1.05	0.92	0.88	0.63	0.62	0.76	0.95	0.61	0.77	1.05
1	ID_316	LRB-BEL-201612-015	Acute	0.62	0.58	1.04	0.98	0.85	0.62	0.73	0.75	1.03	0.62	0.85	1.03
1	ID_324	LRB-BEL-201612-024	Acute	1.15	0.47	1.05	1.02	0.77	0.42	0.63	0.80	1.01	0.54	0.86	1.13
1	ID_333	LRB-BEL-201612-034	Acute	1.18	0.35	1.11	0.97	0.75	0.32	0.59	0.94	1.00	0.43	0.97	1.14
1	ID_335	LRB-BEL-201612-036	Acute	0.88	0.51	1.07	1.00	0.78	0.47	0.63	0.82	0.92	0.58	0.80	1.12
1	ID_341	LRB-BEL-201612-042	Acute	0.70	0.58	0.93	0.91	0.78	0.55	0.73	0.83	0.91	0.66	0.92	0.97
1	ID_361	LRB-BEL-201612-064	Acute	0.58	0.43	1.24	1.05	0.81	0.49	0.74	0.83	1.14	0.51	0.90	1.17
1	ID_363	LRB-BEL-201612-066	Acute	0.67	0.70	1.11	1.08	0.90	0.66	0.73	0.86	1.10	0.71	0.90	1.16
1	ID_366	LRB-BEL-201612-069	Acute	1.04	0.34	1.18	1.07	0.76	0.35	0.54	0.82	1.11	0.41	0.75	1.13
1	ID_384	LRB-BEL-201612-091	Acute	1.06	0.26	1.09	1.09	0.77	0.25	0.38	0.75	1.06	0.39	0.95	1.13
1	ID_399	LRB-BEL-201906-136	Acute	0.73	0.54	1.04	0.96	0.82	0.60	0.65	0.78	0.99	0.58	0.80	1.07
1	ID_400	LRB-BEL-201906-137	Acute	0.74	0.31	1.10	1.11	0.80	0.40	0.74	1.10	1.21	0.48	1.10	1.07
1	ID_408	LRB-BEL-201906-154	Acute	0.37	0.70	1.05	1.01	0.85	0.66	0.66	0.75	0.98	0.70	0.88	1.10
1	ID_411	LRB-BEL-201906-RL013	Acute	0.66	0.71	1.02	0.88	0.79	0.64	0.66	0.79	0.90	0.74	0.85	1.11
1	ID_422	LRB-BEL-201906-RL074	Acute	0.75	0.55	1.04	1.03	0.86	0.69	0.73	1.03	1.05	0.64	0.95	1.12
1	ID_295	MF-KB-TTP11-A	Acute	0.63	0.59	0.91	1.01	0.94	0.62	0.71	0.77	0.93	0.65	0.90	0.90
1	ID_080	HUN1849	Remission	0.40	0.56	1.03	0.86	0.79	0.64	0.68	0.81	1.02	0.68	0.82	0.97
1	ID_036	HUN198	Remission	0.77	0.61	0.97	0.94	0.80	0.66	0.69	0.86	1.00	0.67	0.92	0.98
1	ID_037	HUN198	Remission	0.71	0.51	0.95	0.86	0.75	0.53	0.60	0.82	0.95	0.61	0.92	1.01
1	ID_007	HUN31	Remission	0.41	0.70	0.98	0.95	0.88	0.72	0.71	0.80	0.94	0.71	0.80	0.97
1	ID_096	HUN453	Remission	0.35	0.69	1.06	0.97	0.82	0.66	0.72	0.82	0.98	0.67	0.86	1.04
1	ID_015	HUN65	Remission	0.36	0.68	0.99	0.94	0.81	0.71	0.73	0.83	1.00	0.70	0.78	1.01

Profile	Sample ID	Patient	Phase	Anti-ADAMTS13 IgG (II-1 relative OD)	Anti-spacer IgG (S1-swap/ ADAMTS13 ratio)	Hybrid (hybrid/ADAMTS13 ratio)									
						A	B	C	E	G	J	L	M	N	O
1	ID_053	HUN714	Remission	0.61	0.65	1.02	0.97	0.84	0.57	0.71	0.85	0.96	0.63	0.76	1.04
1	ID_161	TTP015	Remission	0.70	0.47	1.05	0.93	0.76	0.52	0.59	0.82	0.97	0.57	0.79	1.08
1	ID_243	TTP051	Remission	0.62	0.52	1.11	1.03	0.80	0.51	0.62	0.76	1.05	0.54	0.81	1.07
2	ID_064	HUN1003	Acute	0.83	0.30	1.11	0.99	0.74	0.31	0.38	0.55	1.03	0.37	0.63	1.14
2	ID_062	HUN1003	Acute	0.70	0.55	1.03	0.90	0.69	0.49	0.49	0.63	0.92	0.48	0.56	1.01
2	ID_097	HUN430	Acute	0.91	0.30	1.08	0.88	0.66	0.30	0.38	0.57	1.08	0.34	0.73	0.99
2	ID_058	HUN800	Acute	0.95	0.32	1.04	0.94	0.69	0.32	0.45	0.65	1.03	0.36	0.63	1.06
2	ID_319	LRB-BEL-201612-019	Acute	1.01	0.22	0.97	0.78	0.55	0.22	0.35	0.54	0.93	0.25	0.55	0.95
2	ID_322	LRB-BEL-201612-022	Acute	1.03	0.31	1.12	1.13	0.63	0.29	0.44	0.59	1.08	0.28	0.54	1.23
2	ID_332	LRB-BEL-201612-033	Acute	1.04	0.41	1.17	1.09	0.69	0.37	0.55	0.66	1.07	0.39	0.66	1.23
2	ID_340	LRB-BEL-201612-041	Acute	0.40	0.56	0.84	0.81	0.71	0.51	0.58	0.72	0.78	0.62	0.71	0.86
2	ID_349	LRB-BEL-201612-051	Acute	0.64	0.39	1.01	0.98	0.56	0.35	0.41	0.53	1.02	0.39	0.51	1.04
2	ID_352	LRB-BEL-201612-054	Acute	0.52	0.45	0.92	0.76	0.55	0.41	0.52	0.66	0.96	0.50	0.66	1.07
2	ID_354	LRB-BEL-201612-056	Acute	0.75	0.39	0.93	0.85	0.63	0.36	0.40	0.57	0.88	0.42	0.66	0.91
2	ID_356	LRB-BEL-201612-058	Acute	0.87	0.35	0.89	0.93	0.66	0.32	0.41	0.61	0.89	0.38	0.73	1.14
2	ID_357	LRB-BEL-201612-060	Acute	0.98	0.41	1.09	0.99	0.68	0.34	0.50	0.73	1.02	0.45	0.73	1.12
2	ID_358	LRB-BEL-201612-061	Acute	0.64	0.37	1.03	0.91	0.73	0.36	0.47	0.56	1.00	0.41	0.62	1.07
2	ID_371	LRB-BEL-201612-076	Acute	0.53	0.38	0.95	0.85	0.52	0.35	0.48	0.51	0.87	0.39	0.47	1.00
2	ID_379	LRB-BEL-201612-086	Acute	0.68	0.53	1.22	1.10	0.59	0.51	0.60	0.67	1.07	0.50	0.69	1.28
2	ID_381	LRB-BEL-201612-088	Acute	0.88	0.41	1.05	1.01	0.64	0.39	0.45	0.64	1.09	0.42	0.70	1.09
2	ID_403	LRB-BEL-201906-144	Acute	0.67	0.54	0.99	1.08	0.64	0.44	0.52	0.55	1.05	0.41	0.74	1.26
2	ID_412	LRB-BEL-201906-RL015	Acute	1.00	0.43	1.10	1.02	0.74	0.39	0.46	0.60	1.08	0.43	0.74	1.16
2	ID_285	MF-KB-TTP01-A	Acute	0.84	0.43	0.89	0.89	0.69	0.39	0.50	0.56	0.86	0.42	0.71	0.76
2	ID_299	MF-KB-TTP15-A	Acute	0.77	0.40	1.00	0.81	0.66	0.39	0.51	0.60	0.96	0.40	0.73	0.86
2	ID_063	HUN1003	Remission	0.90	0.28	1.05	0.89	0.72	0.30	0.37	0.54	1.01	0.34	0.58	0.97
2	ID_024	HUN113	Remission	0.55	0.47	1.07	0.82	0.70	0.47	0.51	0.60	1.00	0.48	0.61	1.05
2	ID_022	HUN113	Remission	0.54	0.49	1.02	0.85	0.72	0.44	0.60	0.63	1.00	0.52	0.69	1.05
2	ID_079	HUN1849	Remission	0.70	0.27	1.06	0.94	0.68	0.32	0.38	0.60	1.03	0.38	0.71	0.95
2	ID_107	TTP002	Remission	0.52	0.36	1.05	0.88	0.61	0.39	0.43	0.57	0.98	0.45	0.59	1.00
2	ID_104	TTP002	Remission	0.49	0.39	1.09	0.86	0.61	0.42	0.45	0.55	1.04	0.47	0.57	1.07

Profile	Sample ID	Patient	Phase	Anti-ADAMTS13 IgG (II-1 relative OD)	Anti-spacer IgG (S1-swap/ ADAMTS13 ratio)	Hybrid (hybrid/ADAMTS13 ratio)									
						A	B	C	E	G	J	L	M	N	O
2	ID_170	TTP016	Remission	0.38	0.47	1.11	0.84	0.67	0.54	0.55	0.69	1.04	0.55	0.63	1.05
3	ID_087	HUN1129	Acute	1.15	0.41	1.06	0.89	0.75	0.39	0.51	0.69	1.03	0.48	0.84	0.98
3	ID_076	HUN2094	Acute	0.44	0.55	1.18	1.10	0.90	0.60	0.66	0.75	1.13	0.62	0.76	1.06
3	ID_039	HUN280	Acute	0.56	0.62	1.07	1.02	0.83	0.64	0.66	0.70	1.00	0.63	0.81	1.03
3	ID_009	HUN31	Acute	0.39	0.63	1.17	1.17	0.89	0.68	0.67	0.74	1.05	0.67	0.87	1.03
3	ID_047	HUN461	Acute	0.54	0.52	1.06	0.96	0.81	0.61	0.65	0.72	0.97	0.62	0.84	0.99
3	ID_325	LRB-BEL-201612-025	Acute	0.61	0.48	1.01	0.94	0.75	0.50	0.55	0.65	1.00	0.51	0.86	1.05
3	ID_370	LRB-BEL-201612-075	Acute	0.69	0.61	0.96	0.87	0.79	0.51	0.65	0.72	0.95	0.64	0.94	1.10
3	ID_373	LRB-BEL-201612-078	Acute	0.44	0.73	1.02	0.91	0.82	0.63	0.69	0.74	1.07	0.73	1.01	1.27
3	ID_413	LRB-BEL-201906-RL020	Acute	0.64	0.52	0.96	0.94	0.80	0.51	0.60	0.70	1.12	0.60	0.98	1.18
3	ID_421	LRB-BEL-201906-RL072	Acute	0.76	0.68	1.06	1.03	0.77	0.55	0.56	0.72	1.15	0.69	0.83	1.16
3	ID_286	MF-KB-TTP02-A	Acute	0.80	0.30	0.93	0.96	0.85	0.32	0.39	0.73	0.99	0.40	0.84	0.91
3	ID_293	MF-KB-TTP09-A	Acute	0.81	0.38	1.00	0.87	0.88	0.41	0.50	0.73	0.92	0.42	0.84	0.85
3	ID_294	MF-KB-TTP10-A	Acute	0.57	0.49	0.99	1.03	0.85	0.48	0.59	0.64	1.01	0.53	0.78	0.89
3	ID_083	HUN1149	Remission	0.74	0.31	1.10	1.03	0.75	0.35	0.43	0.65	1.04	0.41	0.81	0.96
3	ID_084	HUN1149	Remission	0.47	0.37	1.06	1.00	0.80	0.36	0.46	0.57	1.11	0.45	0.85	1.04
3	ID_140	TTP014	Remission	0.79	0.57	1.02	1.02	0.78	0.58	0.63	0.67	1.00	0.60	0.84	1.07
3	ID_168	TTP016	Remission	0.51	0.60	1.04	0.94	0.78	0.59	0.64	0.73	0.95	0.65	0.84	1.06
3	ID_242	TTP051	Remission	0.60	0.46	1.05	0.97	0.77	0.44	0.57	0.72	1.01	0.51	0.77	1.11
4	ID_310	LRB-BEL-201612-009	Acute	0.55	0.55	0.93	0.92	0.71	0.48	0.56	0.72	0.97	0.59	0.82	1.05
4	ID_315	LRB-BEL-201612-014	Acute	1.12	0.27	1.04	1.00	0.62	0.28	0.41	0.71	0.97	0.35	0.82	1.08
4	ID_317	LRB-BEL-201612-017	Acute	0.83	0.47	1.03	0.85	0.58	0.42	0.73	0.73	1.08	0.53	0.80	1.21
4	ID_318	LRB-BEL-201612-018	Acute	1.15	0.25	1.04	0.91	0.65	0.31	0.38	0.64	0.96	0.30	0.76	1.06
4	ID_320	LRB-BEL-201612-020	Acute	0.99	0.31	1.11	1.07	0.74	0.30	0.37	0.74	1.06	0.36	0.86	1.19
4	ID_334	LRB-BEL-201612-035	Acute	0.96	0.48	1.10	0.94	0.73	0.51	0.60	0.70	0.97	0.53	0.85	1.15
4	ID_345	LRB-BEL-201612-046	Acute	0.95	0.42	0.98	0.86	0.67	0.38	0.54	0.66	0.96	0.47	0.80	1.05
4	ID_390	LRB-BEL-201612-098	Acute	0.98	0.38	1.06	0.99	0.71	0.36	0.39	0.75	1.01	0.39	0.82	1.13
4	ID_394	LRB-BEL-201906-121	Acute	0.66	0.47	1.08	1.03	0.72	0.48	0.57	0.66	1.04	0.53	0.87	1.11
4	ID_404	LRB-BEL-201906-145	Acute	0.67	0.51	0.90	0.89	0.61	0.48	0.61	0.61	1.11	0.59	0.95	1.11
4	ID_287	MF-KB-TTP03-A	Acute	0.89	0.43	0.95	0.85	0.56	0.39	0.66	0.69	0.97	0.54	0.95	0.87

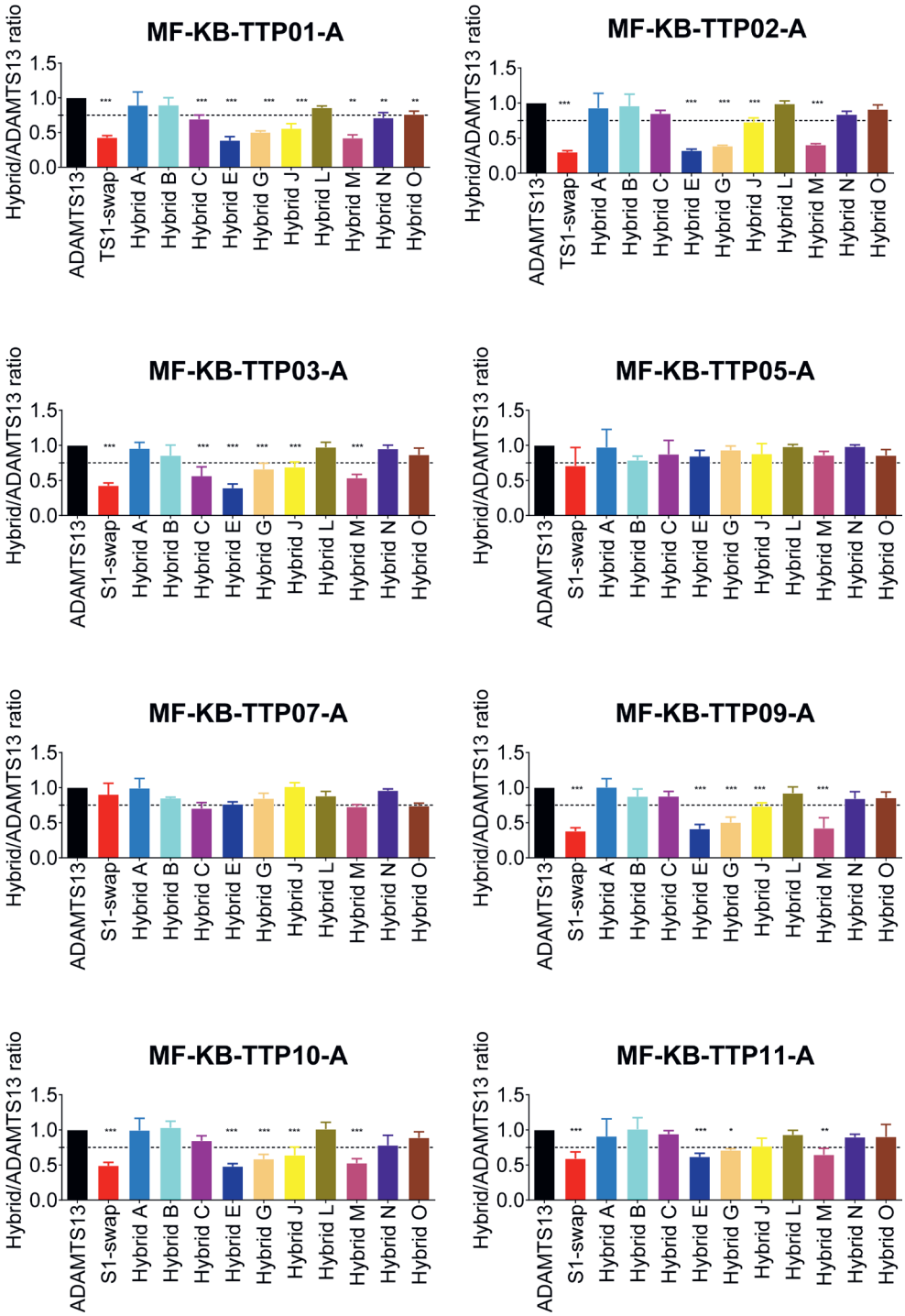
Profile	Sample ID	Patient	Phase	Anti-ADAMTS13 IgG (II-1 relative OD)	Anti-spacer IgG (S1-swap/ ADAMTS13 ratio)	Hybrid (hybrid/ADAMTS13 ratio)									
						A	B	C	E	G	J	L	M	N	O
4	ID_301	MF-KB-TTP17-A	Acute	0.49	0.48	1.07	1.00	0.65	0.55	0.59	0.73	0.90	0.53	0.77	0.90
4	ID_198	TTP044	Acute	0.50	0.49	1.04	0.96	0.69	0.51	0.57	0.60	0.94	0.57	0.82	1.03
4	ID_085	HUN1143	Remission	0.91	0.31	1.04	0.95	0.71	0.33	0.40	0.62	1.00	0.43	0.79	1.03
4	ID_119	TTP006	Remission	0.85	0.39	1.06	0.93	0.72	0.38	0.48	0.68	0.98	0.45	0.87	1.02
4	ID_121	TTP007	Remission	0.83	0.37	0.99	0.93	0.67	0.35	0.45	0.66	0.92	0.42	0.78	0.99
4	ID_173	TTP019	Remission	0.81	0.33	1.06	0.94	0.70	0.33	0.38	0.50	1.00	0.40	0.81	1.05
5	ID_051	HUN684	Acute	1.01	0.61	0.98	0.98	0.87	0.70	0.81	0.81	0.98	0.69	0.92	1.01
5	ID_323	LRB-BEL-201612-023	Acute	0.93	0.69	1.26	1.09	0.80	0.55	1.25	0.82	1.24	0.69	1.27	1.73
5	ID_355	LRB-BEL-201612-057	Acute	0.42	0.56	0.84	0.87	0.78	0.59	0.91	0.93	0.93	0.63	0.96	0.99
5	ID_387	LRB-BEL-201612-094	Acute	0.65	0.70	1.07	1.05	0.88	0.73	0.75	0.84	1.04	0.70	0.89	1.16
5	ID_401	LRB-BEL-201906-140	Acute	0.70	0.68	1.34	0.95	0.76	0.60	0.98	1.03	1.15	0.62	0.95	1.41
5	ID_402	LRB-BEL-201906-142	Acute	0.65	0.67	1.03	0.94	0.80	0.65	0.76	0.83	0.96	0.70	0.85	1.07
5	ID_245	TTP052	Remission	0.90	0.45	1.12	1.01	0.81	0.46	0.86	1.03	1.05	0.63	1.11	1.14
5	ID_274	TTP069	Remission	0.50	0.66	1.05	0.99	0.87	0.70	0.77	0.84	1.03	0.70	0.92	1.05
5	ID_277	TTP072	Remission	0.42	0.63	0.94	0.85	0.80	0.67	0.80	0.79	1.02	0.67	1.11	1.12
5	ID_186	TTP077	Remission	0.97	0.35	1.11	1.02	0.75	0.36	0.79	0.90	1.03	0.61	1.09	1.06
6	ID_023	HUN113	Acute	0.56	0.44	1.00	0.87	0.75	0.49	0.62	0.65	1.08	0.50	0.74	1.05
6	ID_043	HUN396	Acute	0.75	0.37	1.08	1.00	0.82	0.44	0.51	0.62	1.02	0.46	0.60	1.08
6	ID_306	LRB-BEL-201612-002	Acute	0.46	0.55	1.03	1.03	0.75	0.57	0.60	0.67	1.03	0.60	0.67	1.10
6	ID_337	LRB-BEL-201612-038	Acute	0.87	0.37	1.14	1.01	0.75	0.38	0.45	0.69	1.04	0.41	0.74	1.12
6	ID_338	LRB-BEL-201612-039	Acute	0.55	0.33	1.05	0.97	0.76	0.34	0.37	0.60	0.93	0.41	0.65	1.12
6	ID_342	LRB-BEL-201612-043	Acute	0.37	0.60	1.02	0.97	0.75	0.57	0.62	0.65	0.96	0.59	0.69	1.03
6	ID_297	MF-KB-TTP13-A	Acute	0.52	0.41	0.80	0.78	0.78	0.49	0.56	0.62	0.87	0.47	0.60	0.82
6	ID_065	HUN1003	Remission	0.66	0.46	1.05	0.99	0.75	0.45	0.50	0.57	1.00	0.46	0.61	1.02
6	ID_205	TTP032	Remission	0.49	0.60	1.04	1.01	0.77	0.59	0.48	0.66	1.02	0.59	0.66	1.05
7	ID_038	HUN280	Acute	0.99	0.31	1.07	0.97	0.74	0.32	0.60	0.84	0.96	0.53	1.03	1.12
7	ID_309	LRB-BEL-201612-008	Acute	1.29	0.28	1.00	0.96	0.70	0.21	0.35	0.85	1.08	0.32	0.88	1.04
7	ID_350	LRB-BEL-201612-052	Acute	1.16	0.34	0.98	0.89	0.62	0.29	0.45	0.82	0.93	0.45	0.86	0.98
7	ID_369	LRB-BEL-201612-074	Acute	0.98	0.27	1.15	1.06	0.68	0.28	0.55	0.88	1.08	0.48	1.12	1.15
7	ID_377	LRB-BEL-201612-084	Acute	0.98	0.29	1.19	1.11	0.72	0.30	0.53	0.76	1.14	0.34	0.84	1.22

Profile	Sample ID	Patient	Phase	Anti-ADAMTS13 IgG (II-1 relative OD)	Anti-spacer IgG (S1-swap/ ADAMTS13 ratio)	Hybrid (hybrid/ADAMTS13 ratio)									
						A	B	C	E	G	J	L	M	N	O
7	ID_385	LRB-BEL-201612-092	Acute	1.10	0.31	1.17	0.97	0.70	0.31	0.58	0.89	1.05	0.31	0.85	1.24
7	ID_386	LRB-BEL-201612-093	Acute	1.09	0.49	1.24	1.03	0.73	0.33	0.68	0.87	1.13	0.46	1.00	1.29
7	ID_388	LRB-BEL-201612-095	Acute	1.34	0.26	1.19	1.02	0.71	0.27	0.39	0.79	1.10	0.38	0.89	1.19
8	ID_035	HUN198	Acute	0.73	0.35	0.97	0.89	0.61	0.36	0.85	0.88	1.03	0.62	1.01	1.04
8	ID_383	LRB-BEL-201612-090	Acute	0.73	0.60	1.42	1.42	0.70	0.48	0.87	0.81	1.27	0.52	0.93	1.49
8	ID_254	TTP059	Remission	0.90	0.38	1.11	0.97	0.74	0.39	0.76	0.97	1.03	0.53	1.04	1.11
9	ID_392	LRB-BEL-201906-114	Acute	0.52	0.56	1.01	1.00	0.88	0.66	0.93	0.92	1.05	0.75	0.96	1.14
9	ID_136	TTP011	Remission	0.43	0.67	1.10	0.99	0.79	0.70	0.78	0.82	1.04	0.75	0.84	1.06
9	ID_135	TTP011	Remission	0.54	0.69	1.17	1.08	0.78	0.73	0.88	0.91	1.21	0.76	0.93	1.26
10	ID_407	LRB-BEL-201906-152	Acute	0.69	0.53	1.18	1.09	0.71	0.52	0.60	0.76	1.07	0.51	0.60	1.18
10	ID_160	TTP015	Remission	0.69	0.47	0.98	0.91	0.71	0.50	0.54	0.78	0.97	0.57	0.71	0.96
11	ID_308	LRB-BEL-201612-007	Acute	0.84	0.40	0.86	0.72	0.60	0.35	0.56	0.71	0.92	0.39	0.72	1.07
12	ID_307	LRB-BEL-201612-006	Acute	0.89	0.46	1.03	0.91	0.60	0.39	0.95	0.70	0.99	0.47	1.00	1.19
13	ID_328	LRB-BEL-201612-028	Acute	0.96	0.52	1.16	1.08	0.79	0.53	0.76	0.74	1.11	0.63	0.98	1.19
14	ID_331	LRB-BEL-201612-031	Acute	0.67	0.66	0.98	1.00	0.85	0.69	0.80	0.87	0.98	0.70	0.64	1.08
15	ID_368	LRB-BEL-201612-072	Acute	0.53	0.72	1.05	1.06	0.80	0.71	0.70	0.82	1.08	0.75	0.91	1.15
16	ID_001	HUN1	Acute	0.56	0.71	1.09	1.04	0.95	0.75	0.82	0.80	1.00	0.71	0.94	1.09
17	ID_066	HUN1054	Acute	0.41	0.56	1.69	1.54	1.37	0.99	1.07	1.23	1.65	1.10	1.50	1.34
17	ID_326	LRB-BEL-201612-026	Acute	0.44	0.71	1.04	1.06	0.97	0.82	0.99	0.77	1.10	0.77	1.21	1.34
17	ID_360	LRB-BEL-201612-063	Acute	0.58	0.71	1.15	0.98	0.83	0.78	0.86	0.89	0.92	0.78	0.86	1.19
17	ID_364	LRB-BEL-201612-067	Acute	0.50	0.74	1.11	1.01	0.87	0.75	0.92	0.82	1.01	0.75	0.92	1.09
17	ID_289	MF-KB-TTP05-A	Acute	0.94	0.71	0.97	0.79	0.87	0.84	0.93	0.88	0.98	0.86	0.98	0.86
17	ID_041	HUN280	Remission	0.48	0.74	1.08	1.03	0.87	0.76	0.75	0.83	1.00	0.76	0.88	1.08

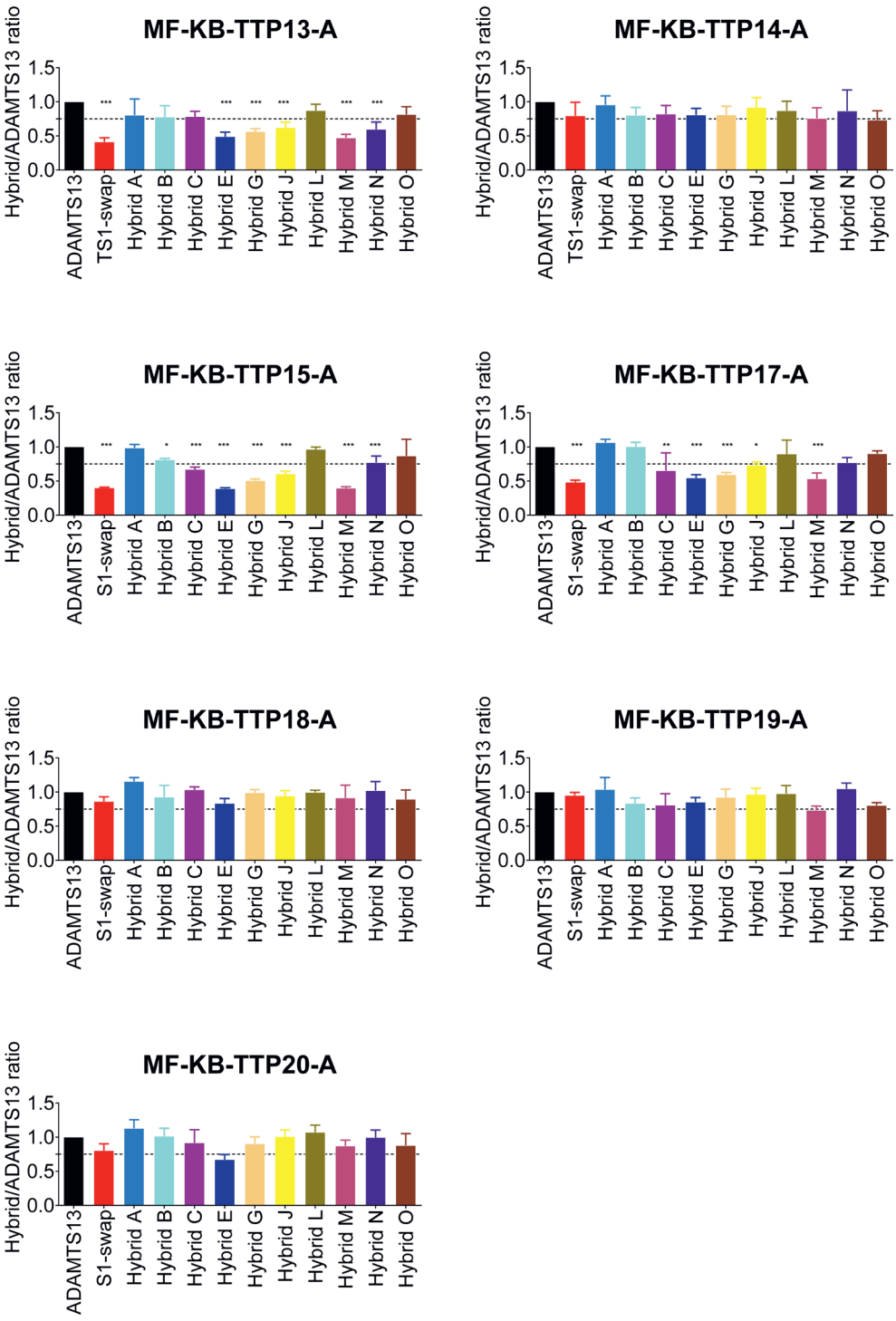
Supporting Table 3. Anti-spacer autoantibody profile remains mainly unchanged during follow-up. Analysis of 5 patients with at least three follow-up samples. The table shows patient ID, date of sampling, disease phase, presence (II-1 relative OD >0.34) or absence (II-1 relative OD ≤0.34) of anti-ADAMTS13 autoantibodies are indicated in green and red boxes, respectively. Presence (S1-swap/ADAMTS13 ratio <0.75, green boxes) or absence (S1-swap/ADAMTS13 ratio ≥0.75, red boxes) of anti-spacer autoantibodies and the anti-spacer autoantibody profile of each sample are indicated. If no anti-ADAMTS13 autoantibodies were detected, anti-spacer autoantibodies were not analyzed as indicated in grey boxes. The amino acid regions involved and not involved in the anti-spacer autoantibody profile are depicted in green boxes (S1-swap/ADAMTS13 ratio <0.75) and red boxes (S1-swap/ADAMTS13 ratio ≥0.75), respectively.

Patiend	Date of sampling	Phase	Anti-ADAMTS13 IgG (II-1 relative OD)	Anti-spacer IgG (S1-swap/ ADAMTS13 ratio)	Hybrid (hybrid/ADAMTS13 ratio)									
					A	B	C	E	G	J	L	M	N	O
HUN1003	08-28-15	Acute	0.70	0.55	1.03	0.90	0.69	0.49	0.49	0.63	0.92	0.48	0.56	1.01
	03-24-16	Remission	0.90	0.28	1.05	0.89	0.72	0.30	0.37	0.54	1.01	0.34	0.58	0.97
	06-10-16	Acute	0.83	0.30	1.11	0.99	0.74	0.31	0.38	0.55	1.03	0.37	0.63	1.14
	08-04-16	Remission	0.66	0.46	1.05	0.99	0.75	0.45	0.50	0.57	1.00	0.46	0.61	1.02
HUN113	11-13-15	Remission	0.54	0.49	1.02	0.85	0.72	0.44	0.60	0.63	1.00	0.52	0.69	1.05
	12-04-15	Acute	0.56	0.44	1.00	0.87	0.75	0.49	0.62	0.65	1.08	0.50	0.74	1.05
	02-21-17	Remission	0.55	0.47	1.07	0.82	0.70	0.47	0.51	0.60	1.00	0.48	0.61	1.05
	03-30-18	Acute	0.11											
HUN280	01-03-12	Acute	0.99	0.31	1.07	0.97	0.74	0.32	0.60	0.84	0.96	0.53	1.03	1.12
	04-15-16	Acute	0.56	0.62	1.07	1.02	0.83	0.64	0.66	0.70	1.00	0.63	0.81	1.03
	08-10-16	Remission	0.33											
	08-09-17	Remission	0.48	0.74	1.08	1.03	0.87	0.76	0.75	0.83	1.00	0.76	0.88	1.08
HUN31	01-23-10	Remission	0.41	0.70	0.98	0.95	0.88	0.72	0.71	0.80	0.94	0.71	0.80	0.97
	08-18-11	Remission	0.23											
	03-13-14	Acute	0.39	0.63	1.17	1.17	0.89	0.68	0.67	0.74	1.05	0.67	0.87	1.03
	01-15-15	Remission	0.53	0.79										
	12-05-15	Acute	0.37	0.55	1.10	1.08	0.91	0.59	0.63	0.86	1.09	0.60	0.96	1.07
	12-14-17	Remission	0.16											
HUN198	09-26-11	Acute	0.73	0.35	0.97	0.89	0.61	0.36	0.85	0.88	1.03	0.62	1.01	1.04
	02-02-16	Remission	0.77	0.61	0.97	0.94	0.80	0.66	0.69	0.86	1.00	0.67	0.92	0.98
	04-18-18	Remission	0.71	0.51	0.95	0.86	0.75	0.53	0.60	0.82	0.95	0.61	0.92	1.01

Supporting Figures



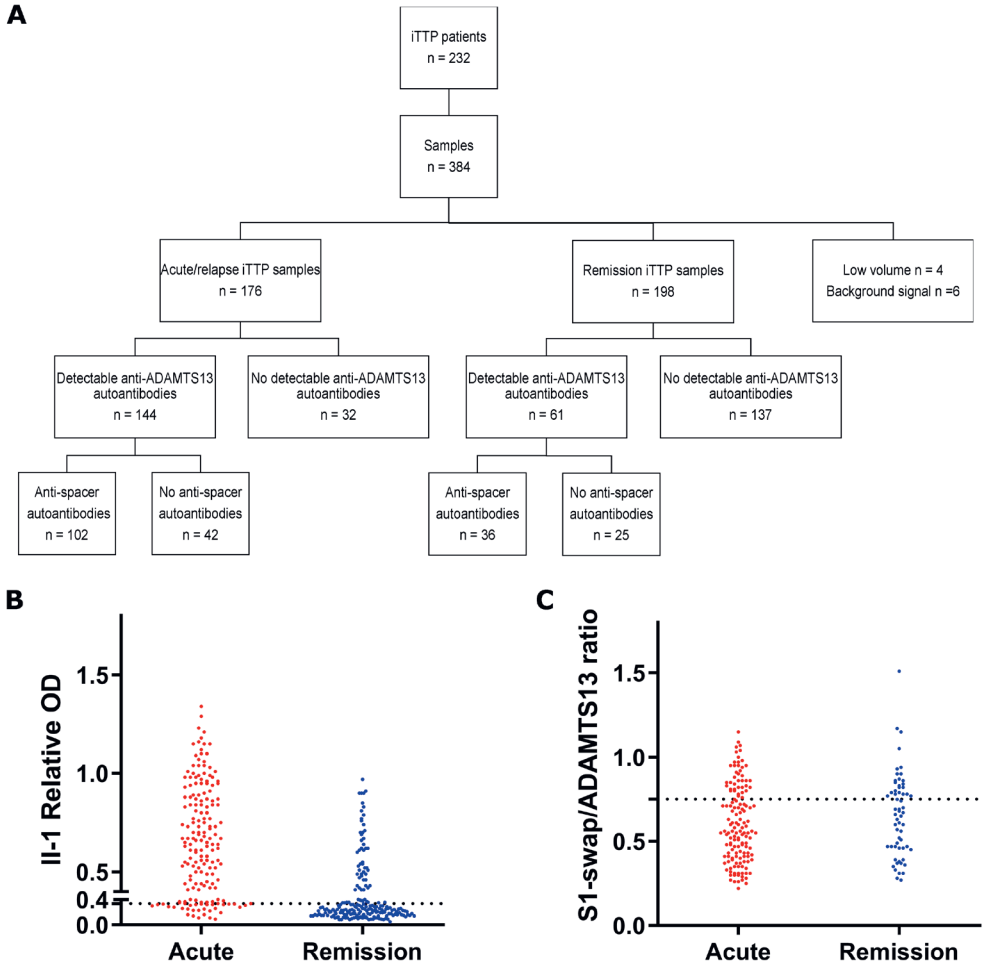
Supporting Figure 1 (continued; legend on page 104).



Supporting Figure 1 (continued; legend on page 104).

Supporting Figure 1 (previous two pages) – A hybrid/ADAMTS13 ratio lower than 0.75 indicates that the exchanged amino acids regions are involved in the epitope of anti-spacer autoantibodies.

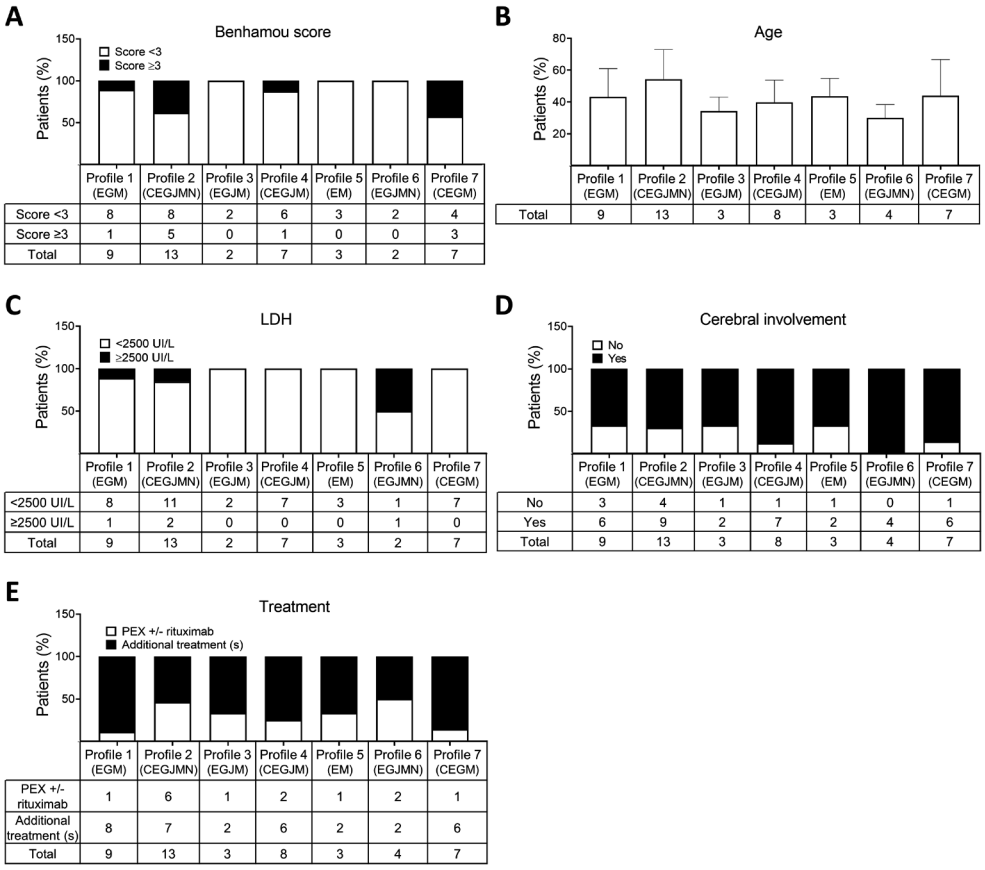
Full-length WT ADAMTS13 or spacer hybrids were captured by mouse monoclonal anti-metalloprotease domain antibody 3H9. Next, plasma was added and bound anti-ADAMTS13 autoantibodies were detected using an HRP conjugated goat anti-human IgG Fc specific antibody. Data are expressed as the ratio of the relative OD of the binding of the patient autoantibodies to the hybrids, to the relative OD of the binding of the patient autoantibodies to WT ADAMTS13 (hybrid/ADAMTS13 ratio). Differences between the binding of plasma anti-ADAMTS13 autoantibodies to WT ADAMTS13 compared to each one of the hybrids were statistically analyzed using one-way ANOVA with Tukey's post-hoc test (*p-value ≤0.05, ** p-value ≤0.01, *** p-value ≤0.001, n=4). The dotted line indicates the ratio of 0.75. Data are expressed as mean ± SD.



Supporting Figure 2 (legend on the next page).

Supporting Figure 2 – Sample selection for epitope mapping of anti-spacer autoantibodies using the full-length spacer hybrids.

A. Flowchart for selection of samples for epitope mapping of anti-spacer autoantibodies. We had access to 384 plasma samples from 232 iTTP patients. However, ten samples were excluded because there was not enough volume to perform the epitope mapping or there was a high background in the assay (defined as a signal higher than the mean signal plus 3 times SD of a healthy donor plasma sample (HD100) to WT ADAMTS13, n=3). **B.** Anti-ADAMTS13 autoantibodies in plasma of iTTP patients during the acute and remission phase. WT ADAMTS13 was captured by mouse monoclonal anti-metalloprotease domain antibody 3H9. Next, plasma was added, and bound anti-ADAMTS13 autoantibodies were detected using HRP conjugated goat anti-human IgG Fc specific antibody (Acute n=176; Remission n=198). OD values compared to II-1 (0.05 µg/mL) were set as 1 to calculate relative OD values. The dotted line indicates the cut-off of the assay to detect anti-ADAMTS13 autoantibodies (II-1 relative OD: 0.34). **C.** Anti-spacer ADAMTS13 autoantibodies in plasma of iTTP patients during the acute and remission phase. Full-length WT ADAMTS13 or spacer hybrid (S1-swap) were captured by mouse monoclonal anti-metalloprotease domain antibody 3H9. Next, plasma was added, and bound anti-ADAMTS13 autoantibodies were detected using HRP conjugated goat anti-human IgG Fc specific antibody (Acute n=144; Remission n=61). The binding of the anti-ADAMTS13 autoantibodies to the S1-swap was calculated relative to the binding to WT ADAMTS13. The dotted line indicates the cut-off ratio (0.75 S1-swap/ADAMTS13 ratio) to define the presence of anti-spacer autoantibodies. A sample was considered to have anti-spacer autoantibodies if the S1-swap/ADAMTS13 ratio was <0.75.



	Profile 1 (EGM)	Profile 2 (CEGJMN)	Profile 3 (EGJM)	Profile 4 (CEGJM)	Profile 5 (EM)	Profile 6 (EGJMN)	Profile 7 (CEGM)
Score <3	8	8	2	6	3	2	4
Score ≥3	1	5	0	1	0	0	3
Total	9	13	2	7	3	2	7

	Profile 1 (EGM)	Profile 2 (CEGJMN)	Profile 3 (EGJM)	Profile 4 (CEGJM)	Profile 5 (EM)	Profile 6 (EGJMN)	Profile 7 (CEGM)
Total	9	13	3	8	3	4	7

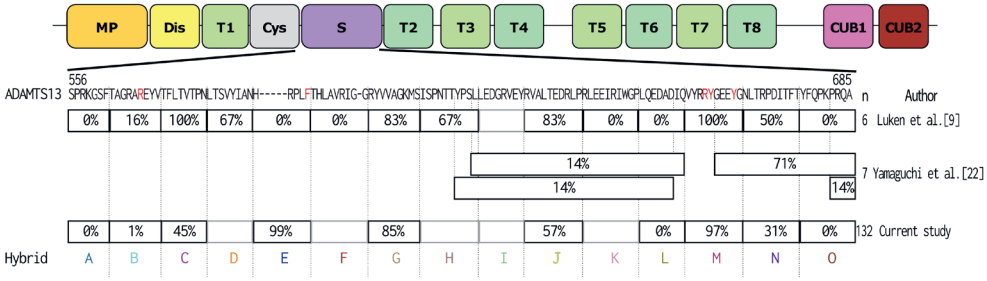
	Profile 1 (EGM)	Profile 2 (CEGJMN)	Profile 3 (EGJM)	Profile 4 (CEGJM)	Profile 5 (EM)	Profile 6 (EGJMN)	Profile 7 (CEGM)
<2500 UI/L	8	11	2	7	3	1	7
≥2500 UI/L	1	2	0	0	0	1	0
Total	9	13	2	7	3	2	7

	Profile 1 (EGM)	Profile 2 (CEGJMN)	Profile 3 (EGJM)	Profile 4 (CEGJM)	Profile 5 (EM)	Profile 6 (EGJMN)	Profile 7 (CEGM)
No	3	4	1	1	1	0	1
Yes	6	9	2	7	2	4	6
Total	9	13	3	8	3	4	7

	Profile 1 (EGM)	Profile 2 (CEGJMN)	Profile 3 (EGJM)	Profile 4 (CEGJM)	Profile 5 (EM)	Profile 6 (EGJMN)	Profile 7 (CEGM)
PEX +/- rituximab	1	6	1	2	1	2	1
Additional treatment (s)	8	7	2	6	2	2	6
Total	9	13	3	8	3	4	7

Supporting Figure 3 – Anti-spacer autoantibody profiles are not linked with disease severity.

Distribution of acute iTTP patients of the TTP samples from Paris (n=47) according to anti-spacer autoantibodies profiles for **A.** Benhamou score (score <3, white bars; score ≥3, black bars),[19] (B-E) parameters included in the Benhamou score **B.** age (mean and SD), **C.** LDH level (<2500 IU/L, white bars; elevated level: ≥2500 IU/L, black bars) and **D.** cerebral involvement (no, white bars; yes, black bars) and for **E.** treatment with plasma exchange (PEX) with (+)/without (-) rituximab (white bars) or PEX with/without rituximab and additional treatments(s) (black bars). All analyses included n=47 iTTP patients, except for Benhamou score and LDH where n=43. No statistical differences were observed between anti-spacer autoantibody profile and Benhamou score, LDH level, cerebral involvement, treatment (Fisher's exact test; *p*-value >0.05) or age (one-way ANOVA; *p*-value >0.05)



Supporting Figure 4 – Overview of epitope mapping studies of the ADAMTS13 spacer domain.

Schematic representation of full-length ADAMTS13, consisting of the metalloprotease (MP), a disintegrin-like (Dis), a first thrombospondin type-1 repeat (T1), a cysteine-rich (Cys), a spacer (S), seven additional T1 repeats (T2–8), and two CUB domains. Under the domain structure of ADAMTS13, the amino acid sequence of the spacer domain is displayed and amino acid residues described in alanine scanning studies are represented in red [10, 20, 21]. The percentage of patients with autoantibodies binding to a specific region in the spacer domain in a certain study is indicated [9, 22]. The dotted lines show the boundaries of the regions investigated in each study, gray boxes correspond to regions not tested in the study. At the bottom of the figure, the names of the hybrids are mentioned.