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ORIGINAL ARTICLE

ADAMTS-13 conformation influences autoimmune recognition in immune thrombotic thrombocytopenic purpura

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Abstract

Background: Patients with immune-mediated thrombotic thrombocytopenic purpura (iTTP) have anti–ADAMTS-13 immunoglobulin G (IgG) autoantibodies that enhance ADAMTS-13 clearance and/or inhibit its function. ADAMTS-13 normally circulates in a closed conformation, which is manifested by the interaction of the CUB domains with the central spacer domain. Disruption of the spacer–CUB interaction opens ADAMTS-13, which augments its proteolytic function but may also expose cryptic autoimmune epitopes that promote further autoantibody recognition.

Objectives: To explore differences in autoantibody binding to ADAMTS-13 in its closed or open conformations in patients with iTTP and to correlate these differences with disease-related parameters.

Methods: We developed a novel assay to measure autoantibodies binding to closed and open ADAMTS-13. Autoantibody titer and IgG subclass binding to open or closed ADAMTS-13 were measured in 70 iTTP first presentation samples and correlated with clinical data, remission, and relapse.

Results: In 70 patients with iTTP, the mean autoantibody titer against open ADAMTS-13 was, on average, approximately 2-fold greater than that against closed ADAMTS-13, suggesting that ADAMTS-13 opening increases epitope exposure and immune complex formation. Autoantibody titer against closed/open ADAMTS-13 and IgG subclass did not correlate with ADAMTS-13 antigen at presentation. Two patients with iTTP and persistent autoantibodies lost specificity for closed ADAMTS-13 in remission. Recognition of closed/open ADAMTS-13 and autoantibody IgG subclass between the first and second iTTP episodes were very similar.

Conclusion: ADAMTS-13 autoantibody binding is highly influenced by ADAMTS-13 conformation. Although this does not appear to modify the pathogenicity of autoantibodies, the autoantibody signature at relapse suggests that relapse represents re-emergence of the original autoimmune response rather than de novo presentation.

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KEYWORDS
autoantibodies, autoimmune disease, purpura, thrombosis, thrombotic thrombocytopenic, von Willebrand factor

1 | INTRODUCTION

The metalloprotease ADAMTS-13 proteolytically controls the multimeric size of von Willebrand factor (VWF) in plasma, which modulates its platelet-binding potential. Severe ADAMTS-13 deficiency (<10%) causes the persistence of ultralarge VWF (UL-VWF). Unraveling of UL-VWF in circulation without ADAMTS-13–dependent control can cause VWF/platelet-rich microvascular thrombosis and thrombotic thrombocytopenic purpura (TTP) [1–4]. TTP is caused by either congenital *ADAMTS-13* deficiency or an immune-mediated mechanism associated with anti–ADAMTS-13 autoantibodies [5,6]. Approximately 95% of TTP cases are immune-mediated TTP (iTTP) [7]. These patients are treated with plasma exchange (PEX), which provides a source of ADAMTS-13, removes circulating UL-VWF, and reduces circulating anti–ADAMTS-13 immunoglobulin G (IgG) levels. Steroids and rituximab are frequently used to target the autoimmune component of the disease. Although these treatments facilitate remission in many patients with iTTP, relapse remains a significant problem requiring long-term monitoring.

The autoimmune response in iTTP involves the development of polyclonal, predominantly IgG antibodies that recognize ADAMTS-13 [8–12]. Characterization of the domain specificity of these antibodies and their inhibitory effects has helped elucidate the pathogenic roles of anti–ADAMTS-13 autoantibodies in TTP. In some patients, autoantibodies bind to ADAMTS-13 and inhibit its proteolytic function to varying degrees to induce deficiency. This does not appear to be the primary pathogenic mechanism as, more frequently, autoantibodies also induce ADAMTS-13 deficiency by the formation of immune complexes that promote ADAMTS-13 clearance [8,13]. This is an important disease process as ADAMTS-13 antigen levels are frequently severely reduced in iTTP, and ADAMTS-13 antigen levels at presentation are significantly lower in patients who die from an episode than in those who survive [8,14].

ADAMTS-13 exists in at least 2 distinct conformations, termed open and closed [15,16]. In circulation, ADAMTS-13 adopts its closed conformation [17]. In this form, the C-terminal CUB domains of ADAMTS-13 fold back and interact with the central spacer domain [15,16,18]. This interaction stabilizes ADAMTS-13 in a conformation that is functionally less active than the open form due to long-range structural constraints upon the metalloprotease domain [19]. The spacer–CUB interaction can be naturally disrupted when ADAMTS-13 interacts with the VWF D4-CK domains [15,16]. Disruption of the spacer–CUB interaction causes ADAMTS-13 to adopt its open conformation in which its spacer domain is fully exposed, and the enzyme is more proteolytically active [15,16,18,20]. These same conformational changes in ADAMTS-13 can also be induced by specific mouse monoclonal antibodies, some autoantibodies in patients with iTTP, and reductions in pH [15,16,21]. These approaches augment ADAMTS-13 proteolytic function in a similar manner.

The differential exposure of the spacer domain associated with the open and closed conformations of ADAMTS-13 may be particularly pertinent to iTTP as the spacer domain is the region most commonly recognized by autoantibodies from patients with iTTP [8]. Moreover, the epitopes to which some iTTP antibodies have been revealed to bind appear to overlap with the region of the spacer domain that interacts with the CUB domains [22,23]. This could suggest that many antibodies from patients with iTTP may not efficiently recognize ADAMTS-13 in its closed form. Consistent with this, an antibody (II-1) derived from patients with iTTP that recognizes a spacer domain epitope coimmunoprecipitates with ADAMTS-13 more efficiently when in its open conformation [16]. Similarly, 3 anti–ADAMTS-13 autoantibodies isolated from patients with iTTP recognize cryptic epitopes exposed only after ADAMTS-13 adopts its open conformation [17]. It is unknown whether this is a common feature of the autoimmune response in iTTP. This raises the hypothesis in iTTP, like certain other autoimmune disorders, that autoantibodies may specifically/preferentially recognize conformational epitopes in ADAMTS-13 and that the existence of different ADAMTS-13 conformers may be one of the underlying reasons why ADAMTS-13 might become susceptible to autoimmune recognition.

We hypothesized that patient autoantibody binding to ADAMTS-13 depends on its conformation, and those that recognize the closed form may be more pathogenic than those binding with the open form. We also hypothesized that autoantibody subclass may play a role in ADAMTS-13 clearance as this determines effector function [24–26]. Anti–ADAMTS-13 IgG subclass has been investigated previously in the context of TTP [27–29], with one study finding that patients with higher IgG1 proportions had lower antigen levels than those with high IgG4 levels [28], but these studies did not take into account the conformation of ADAMTS-13, which may be important for antibody effector function. We, therefore, aimed to determine the ability of antibodies from patients with iTTP to bind to closed and/or open ADAMTS-13 and whether this correlates with clinical parameters.

2 | METHODS

2.1 | Patients with iTTP

Citrated plasma samples from patients at acute first presentation of iTTP from the UK TTP registry were used for analysis along with selected remission samples from these patients or subsequent acute clinical relapse samples. The protocol was approved by a regional ethics committee (Multicenter Research Ethics Committee: 08/H0810/54 and 08/

H0716/72). Acute iTTP was diagnosed as previously described [30]. Anti–ADAMTS-13 IgG antibody titers, ADAMTS-13 activity, and antigen were measured as previously described [14] and as part of previous studies [8,14]. The cutoff for assigning anti–ADAMTS-13 IgG positivity was 6%. The detection limit for ADAMTS-13 activity was 5% (normal range, 64%-134%), and the detection limit for ADAMTS-13 antigen was 0.5% (normal range, 74%-134%). Pooled normal plasma was used to make standard curves to quantify ADAMTS-13 antigen and activity and was arbitrarily set to 100%. Samples from patients with iTTP are presented as a percentage relative to this. Plasma samples at acute presentation before the initiation of treatment were tested. After diagnosis, all patients received corticosteroids and rituximab in addition to PEX, as previously described [31]. Each patient was treated with steroids and rituximab for their first and second episodes. Two patients received caplacizumab (patient 4/episode 2 and patient 66) for their episodes as part of the HERCULES trial [32]. Age, relapse, and patient outcomes (survival/death) were used as comparators. Anti–ADAMTS-13 titers against the N-terminal domains of ADAMTS-13 (MDTCS) or the C-terminal tail were available from a previous study for some of these patients (n = 55) [8].

2.2 | Recombinant ADAMTS-13 expression

Human recombinant ADAMTS-13 with a C-terminal 6×His tag was stably expressed in mammalian human embryonic kidney cells 293 cells, purified using a Ni^{2+}-HiTrap column (GE Healthcare), and dialyzed into 20-mM HEPES, 150-mM NaCl, and 2-mM $CaCl_2$ (pH 7.4) (HEPES buffer), as previously described [33].

2.3 | Patient autoantibody recognition of open and closed ADAMTS-13

Recombinant ADAMTS-13 (30 nM) was captured at the surface of Ni^{2+}-coated plates (Thermo Fisher Scientific) via its C-terminal 6×His tag in its closed conformation. To induce ADAMTS-13 to open, affinity-isolated rabbit anti–ADAMTS-13 metalloprotease and disintegrin domains (anti-MP-Dis) were added to wells [34]. Following this, patient plasma diluted 1:50 or 1:25 was added to wells for 1 hour to enable antibody binding. Bound patient antibodies were detected using a polyclonal antihuman Fc antibody conjugated to horseradish peroxidase (Sigma). Plates were washed 5 times with HEPES buffer, 0.1% Tween, and 5-mM imidazole. The intra-assay coefficient of variation was 3% to 9%, whereas the interassay coefficient was 10%. Two patients with iTTP, both with known high titers of anti–ADAMTS-13 antibodies (105% and 99%) were analyzed on each plate to enable standardization between plates. Open or closed anti–ADAMTS-13 titers are expressed as a percentage of these patients' IgG for the open titer. Normal plasma was included on each plate as a negative control, and background was subtracted from patients' plasma signal. Results are displayed as an open titer (antibodies binding to open ADAMTS-13), closed titer (antibodies binding to closed ADAMTS-13), or open-only titer (ie, open titer minus closed titer).

To verify that the ADAMTS-13 in the enzyme-linked immunosorbent assay (ELISA) represented the closed and open forms, an antispacer domain antibody cloned from a patient with iTTP (II-1) was added to wells both with and without the anti–MP-Dis antibody. This antibody recognizes a partially cryptic epitope in the spacer domain whose binding is markedly increased by ADAMTS-13 opening [16,17]. As a control, an anti–ADAMTS-13 thrombospondin repeat domains 2 to 4 antibody, which recognizes both open and closed forms equally, was used [34].

2.4 | IgG subclass of antibodies recognizing closed and open ADAMTS-13

To detect the IgG subclass of anti–ADAMTS-13 autoantibodies from patients with iTTP, the aforementioned ELISAs were modified using monoclonal subclass-specific mouse antihuman IgG1 to IgG4 antibodies for detection (Thermo Fisher Scientific and Abcam). These antibodies were detected using an antimouse horseradish peroxidase–conjugated antibody. As before, the plasmas from the same 2 patients with high titer IgG antibodies were analyzed on each plate to standardize between plates, and normal plasma was run on each plate as a negative control. Normalized absorbance values are shown. Monoclonal antibodies were used to ensure stoichiometric binding of the secondary antibody to each IgG subclass to enable measurement of the relative proportions of each subclass. This was verified using a standard with known concentrations of each IgG subclass.

2.5 | Statistical analysis

Statistical analyses were performed using GraphPad Prism. Spearman's rank correlation coefficient (2-tailed) was used for the following correlations: i) closed IgG titer and open IgG titer, ii) N-terminal and closed titer, iii) N-terminal and open titer, iv) ADAMTS-13 antigen levels and closed titer, and v) ADAMTS-13 antigen levels and open-only titer. An unpaired t-test was used to test differences between antigen levels in patients with the lowest (quartile 1 [Q1]) and highest (quartile 4 [Q4]) quartiles for individual subclass titers (IgG1-4) recognizing closed ADAMTS-13. Mann–Whitney U-test was used to compare closed and open-only titers in patients who survived vs died from an episode.

3 | RESULTS

3.1 | Development of an assay to study conformational sensitivity of anti–ADAMTS-13 IgG

Previous studies that have explored open and closed ADAMTS-13 have specifically quantified the relative amounts of open ADAMTS-13

present in plasmas of patients with iTTP [35], as opposed to quantifying the autoantibodies recognizing these 2 forms. In that study [35], a monoclonal antibody (mAb) that only recognizes open ADAMTS-13 was used to capture ADAMTS-13. The assays were performed in the presence and absence of an additional antibody that specifically opens all ADAMTS-13 to enable quantitation of the proportion of total ADAMTS-13 in a plasma sample that is open [17,35]. Therefore, the assay has been used primarily to assess the ability of autoantibodies from patients with iTTP to induce opening of ADAMTS-13 in plasma [17,35]. In contrast, we developed an ELISA to specifically measure the ability of anti–ADAMTS-13 autoantibodies from patients with iTTP to recognize ADAMTS-13 when in either its closed or open conformation.

Ordinarily, anti–ADAMTS-13 titer assays involve ADAMTS-13 being first adsorbed to the surface of a microtiter well. This causes ADAMTS-13 to open and reveal epitopes that might naturally remain concealed when in its closed conformation. Therefore, we specifically captured recombinant ADAMTS-13 to microtiter wells via its C-terminal 6×His tag, hypothesizing that this would capture and maintain ADAMTS-13 in its closed conformation. In parallel, we performed the same assay but included affinity-isolated anti–MP-Dis antibodies purified from a polyclonal anti–ADAMTS-13 to ADAMTS-13 [34]. Anti–MP-Dis antibodies were used as certain anti-MP antibodies have been previously shown to induce ADAMTS-13 opening [36]. Moreover, as the MP-Dis domains of ADAMTS-13 are less frequently targeted by iTTP autoantibodies, we rationalized that this would reduce the influence of any epitope competition with autoantibodies from patients with iTTP.

To verify that this assay specifically captures ADAMTS-13 in its closed form and that the anti–MP-Dis antibodies induce opening, an antispacer domain antibody (II-1) previously demonstrated to recognize a partially cryptic epitope in the spacer domain was added to wells [16,36]. As expected, there was an appreciable increase in the binding of this antibody to the open form compared with the closed form. As a control, an anti–thrombospondin repeat domains 2 to 4 antibody was added to different wells in parallel. As this antibody recognizes both open and closed forms of ADAMTS-13 similarly, no increase in signal was measured when ADAMTS-13 was opened (Figure 1A).

Although the majority of ADAMTS-13 normally exists in plasma in its closed conformation [17], this does not exclude the possibility that ADAMTS-13 may naturally be in equilibrium between closed and open forms, which we investigated further. For this, plasma from 2 patients with iTTP (TTP-A and TTP-B) was incubated in our assay for either 1 hour or 16 hours. If ADAMTS-13 were naturally in dynamic equilibrium, one might expect the proportion of antibodies recognizing closed ADAMTS-13 to increase with incubation time. However, the relative proportion of antibodies recognizing the 2 forms did not increase with incubation time (Figure 1B), suggesting that ADAMTS-13 does not naturally open and close in a dynamic manner. We next tested whether the recognition of the open and closed forms of ADAMTS-13 was influenced by the plasma dilution/anti–ADAMTS-13 IgG concentration. For this, we diluted plasmas 1:50, 1:25, and 1:10. Although (and as expected) anti–ADAMTS-13 titers increased as plasma dilutions decreased, the proportions of antibodies in each patient recognizing open and closed forms remained very similar (Figure 1C). It is important to note that using high concentrations of ADAMTS-13 (30 nM) and high dilutions of plasma (1:50 to 1:25) diminished the potential influence of any patient antibodies that themselves may open ADAMTS-13 to appreciably change the conformation of the majority of the closed ADAMTS-13 captured at the plate surface.

3.2 | Autoantibody binding in patients with iTTP increases when ADAMTS-13 is in its open conformation

To investigate the ability of antibodies to bind to the closed and open forms of ADAMTS-13, citrated plasma samples from the first presentation episodes of 70 patients with acute iTTP were analyzed (Figure 1D). At the first presentation, all patients with iTTP had antibodies recognizing open ADAMTS-13 to varying degrees. Except for plasmas from 2 patients (1 and 2), the plasmas from all other patients with iTTP also contained antibodies that recognized the closed ADAMTS-13. On average, 55% of the anti–ADAMTS-13 IgG in each sample from patients with iTTP recognized cryptic epitopes (median, 54%; range, 20%-100%) that were only exposed when ADAMTS-13 was opened (ie, open only: open titer minus closed titer). No patient with iTTP had higher antibody binding to closed ADAMTS-13. Plotting the open against closed titers revealed a strong and significant positive correlation (Spearman's rank correlation coefficient, $r = 0.913$; $P < .0001$; Figure 1E). There was a good correlation ($r = 0.585$; $P < .0001$) between the open ADAMTS-13 ELISA used here, and the standard ADAMTS-13 ELISA using ADAMTS-13 adsorbed to a microtiter well (not shown).

The ADAMTS-13 spacer domain is the region most commonly recognized by autoantibodies in iTTP [8,10]. Previous studies have revealed that the majority of antibodies recognizing the MDTCS bind to the spacer domain specifically [8,10]. Given that the spacer domain interacts with the C-terminal CUB domains in closed ADAMTS-13 [15,16,18,19], we hypothesized that there may be a stronger correlation between antibody titer against MDTCS and open ADAMTS-13 due to exposure of cryptic epitopes within the spacer domain. However, we found that the correlations between closed titer and MDTCS titer and open titer and MDTCS titer were very similar. Closed and open ADAMTS-13 titer correlated strongly and positively with MDTCS specificity (Figure 1F, G), suggesting that although cryptic epitopes exist in the spacer domain, there are epitopes in the N-terminal domains that are recognized by autoantibodies when ADAMTS-13 is in its closed form.

3.3 | Autoantibody titer against closed ADAMTS-13 does not correlate with ADAMTS-13 antigen levels or disease severity

To explore whether autoantibodies recognizing closed ADAMTS-13 might exert increased pathogenic effects, we correlated the closed ADAMTS-13 titer with disease-related parameters. We first hypothesized that autoantibodies binding to closed ADAMTS-13 may have a

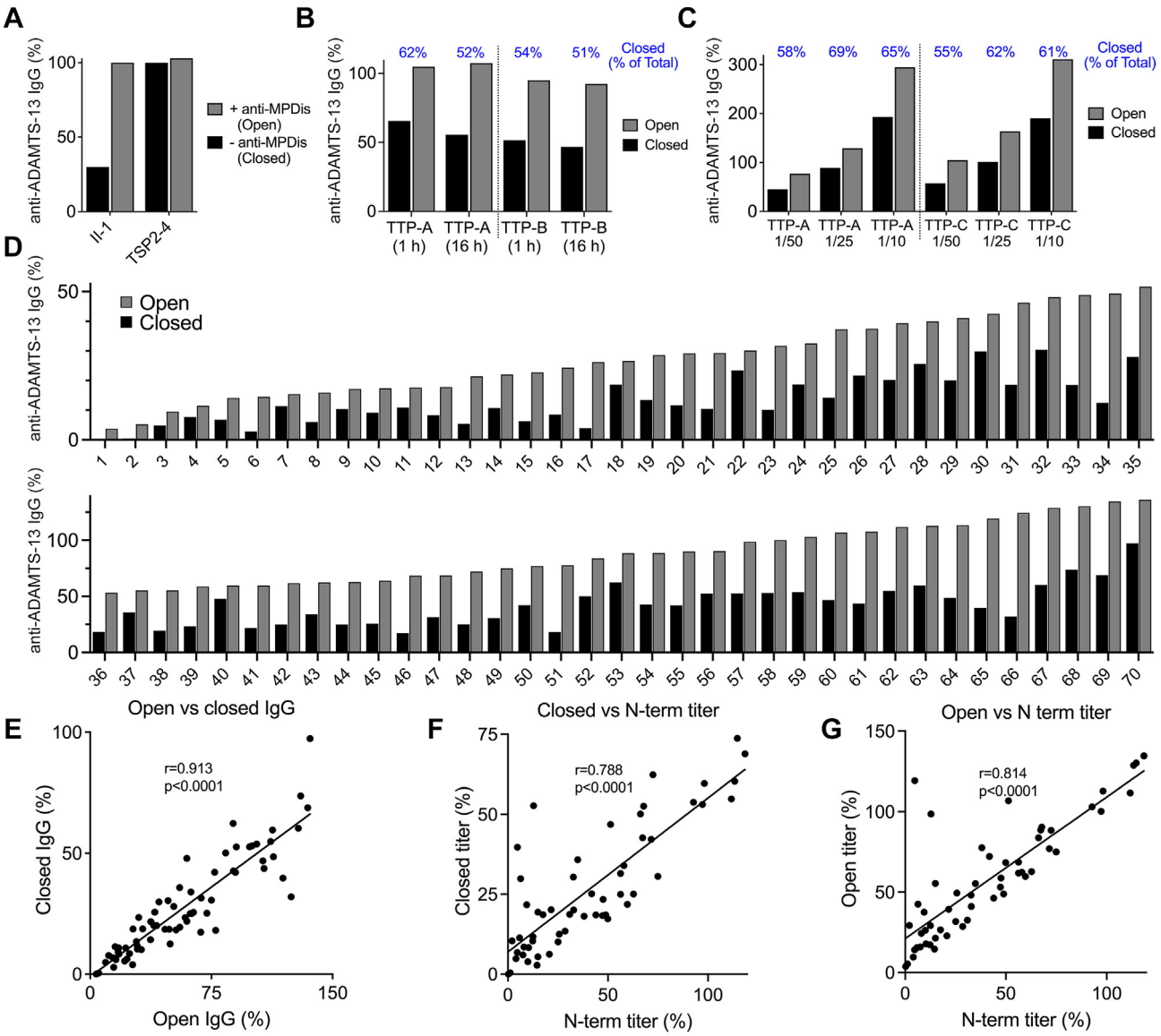

FIGURE 1 Thrombotic thrombocytopenic purpura (TTP) autoantibody binding to ADAMTS-13 is conformation-dependent. (A) Recombinant ADAMTS-13 (30 nM) was captured in its closed conformation via its His tag and preincubated without (black) or with an anti–ADAMTS-13 metalloprotease and disintegrin domain (anti-MP-Dis) antibody (grey). To ascertain whether the ADAMTS-13 was captured in its closed and open forms, partially conformation-dependent antispacer domain (II-1) or antithrombospondin repeat domains 2 to 4 (TSP2-4) (not conformation-dependent) antibodies were used to detect both forms. Opening of ADAMTS-13 with the anti-MP-Dis antibody specifically augmented the binding of II-1 (data presented are means of duplicate samples). (B) To ascertain whether ADAMTS-13 naturally opens/closes over time, the assay in (A) was repeated using plasmas from 2 patients with immune-mediated TTP (iTTP) incubated on the plate for either 1 hour or 16 hours. Recognition of open and closed ADAMTS-13 by patient antibodies remained similar over time (data presented are means of duplicate samples). (C) To ascertain whether antibody concentration in patients with iTTP changes ADAMTS-13 conformation, increasing dilutions of plasmas from 2 patients with TTP were used in our enzyme-linked immunosorbent assay. As plasma dilutions were decreased from 1:50 to 1:10, the anti–ADAMTS-13 titer increased, but the proportion of antibodies recognizing closed and open ADAMTS-13 remained constant (data presented are means of duplicate samples). (D) Plasma samples from 70 patients with iTTP at their first episode were analyzed for binding to the open and closed forms of ADAMTS-13. Each patient is represented by a number and ordered according to open titer (data presented are means of duplicate samples). (E) The titer against closed ADAMTS-13 is significantly and positively associated with the titer against open ADAMTS-13. (F and G) The titer against closed and open ADAMTS-13 also positively and significantly correlated with autoantibody titer against the N-terminal domains of ADAMTS-13. IgG, immunoglobulin G.

larger impact on the rate of clearance, as they could bind to ADAMTS-13 in its normal, circulating closed conformation. We used ADAMTS-13 antigen levels at presentation as a measure of the extent of antibody-induced clearance in these patients. There was no correlation between the closed titer and ADAMTS-13 antigen at presentation (Figure 2A). Open ADAMTS-13 titer also did not correlate with antigen levels (Figure 2B), as previously shown [8]. We then looked for correlations between closed titer and patient clinical

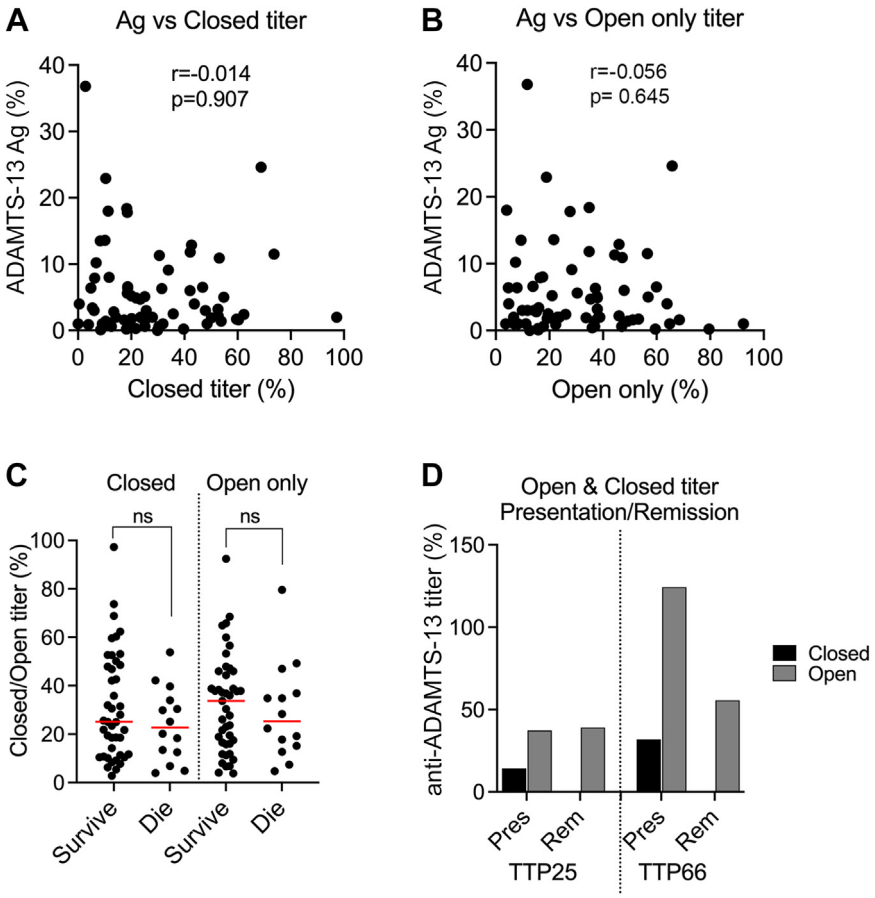

FIGURE 2 Patient clinical features are not dependent on closed antibody titer. (A) ADAMTS-13 antigen levels are plotted against antibody titer recognizing closed ADAMTS-13. (B) ADAMTS-13 antigen levels against antibody titer recognizing open ADAMTS-13 only (ie, open titer minus closed titer). (C) There was no difference in the anti–ADAMTS-13 titer against open or closed ADAMTS-13 in patients who survived or died. (D) In 2 patients with immune-mediated thrombotic thrombocytopenic purpura (TTP) (26 and 67) who entered remission (Rem) but had persistent anti–ADAMTS-13 autoantibodies, the autoantibodies in the remission samples (unlike at presentation [Pres]) did not recognize ADAMTS-13 in its closed conformation. Ag, antigen; ns, not significant.

features. There was no relationship between closed titer and age at TTP presentation, gender (data not shown), or episode outcome (survival vs death) (Figure 2C). Out of the 70 first-episode samples analyzed, 2 patients (TTP25 and TTP66) went into remission (and without subsequent relapse) but had persistent anti–ADAMTS-13 autoantibodies (15 and 16 days after the final PEX, respectively). Interestingly, in remission, only autoantibodies that recognized open ADAMTS-13 (ie, that recognized cryptic epitopes) could be detected, whereas the titer against closed ADAMTS-13 was undetectable (Figure 2D). At these times, patient TTP66 had ADAMTS-13 antigen and activity of approximately 40%, suggesting that the autoantibodies were noninhibitory. Patient TTP25 had ADAMTS-13 antigen levels in the normal range but ADAMTS-13 activity of approximately 6%, indicating persistent inhibitory autoantibodies.

3.4 | IgG subclass of bound antibodies and rate of ADAMTS-13 clearance

As anti–ADAMTS-13 titers recognizing open or closed ADAMTS-13 did not correlate with ADAMTS-13 antigen levels at presentation, we hypothesized that the IgG subclass of these antibodies may be important. IgG subclass is a determinant of IgG effector function [25]; therefore, differences between anti–ADAMTS-13 IgG subclass titers recognizing closed ADAMTS-13 might influence ADAMTS-13 clearance and thus correlate more strongly with ADAMTS-13 antigen levels at presentation. For IgG subclass analysis, citrated plasmas from 52 of the 70 acute iTTP first presentation episodes were analyzed. Anti–ADAMTS-13 titer for each IgG subclass was determined, and normalized absorbance values for each subclass were added to depict titer and IgG subclass proportion. For each individual patient with iTTP, the proportions of different IgG subclasses recognizing open or closed ADAMTS-13 were very similar (Figure 3A, B). Most patients had detectable anti–ADAMTS-13 IgG1. Anti–ADAMTS-13 IgG3 and IgG4 subclasses were also frequently detected, but very few patients with iTTP in this cohort had autoantibodies of the IgG2 subclass that recognized either open or closed ADAMTS-13. To explore the influence of IgG subclass upon ADAMTS-13 clearance, we separated patients with iTTP into quartiles for each IgG subclass (Q1 being the lowest titer and Q4 being the highest titer) and compared ADAMTS-13 antigen levels between these groups (Figure 3C–F). Classically, IgG1 and IgG3 have been more closely linked with effector functions that might promote clearance of immune complexes containing these subclasses [25]. One might, therefore, predict that for patients with iTTP in Q4, either IgG1 or IgG3 might have lower ADAMTS-13 antigen levels at presentation than for the patients in Q1. This, however, was not the case, as there was no significant difference in ADAMTS-13 antigen levels in Q1 and Q4 for either subclass. Although there was no difference in ADAMTS-13

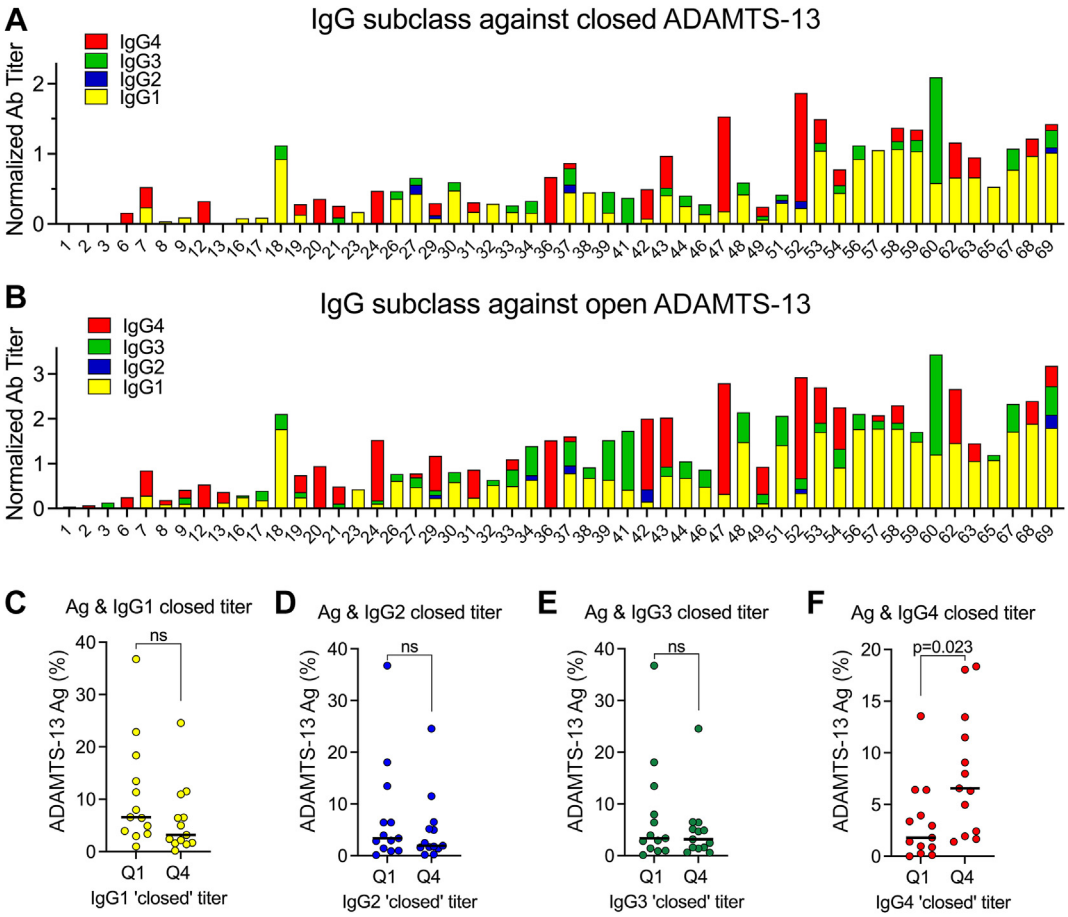

FIGURE 3 Immunoglobulin G (IgG) subclass against closed and open ADAMTS-13 in patients with immune-mediated thrombotic thrombocytopenic purpura (iTTP). The patients with iTTP analyzed in Figure 1 were assessed for the proportions of different IgG subclasses (IgG1-4) recognizing either (A) closed or (B) open ADAMTS-13. For each IgG subclass, patients were separated into quartiles according to the titer of each IgG subclass recognizing closed ADAMTS-13; quartile 1 (Q1) represents the lowest IgG subclass titer, and quartile 4 (Q4) represents the highest IgG subclass titer. There were no differences between the ADAMTS-13 antigen levels between Q1 and Q4 for (C) IgG1, (D) IgG2, or (E) IgG3. (F) However, ADAMTS-13 antigen levels were significantly higher in patients with TTP in Q4 for IgG4. Ab, antibody; Ag, antigen; ns, not significant.

antigen in Q1 and Q4 for IgG2, few patients were identified with anti–ADAMTS-13 IgG2. Patients in Q4 for anti–ADAMTS-13 IgG4 had significantly (albeit modestly) higher ADAMTS-13 antigens than those in Q1. Given that IgG4 has a severely reduced ability to clear antigens due to the markedly reduced affinity for Fcγ receptors, the primary effect of pathogenic IgG4 antibodies may be to inhibit ADAMTS-13 function, suggesting that patients with iTTP and higher IgG4 levels that inhibit ADAMTS-13 may present prior to their ADAMTS-13 antigen levels decreasing to as low as those in Q1.

3.5 | Changes in recognition of closed/open ADAMTS-13 and IgG subclass at relapse

Finally, we explored whether there were discernible changes in the recognition of closed/open ADAMTS-13 or IgG subclasses in patients who relapsed. Plasma samples at acute presentation before the initiation of treatment were tested. Each patient was treated with steroids and rituximab for their first and second episode. We specifically compared patient samples between first and second iTTP episodes (Figure 4A). There were no clear patterns associated with the closed and open titer between first and second episodes, except that the proportion of antibodies recognizing closed ADAMTS-13 remained similar. There were no patterns in the overall titers recognizing either closed or open-only anti–ADAMTS-13 between episodes (Figure 4B, C). The proportions of each IgG subclass recognizing either closed or open ADAMTS-13 were strikingly similar when comparing first and second episodes for the 4 patients for whom we performed subclass analysis (Figure 4D), strongly supporting the contention that iTTP relapse represents the re-emergence of the original autoimmune response, rather than de novo development of the disease.

4 | DISCUSSION

Autoantibody recognition of conformational epitopes is a characteristic of several autoimmune diseases, particularly those in which soluble (rather than cell surface) antigens are targeted. These include

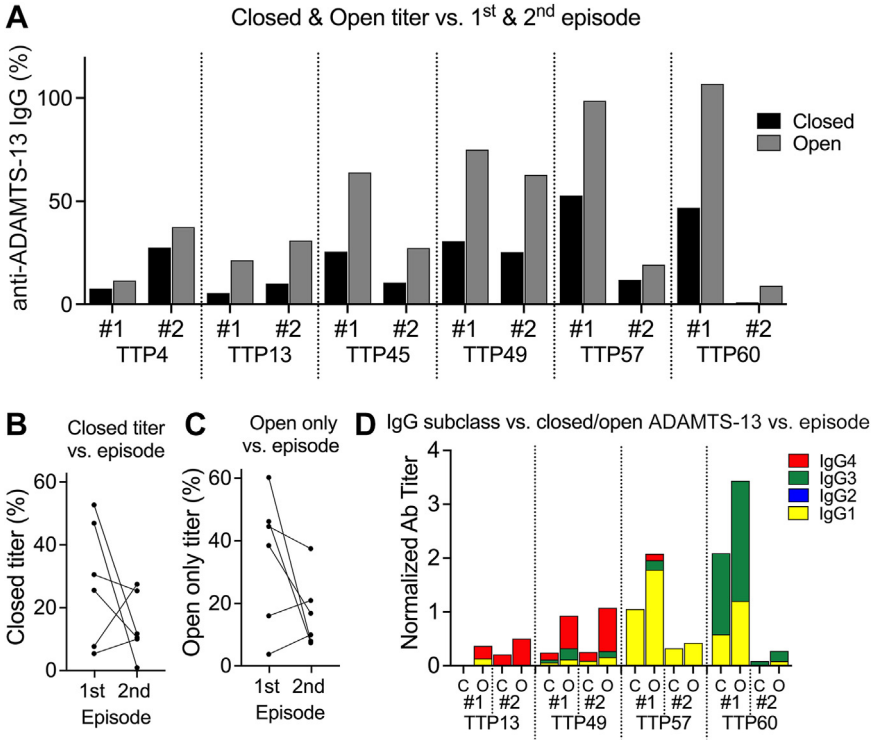

FIGURE 4 Open and closed anti–ADAMTS-13 titer and immunoglobulin G (IgG) subclass distribution in first and second episodes of patients with immune-mediated thrombotic thrombocytopenic purpura (TTP). (A) Anti–ADAMTS-13 titers against closed (black) and open (grey) ADAMTS-13 were measured in 7 patients with immune-mediated TTP at both first (#1) and second (#2, relapse) presentation samples. Changes in (B) closed titer and (C) open-only titer in each patient with immune-mediated TTP at the first and second presentations are presented. (D) The IgG subclass distribution of the autoantibodies recognizing closed (C) and open (O) ADAMTS-13 at the first and second presentations are shown for 4 of the 6 patients with immune-mediated TTP. Ab, antibody.

disorders such as heparin-induced thrombocytopenia, antiphospholipid syndrome, Wegener's granulomatosis, and iTTP. In heparin-induced thrombocytopenia, patient antibodies recognize cryptic epitopes on platelet factor 4 exposed after conformational changes induced by heparin binding [37]. Similarly, in antiphospholipid syndrome, the autoimmune targets include several different proteins that are only recognized when bound to phospholipid surfaces. The prime exemplars are antibodies directed towards β2 glycoprotein I, which are the most pathogenic and are associated with thrombosis [38,39]. Antibodies most commonly recognize cryptic epitopes in domain I of β2 glycoprotein I that are only revealed upon binding to phospholipids [40–42]. In Wegener's granulomatosis/granulomatosis with polyangiitis, autoantibody binding to activated proteinase-3 (but not the zymogen form) is frequently detected [43–45]. Based on these findings, it is tempting to hypothesize that the existence of these different conformational states may predispose certain antigens to increased susceptibility to autoimmune targeting. That ADAMTS-13 is a conformationally sensitive protein capable to exposure of cryptic epitopes when it naturally, albeit transiently, transitions from its closed to open states during the process of VWF proteolysis [16,36] may suggest that this could be a fundamental attribute that increases its susceptibility to autoimmune recognition. The finding that approximately 4% of healthy individuals harbor low-titer nonpathogenic autoantibodies against ADAMTS-13 provides some indication of the frequency with which ADAMTS-13 may be targeted [9].

To ascertain the frequency with which cryptic epitopes in ADAMTS-13 are recognized by autoantibodies of patients with iTTP, we developed a novel ELISA-based assay. Frequently, when flexible proteins are adsorbed onto microtiter wells, this leads to a degree of opening. This occurs with many proteins, including VWF and β2 glycoprotein I. This means that the standard assays used to measure anti–ADAMTS-13 autoantibody titer measure immunoreactivity against open ADAMTS-13. To capture ADAMTS-13 in its closed form, we immobilized it via a C-terminal His tag. Thereafter, we used the II-1 mAb originally cloned from a patient with TTP that preferentially recognizes the open form of ADAMTS-13 [16]. This antibody is slightly unusual as it binds to closed ADAMTS-13, but with a seemingly lower affinity than that to open ADAMTS-13, which is supported by previous studies [16]. Although it could be contended that a proportion of the ADAMTS-13 captured may be in an open conformation, this is argued against by the finding that some patient samples exhibited no immunoreactivity against ADAMTS-13 captured via its His tag (Figure 1D, patients 1 and 2, and 2D), supporting the contention that ADAMTS-13 captured in this way was uniformly in its closed conformation. Opening ADAMTS-13 with an anti–MP-Dis antibody was demonstrated to increase the binding of II-1, consistent with this inducing opening in a similar manner to the anti-MP mAb, 3H9 [36]. That this promoted ADAMTS-13 to open was further corroborated by our finding that every patient with iTTP tested exhibited increased immunoreactivity against ADAMTS-13 incubated with the anti–MP-Dis antibody (Figure 1D). We did not measure antibody binding to ADAMTS-13 in the presence of physiological shear stresses, so we were unable to rule out any influence that shear itself might exert upon ADAMTS-13 conformation and subsequent antibody binding. Although physiological shear impacts VWF, to date, there is no evidence to suggest it also affects ADAMTS-13 function. Assays to date

investigating ADAMTS-13 conformation have been measured in the absence of shear stress, suggesting that at least some of these dynamic changes do not require shear forces.

All of the 70 iTTP acute first presentation samples analyzed exhibited increased anti–ADAMTS-13 titers against open ADAMTS-13, revealing that an appreciable proportion of the autoantibodies specifically/preferentially bind to epitopes that are revealed when ADAMTS-13 is in its open conformation. Indeed, on average, 55% of the anti–ADAMTS-13 titer (range, 20%-100%) was attributable to antibodies that preferentially bind cryptic epitopes that are only exposed when ADAMTS-13 opens. Although this does not demonstrate that cryptic epitopes are the reason for autoimmune recognition, it clearly shows that they are a major component of autoimmune targeting. Consistent with this, 3 monoclonal antibodies cloned/isolated from patients with iTTP [36] were previously demonstrated to recognize cryptic epitopes in ADAMTS-13. In most cases, such antibodies bind the spacer domain. Given that the ADAMTS-13 CUB domains shield an extensive patch on the spacer domain when in its closed conformation [18], it is likely that the regions at or close to the spacer–CUB interface may represent the primary epitopes for such antibodies.

Our data, therefore, suggest the existence of 2 populations of autoantibodies: those that recognize the circulating closed form of ADAMTS-13 (and likely also the open form) and those that may only/preferentially bind to ADAMTS-13 when it is opened (ie, either during VWF proteolysis or by other autoantibodies capable of inducing this transition). Conceptually, these 2 populations might contribute differently to iTTP pathogenesis. We rationalized that autoantibodies binding to closed ADAMTS-13 might be more pathogenic due to their ability to bind the native closed/circulating form of ADAMTS-13. Given that antibody-mediated clearance of ADAMTS-13 antigen is a major pathogenic mechanism in iTTP, autoantibodies binding closed ADAMTS-13 might more efficiently promote the reduction in ADAMTS-13 antigen. However, when we correlated the closed anti–ADAMTS-13 IgG titer with ADAMTS-13 antigen levels at presentation (used as a marker of clearance), we found no evidence to support this contention (Figure 2A, B). This analysis may of course be too simplistic as it does not take into account the other major pathogenic deficiency mechanism, the functional inhibition of ADAMTS-13, which may happen to greater or lesser extents in different patients with iTTP. The closed ADAMTS-13 titer, just like the previously reported open ADAMTS-13 titer, did not correlate with any other parameters tested (age, gender, or survival). The lack of correlation of the closed titer with either ADAMTS-13 antigen or patient outcome suggests that this population of autoantibodies is not distinct (pathologically) from those that bind to open ADAMTS-13.

As closed titer did not correlate with ADAMTS-13 antigen levels, we investigated whether anti–ADAMTS-13 IgG subclass may play a role in clearance. Anti–ADAMTS-13 IgG may increase ADAMTS-13 clearance via antibody effector function, which is likely determined by the antibody Fc region. There are 4 IgG subclasses differing in their Fc regions: IgG1 and IgG3 antibodies have an increased affinity for activating Fcγ receptors compared with IgG2 and IgG4 [24,25]. Immune complexes of IgG1 and IgG3 subclasses may play a more prominent role in antigen clearance. We, therefore, measured the proportion of each IgG subclass recognizing either closed or open ADAMTS-13 for 52 of 70 patients with iTTP. As expected, the proportions of each IgG subclass recognizing closed and open ADAMTS-13 were strikingly similar for each individual patient. The proportion of IgG2 subclass recognizing ADAMTS-13 was very low, as previously described [29], consistent with the primary targeting of polysaccharides by IgG2 [24,25]. We again explored whether anticlosed ADAMTS-13 titer for each subclass might correlate with ADAMTS-13 antigen levels at presentation, but we observed no significant difference between the highest and lowest quartile for either IgG1 or IgG3 in ADAMTS-13 antigen levels at presentation. We detected a significant difference in ADAMTS-13 antigen levels when comparing the lowest and highest quartiles for IgG4, but the effect size was rather modest. There were a small number of patients with iTTP who exhibited autoantibodies of a single subclass against closed ADAMTS-13 (Figure 3A). These included patient 20, IgG4, 8% ADAMTS-13 antigen (Ag); patient 12, IgG4, 13% ADAMTS-13 Ag; patient 36, IgG4, 18% ADAMTS-13 Ag; patient 41, IgG3, 5% ADMTS-13 Ag; patient 57, IgG1, 2% ADAMTS-13 Ag; and patient 65, IgG1, <0.5% ADAMTS-13 Ag. Anecdotally, these may suggest that IgG1 and IgG3 might be more effective in clearing ADAMTS-13 than IgG4, but more extensive studies are required to explore this.

Two additional interesting findings also arose from our studies with respect to remission and relapse in patients with iTTP. First, some patients with iTTP go into remission despite the persistence of autoantibodies against ADAMTS-13. In our cohort of 70 patients who experienced their first iTTP episode, there were 2 that entered into clinical remission but remained positive for anti–ADAMTS-13 autoantibodies; both patients completely lost reactivity against closed ADAMTS-13 but remained strongly positive for antibodies recognizing open ADAMTS-13. Whether this is generalizable to other patients with detectable anti–ADAMTS-13 IgG in clinical remission requires further investigation. Given that increased proportions of "open" ADAMTS-13 in remission are associated with relapse risk [35,46], it would be interesting to investigate both the ADAMTS-13 conformation and anti–ADAMTS-13 open/closed antibody profile in such patients during remission to gauge any potential correlation. The second finding was associated with relapse. The analyses of presentation samples from the first and second episodes with respect to open and closed titer and autoantibody IgG subclass revealed that although the titers differed at presentation between episodes, the proportion of the different IgG subclasses for each patient was strikingly similar when comparing first and second presentations. Given that the IgG subclass proportions for each patient varied appreciably, each patient might have a particular "signature" for their autoimmune response. The similarities between first and second presentation subclass signatures support the contention that iTTP relapse most commonly occurs due to re-emergence of the clones responsible for the initial presentation as opposed to a *de novo* autoimmune response.

In conclusion, we have shown that antibody binding to ADAMTS-13 is highly dependent on its conformation, with many antibodies unable to bind to ADAMTS-13 when it is in its native closed conformation. On average, more than half of the anti–ADAMTS-13 autoantibodies recognize epitopes that are preferentially exposed in open ADAMTS-13. Although differences in these 2 populations of antibodies do not appear to correlate with iTTP pathogenesis, this finding makes it tempting to speculate that the existence of different ADAMTS-13 conformations increases the likelihood of its autoimmune recognition.

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AUTHOR CONTRIBUTIONS

M.I.U. performed experiments, analyzed the data, prepared the figures, and wrote the manuscript. M.R.T. analyzed the data and wrote the manuscript. M.A.S. analyzed the data and wrote the manuscript. J.T.B.C. analyzed the data, prepared the figures, and wrote the manuscript.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

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