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Generation of anti-idiotypic antibodies to detect anti-spacer antibody idiotopes in acute thrombotic thrombocytopenic purpura patients

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ABSTRACT

In autoantibody-mediated autoimmune diseases, autoantibody profiling allows patients to be stratified and links autoantibodies with disease severity and outcome. However, in immune-mediated thrombotic thrombocytopenic purpura (iTTP) patients, stratification according to antibody profiles and their clinical relevance has not been fully explored. We aimed to develop a new type of autoantibody profiling assay for iTTP based on the use of anti-idiotypic antibodies. Anti-idiotypic antibodies against 3 anti-spacer autoantibodies were generated in mice and were used to capture the respective anti-spacer idiotopes from 151 acute iTTP plasma samples. We next deciphered these anti-spacer idiotope profiles in iTTP patients and investigated whether these limited idiotope profiles could be linked with disease severity. We developed 3 anti-idiotypic antibodies that recognized particular idiotopes in the anti-spacer autoantibodies II-1, TTP73 or I-9, that are involved in ADAMTS13 binding; 35%, 24% and 42% of patients were positive for antibodies with the II-1, TTP73 and I-9 idiotopes, respectively. Stratifying patients according to the corresponding 8 anti-spacer idiotope profiles provided a new insight into the anti-spacer II-1, TTP73 and I-9 idiotope profiles in these patients. Finally, these limited idiotope profiles showed no association with disease severity. We successfully developed 3 anti-idiotypic antibodies that allowed us to determine the profiles of the anti-spacer II-1, TTP73 and I-9 idiotopes in iTTP patients. Increasing the number of patients and/or future development of additional anti-idiotypic antibodies against other anti-ADAMTS13 autoantibodies might allow idiotope profiles of clinical, prognostic value to be identified.

Introduction

In autoantibody-mediated autoimmune diseases, patients develop autoantibodies against self-antigens.[1] The autoantibody response can be directed to multiple self-antigens like in systemic sclerosis,[2] Sjögren syndrome,[3] and type 1 diabetes[4] or to a single self-antigen like myasthenia gravis[5] and Graves disease.[6] Patients suffering from the autoimmune disorder immune-mediated thrombotic thrombocytopenic purpura (iTTP) present with an autoantibody response against one antigen: the von Willebrand factor (VWF) cleaving protease ADAMTS13 (A Disintegrin And

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Metalloprotease with ThromboSpondin type 1 repeats, member 13).[7,8] Deficiency in ADAMTS13 leads to accumulation of hyper-active ultra-large VWF multimers that spontaneously interact with platelets. The resulting microthrombi block arterioles and capillaries, which leads to severe thrombocytopenia, hemolytic anemia and organ failure. The VWF cleaving protease ADAMTS13 consists of 14 domains: the metalloprotease (M), disintegrin-like (D), cysteine-rich (C) and spacer (S) domains, 8 thrombospondin type 1 repeats (T1-8) and 2 CUB domains.[9] It is known that the anti-ADAMTS13 autoimmune response in iTTP patients is polyclonal but 80-100% of patients possess autoantibodies targeting the cysteine-rich and spacer domain.[7,10-12] The standard treatment for iTTP is plasma exchange (PEX), often in combination with immunosuppressive agents (mainly steroids and rituximab).[8] Recently, the anti-VWF nanobody caplacizumab, used as front-line therapy together with PEX, hastened TTP recovery, opening promising perspectives to improve the prognosis of the disease.[13,14] Splenectomy is only performed in the most severe patients, when other measures have failed.[8,15]

Since autoimmune diseases manifest differently among patients and have a chronic course with recurring acute bouts, biomarkers are identified that allow patient stratification to predict disease outcome and prognosis and to adapt specific treatment.[16] Obviously, autoantibodies are useful biomarkers in autoimmune diseases and autoantibody profiling has been shown to be valuable in stratifying patients with autoimmune disorders.[17,18] On the one hand, autoantibody profiling approaches are based on the binding of the patient autoantibodies to the disease causing antigen (recombinant proteins, fragments of these, or peptides).[19,20] Whereas, on the other hand, autoantibody profiling can be performed independently of the antigen using anti-idiotypic antibodies that recognize autoantibodies that bind to the antigen (Figure 1).[21] Anti-idiotypic antibodies can be generated by immunizing mice with purified or cloned antigen-binding antibodies.[22-24] Antibodies that bind to particular idiotopes involved in antigen binding can then be used to detect specific autoantibodies in patient plasma or serum.[21] Finally, even if the disease-causing antigen is not known, antibody profiling can lead to the identification of disease-linked peptides using next-generation sequencing[25] and mass spectrometry[26,27] of the total antibody response in autoimmune disease patients.

Furthermore, iTTP is a chronic disease with a variable disease outcome and risk of relapse.[28] Levels of ADAMTS13 activity, anti-ADAMTS13 autoantibody subtypes, ADAMTS13 antigen levels or a combination of these have been used to identify patient groups with a worse disease outcome or a higher risk of relapse.[28-35] Although the outcome of the different studies varies, it has been shown, for example, that an ADAMTS13 activity <10% during acute disease is linked with an increased risk of relapse,[35] and that presenting anti-ADAMTS13 autoantibody and ADAMTS13 antigen levels predict prognosis.[31] In addition, prognostic scoring systems based on clinical and/or laboratory parameters have been set up to predict severe cases and patients at risk; from 1987 with the Rose index,[36,37] to more recently with the PLASMIC

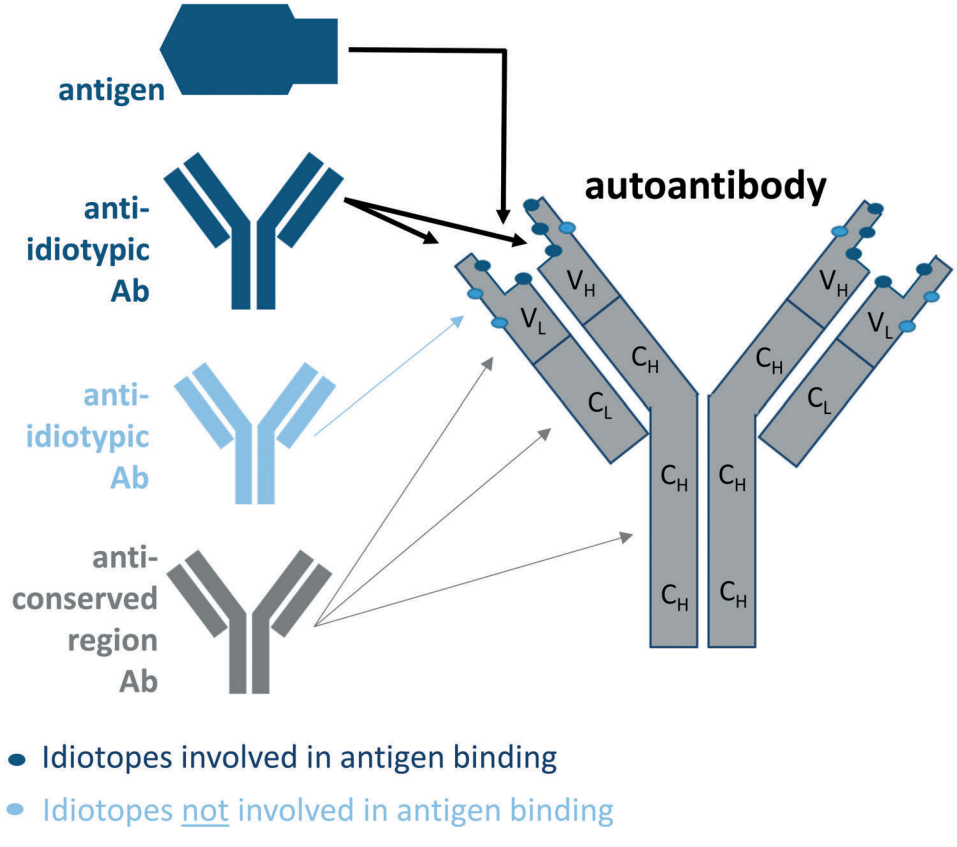


Figure 1. Anti-idiotypic antibodies directed against different idiotopes in autoantibodies. A representative autoantibody is illustrated with the variable regions of heavy (VH) and light (VL) chains and the constant regions of heavy (CH) and light (CL) chains. Variable regions consist of framework regions and complementarity determining regions (CDRs). The CDRs are unique among antibodies and consist of idiotopes that are involved in binding to the (self)-antigen (dark blue dots) and idiotopes that are not involved in binding to the (self)-antigen (light blue dots). All other regions are conserved regions (gray) between different antibodies, and make up the framework regions of the VH and VL and the constant regions of CH and CL chains. Anti-idiotypic antibodies (Abs) bind to idiotopes involved in (self)-antigen binding, shown in dark blue, whereas anti-idiotypic antibodies that bind to idiotopes not involved in binding to the (self)-antigen are shown in light blue. Anti-conserved region antibodies are in gray.

score,[38] and the score by Benhamou *et al.*[39] The predictive model set up by Benhamou *et al.* takes into account age, lactate dehydrogenase (LDH) levels, and cerebral involvement, and detects early death in acquired severe ADAMTS13 deficiency-associated idiopathic TTP.[39] However, in iTTP, autoantibody profiling to stratify patients has not yet been fully explored.

In this study, we developed an autoantibody profiling assay for iTTP using anti-idiotypic antibodies that recognize particular idiotopes on anti-ADAMTS13 autoantibodies, idiotopes that are involved in ADAMTS13 binding (Figure 1). Since the ADAMTS13 spacer domain seems to be the main immunogenic region targeted in these patients,[29] we generated an anti-idiotypic antibody against 3 available cloned human anti-spacer autoantibodies. The selected anti-idiotypic antibodies were then used to screen 151 iTTP plasmas for the presence of autoantibodies with the same idiotopes across patients, which resulted in stratification of iTTP patients according to these anti-spacer idiotope profiles. We next investigated in a subgroup of 95 patients whether certain anti-spacer idiotope profiles could be linked with disease severity.

Methods

Immunization strategy and characterization of anti-II-1, anti-TTP73 and anti-I-9 antibodies

Anti-II-1, anti-TTP73 and anti-I-9 antibodies were developed by immunizing BALB/c mice (Janvier Labs, Le Genest-Saint-Isle, France) with the cloned human anti-spacer autoantibodies II-1,[40] TTP73, or I-9,[41] respectively (see the immunization strategy in the *Online Supplementary Appendix*). The binding of purified anti-II-1, anti-TTP73 or anti-I-9 antibodies to II-1, TTP73, and I-9, respectively, and to the conserved regions (Figure 1, gray) in human immunoglobulin G (IgG) antibodies were identified using ELISA.

ELISA to identify anti-II-1, anti-TTP73 and anti-I-9 antibodies that inhibit the binding of anti-spacer autoantibodies II-1, TTP73, or I-9 to ADAMTS13, respectively

Human anti-spacer autoantibodies II-1, TTP73 or I-9 (constant final EC50: 0.04, 0.85 and 0.04 μg/mL, respectively) (see *Online Supplementary Methods*), were pre-incubated with a 1:2 dilution series of murine anti-II-1, anti-TTP73, or anti-I-9 antibodies (final start concentration 10 μg/mL) respectively, in a pre-blocked plate. After 30 minutes, samples were transferred to a recombinant human (rh)ADAMTS13 [2.7 μg/mL in phosphate buffered saline (PBS)] coated plate. Bound human anti-spacer autoantibodies II-1, TTP73, or I-9 were detected using a mixture of HRP-labeled anti-human $IgG_{1\text{-}4}$ (IgG1: 1/20,000 and $IgG_{2\text{-}4}$: 1/2,000; Sanquin, Amsterdam, the Netherlands).

ELISA to study the binding of the anti-idiotypic antibodies to the anti-spacer idiotopes of II-1, TTP73, and I-9

Murine anti-idiotypic antibodies 17H9 (anti-II-1 antibody), 9G12 (anti-TTP73 antibody) and 7D10 (anti-I-9 antibody) were coated at 5 μg/mL in carbonate/bicarbonate coating buffer (50 mM Na_2CO_3/$NaHCO_3$, pH 9.6). After blocking, human anti-spacer autoantibodies II-1, TTP73, and I-9 were added at a starting concentration of 1 μg/mL and further diluted 1:2. Bound anti-spacer autoantibodies were detected by adding a mixture of HRP-labeled anti-human $IgG_{1\text{-}4}$ antibodies (Sanquin).

Patients' samples

Detailed information about the 151 iTTP plasma samples can be found in the *Online Supplementary Methods*. The study protocol was approved by the Medical Ethical Committee of the University Medical Center Utrecht (Utrecht, the Netherlands), the Ethics Committee of Hospital Pitié-Salpêtrière and Hospital Saint-Antoine (Paris, France), and the Ethics Committee of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico (Milan, Italy). The study was carried out in accordance with the Declaration of Helsinki.

ELISA to identify the presence of anti-spacer idiotope profiles in plasmas of acute iTTP patients using the newly developed anti-idiotypic antibodies

Murine anti-idiotypic antibody 17H9 (anti-II-1 antibody), 9G12 (anti-TTP73 antibody), or 7D10 (anti-I-9 antibody) were coated at 5 μg/mL. After blocking, patient plasma (starting dilution 10%, v/v) was added and diluted 1:2. Bound patient antibodies were detected with HRP-labeled anti-human $IgG_{1\text{-}4}$ antibodies (Sanquin).

Statistical analysis

Graphpad Prism v.5.03 software (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis. Further details of the methods used are available in the *Online Supplementary Methods.*

Results

Development of anti-idiotypic antibodies against idiotopes in anti-spacer autoantibodies II-1, TTP73 or I-9 involved in ADAMTS13 binding

To generate anti-idiotypic antibodies recognizing particular idiotopes in anti-spacer autoantibodies involved in ADAMTS13 binding, 3 cloned human anti-spacer autoantibodies with different epitopes and inhibitory characteristics were available: II-1,[40] TTP73[42] and I-9[41] (see *Online Supplementary Methods*) and were used to immunize BALB/c mice. As the injected anti-spacer autoantibodies are full IgG antibodies in which the variable regions are grafted onto a human IgG1 constant region,[40,41] the mice developed antibodies that either recognized conserved regions (e.g. constant regions: C_H and C_L and framework regions in V_H and V_L) (Figure 1, gray parts) or idiotopes in the complementarity determining regions (CDRs) of the V_H and V_L of II-1, TTP73 and I-9 (Figure 1, dark and light blue dots). We obtained 1 mouse monoclonal antibody that recognized anti-spacer autoantibody II-1, 2 that recognized anti-spacer autoantibody TTP73 and 10 that recognized anti-spacer autoantibody I-9 (Figure 2A) as the generated antibodies bound to the coated anti-spacer autoantibodies II-1, TTP73 or I-9, respectively. To identify which of the generated monoclonal antibodies recognized the conserved part of the human autoantibodies (C_H, C_L and framework regions in V_H and V_L) (Figure 1, gray parts), their binding to a pool of purified human IgG antibodies was studied. Monoclonal antibody 17H9 recognizing anti-spacer autoantibody II-1 did not recognize the conserved part of the coated human IgG antibodies (Figure 2B), while 1 of the monoclonal antibodies (20H3) recognizing anti-spacer autoantibody TTP73 and 9 of the monoclonal antibodies (1E6, 5C8, 6C9, 7E8, 9F9, 9G9, 9H4, 11F7, and 14G6) recognizing anti-spacer autoantibody I-9 did bind to the conserved part of the coated human IgG antibodies

(Figure 2B). Therefore, monoclonal antibodies 17H9, 9G12 and 7D10 are anti-idiotypic antibodies that target idiotopes in the CDRs of V_H and V_L of anti-spacer autoantibody II-1, TTP73, or I-9, respectively (Figure 2B).

We next aimed to identify if the anti-idiotypic antibodies recognizing particular idiotopes in the anti-spacer autoantibodies II-1, TTP73, and I-9 are anti-idiotypic antibodies that are involved in ADAMTS13 binding (Figure 1, dark blue antibody). To do so, we used a competition ELISA where we studied if the binding of anti-spacer autoantibodies II-1, TTP73 and I-9 could be inhibited by their respective anti-idiotypic antibody. The 3 developed anti-idiotypic antibodies (17H9, 9G12 and 7D10) inhibited the binding of their respective anti-spacer autoantibodies (II-1, TTP73 and I-9) to rhADAMTS13 (Figure 2C).

In conclusion, we developed 3 anti-idiotypic antibodies that recognize particular idiotopes in the anti-spacer autoantibodies II-1, TTP73 and I-9 that are involved in ADAMTS13 binding, as they strongly inhibit the binding of anti-spacer autoantibodies II-1, TTP73 or I-9, respectively, to rhADAMTS13.

Anti-idiotypic antibodies and their binding to idiotopes in II-1, TTP73 and I-9

Since anti-spacer autoantibodies II-1 and I-9 have overlapping but different epitopes (see *Online Supplementary Methods*),[43] they will have both shared and unique idiotopes. We, therefore, investigated whether anti-idio-

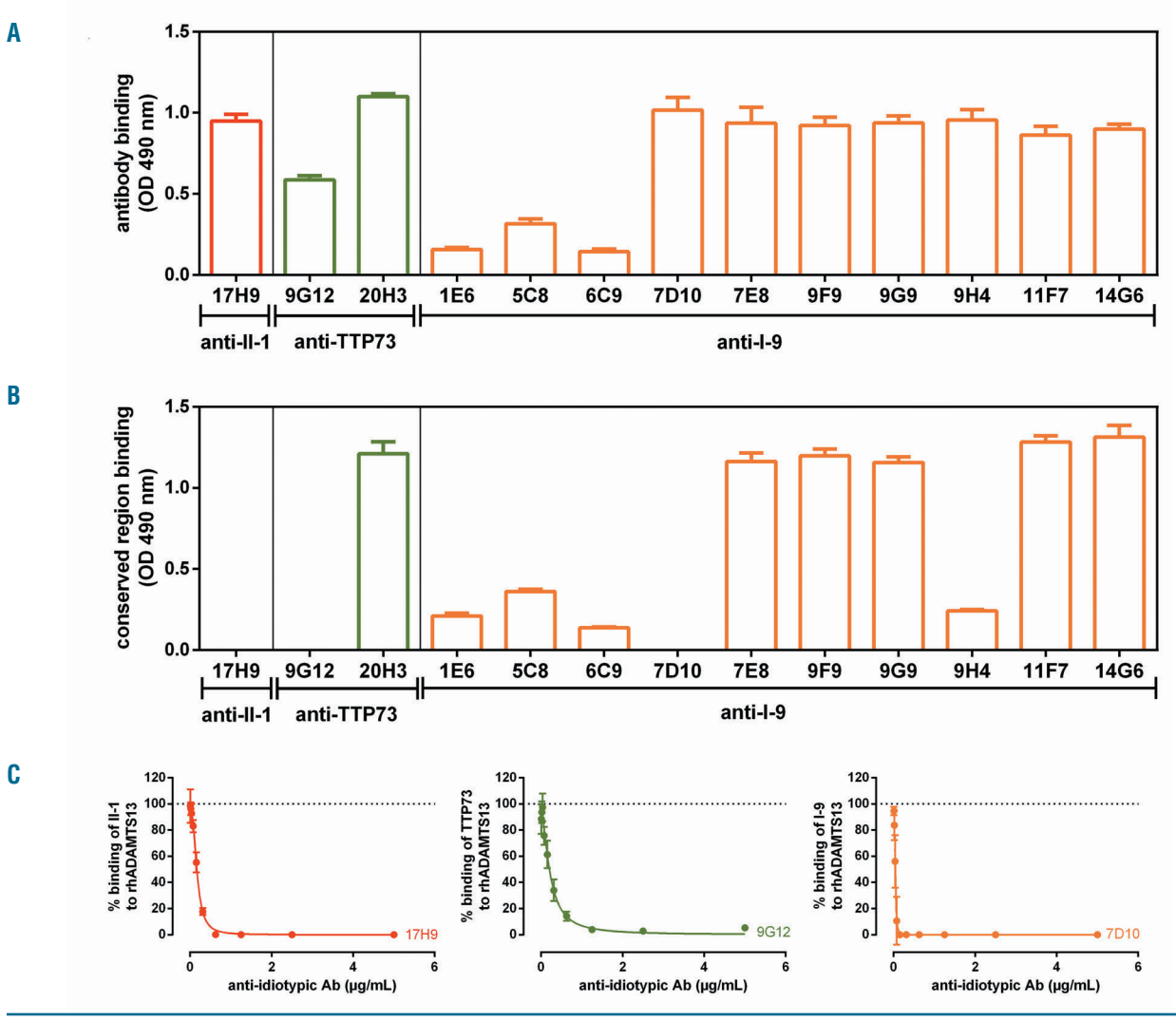


Figure 2. Development and characterization of anti-idiotypic antibodies that inhibit the binding of respectively anti-spacer autoantibody II-1, TTP73 or I-9 to rhADAMTS13. (A) Binding of purified murine anti-II-1 (red), anti-TTP73 (green) and anti-I-9 (orange) antibodies to coated human anti-spacer autoantibodies II-1, TTP73 or I-9. Bound murine anti-II-1, anti-TTP73 and anti-I-9 antibodies were detected using GAM-HRP. Murine anti-II-1, anti-TTP73 or anti-I-9 antibody binding was expressed as relative absorbance values [mean ± Standard Deviation (SD), n=3] with absorbance of the respective positive controls (sera of mice immunized with either II-1, TTP73 or I-9) set as 1. (B) Binding of purified murine anti-II-1 (red), anti-TTP73 (green) and anti-I-9 (orange) antibodies to a coated human IgG pool. Bound murine anti-II-1, anti-TTP73 and anti-I-9 antibodies were detected using GAM-HRP. Murine anti-II-1, anti-TTP73 or anti-I-9 antibody binding was expressed as relative absorbance values (mean±SD, n=3) with absorbance of the respective positive controls (sera of mice immunized with either II-1, TTP73 or I-9) set as 1. Binding to coated human IgG pool indicates that the murine antibodies bind to the conserved regions of antibodies. (C) Inhibition of anti-spacer autoantibody binding to rhADAMTS13 by anti-idiotypic antibodies. A 1:2 dilution of murine anti-II-1 (red), anti-TTP73 (green) or anti-I-9 antibodies (orange) were pre-incubated with constant amounts of anti-spacer autoantibody II-1, TTP73 and I-9, respectively, before addition to a rhADAMTS13 coated 96-well microtiter plate. Bound II-1 (red), TTP73 (green) and I-9 (orange) antibodies were detected using a mixture of anti-human IgG_{1-4}-HRP. Data are expressed as % binding (mean±SD, n=3) of anti-spacer autoantibodies II-1 (red), TTP73 (green) or I-9 (orange) to rhADAMTS13 in the presence of the competing murine anti-II-1 (17H9), anti-TTP73 (9G12) or anti-I-9 (7D10) antibody relative to the binding of II-1, TTP73 or I-9 in the absence of anti-idiotypic antibodies (dotted lines). OD: optical density.

typic antibodies developed against anti-spacer autoantibody II-1 (17H9) and I-9 (7D10) recognized shared or unique idiotopes in II-1 and I-9. As a control, we included the anti-idiotypic antibody 9G12 developed against the anti-spacer autoantibody TTP73, which does not have an overlapping epitope with II-1 and I-9.

The anti-idiotypic antibody against anti-spacer autoantibody II-1 (17H9) recognized a unique idiotope in II-1 as it only captured II-1 and not anti-spacer autoantibodies I-9 and TTP73 (Figure 3A). As expected, the anti-idiotypic antibody against TTP73 (9G12) also recognized a unique idiotope in anti-spacer autoantibody TTP73 as it only captured TTP73 and not anti-spacer autoantibodies II-1 and I-9 (Figure 3B). In contrast, the anti-idiotypic antibody (7D10) against the anti-spacer I-9 idiotope captured both anti-spacer autoantibody I-9 and II-1 (Figure 3C) showing that anti-idiotypic antibody 7D10 recognizes a common idiotope in II-1 and I-9.

In conclusion, these data show that the anti-idiotypic antibodies against anti-spacer autoantibody II-1 (17H9) and TTP73 (9G12) recognize a unique idiotope in II-1 and TTP73, respectively, whereas the anti-idiotypic antibody developed against anti-spacer autoantibody I-9 (7D10) recognizes an idiotope present in both anti-spacer autoantibodies II-1 and I-9 (Figure 3).

Identification of anti-spacer idiotope profiles in plasmas of acute iTTP patients using the newly developed anti-idiotypic antibodies

As a first step, we screened the plasma of 151 iTTP patients for the presence or absence of the anti-spacer II-

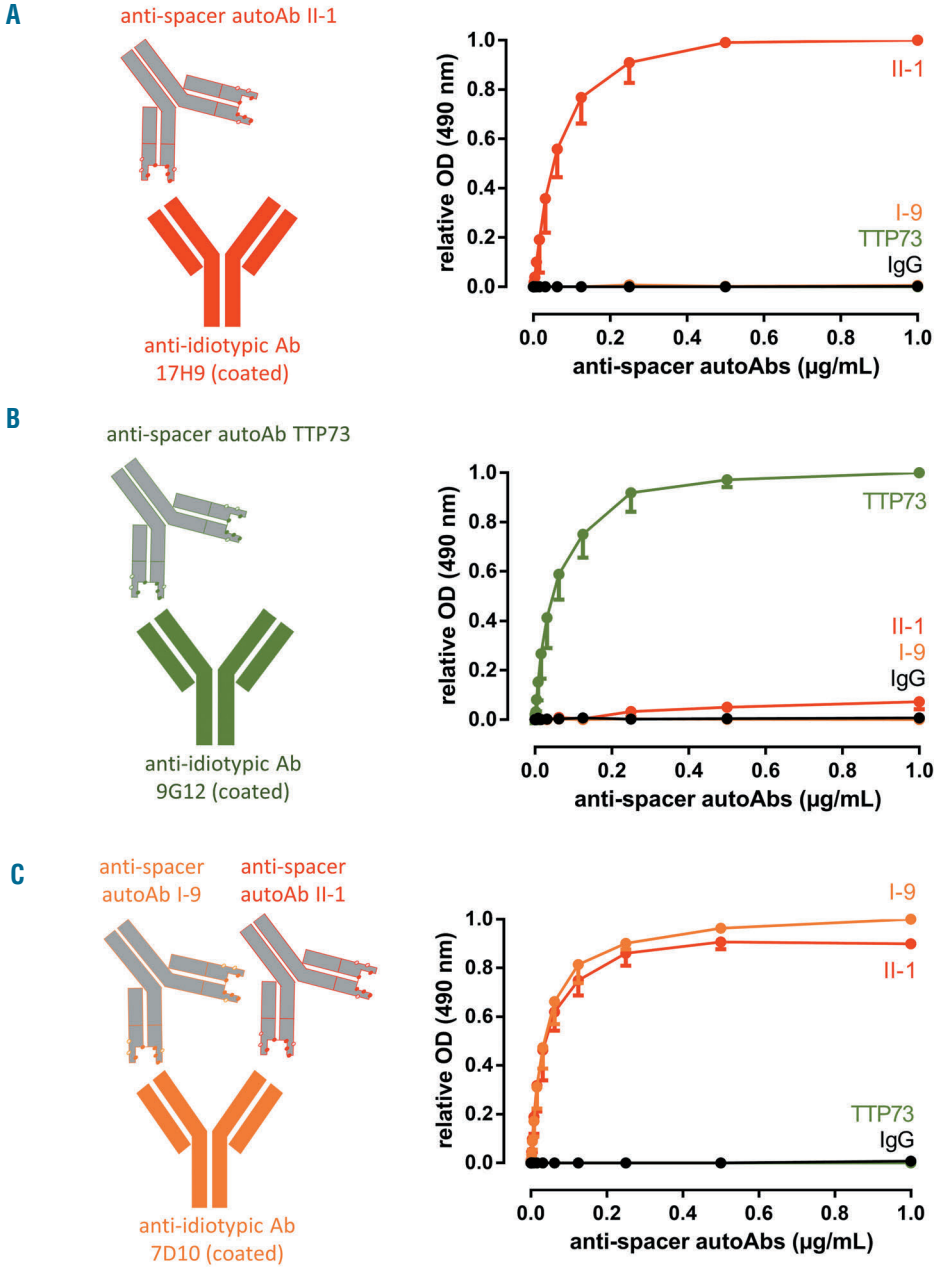


Figure 3. Anti-idiotypic antibodies and their binding to the anti-spacer idiotopes of II-1, TTP73 and I-9. Binding of human anti-spacer autoantibodies (autoAbs) II-1 (red), TTP73 (green), I-9 (orange) and of a pool of human IgG antibodies (negative control, black) to coated murine anti-idiotypic antibody (Ab) 17H9 developed against II-1 (A), 9G12 developed against TTP73 (B) and 7D10 developed against I-9 (C). Bound human anti-spacer autoantibodies II-1, TTP73 and I-9 were detected using a anti-human IgG_{1-4}. Data are expressed as relative absorbance values (mean+Standard Deviation, n=3) [da sottolineare il simbolo +]with absorbance of binding of II-1, TTP73 and I-9 at 1 µg/mL to their respective anti-idiotypic antibodies set as 1. OD: optical density.

1, TTP-73 and I-9 idiotopes using the 3 newly developed anti-idiotypic antibodies. In a second step, we stratified the patients according to their anti-spacer idiotope profile.

The 151 iTTP plasma samples were all collected during an acute iTTP episode (see the *Online Supplementary Methods* for details). All patients presented with severe ADAMTS13 deficiency (< 10% activity) and detectable anti-ADAMTS13 IgG titers. Anti-ADAMTS13 IgG titers ranged from 16 to ≥100 IU/mL (median: 87 IU/mL) (Figure 4). Of the 151 iTTP patients, 34% (52 out of 151) were positive for antibodies with the anti-spacer II-1 idiotope (recognized by anti-idiotypic antibody 17H9) (Figure 4A, red dots) with median anti-spacer II-1 idiotope levels of 47 ng/mL (Figure 4A, red squares). Twenty-five percent (37 out of 151) of the patients were positive for antibodies with anti-spacer TTP73 idiotope (recognized by anti-idiotypic antibody 9G12) (Figure 4B, green dots) with median anti-spacer TTP73 idiotope levels of 174 ng/mL (Figure 4B, green squares). Forty-two percent (63 out of 151) of the patients were positive for antibodies with anti-spacer I-9 idiotope (recognized by anti-idiotypic antibody 7D10) (Figure 4C, orange dots) with median anti-spacer I-9 idiotope levels of 57 ng/mL (Figure 4C, orange squares).

We next stratified the acute iTTP patients according to their anti-spacer idiotope profile (Figure 5). The 8 possible profiles correspond to the presence of either 1, 2, 3 or none of the 3 anti-spacer idiotopes. All 8 anti-spacer idiotope profiles were identified in the iTTP patient cohort (n=151) (Figure 5). In 28% (42 out of 151) of the patients, only one particular idiotope could be detected in the plasma, with 8% (12 out of 151) having the II-1 idiotope (profile 1), 4% (6 out of 151) having the TTP73 idiotope (profile 2), and 16% (24 out of 151) having the I-9 idiotope (profile 3). In 19% (28 out of 151) of the patients, 2 idiotopes were identified in their antibody repertoire, with 5% (7 out of 151) having II-1 and TTP73 idiotopes (profile 4), 10% (15 out of 151) having II-1 and I-9 idiotopes (profile 5), and 4% (6 out of 151) having I-9 and TTP73 idiotopes (profile 6). In 12% (18 out of 151) of the patients, all 3 idiotopes were present in their antibody repertoire (profile 7). In 42% (63 out of 151) of the patients, none of the 3 idiotopes were detected (profile 8).

In conclusion, using the 3 developed anti-idiotypic antibodies, we here for the first time unraveled the specific II-1, TTP73, and I-9 idiotope profiles in iTTP patients and showed that 58% of the patients had antibodies with II-1, TTP73, and I-9 idiotopes in their plasma, and this in different combinations, while 42% of the patients were negative for these idiotopes.

Anti-spacer idiotope profiles and their possible link with disease severity

We next investigated whether the identified anti-spacer idiotope profiles (Figure 5) could be linked with disease severity, although the number of patients per profile group was rather low and we only screened for the presence or absence of 3 anti-spacer idiotopes. As a measure of disease severity, we studied disease outcome and applied treatment strategy. This part of the study was performed on the 95 patients of the French Reference Center for Thrombotic Microangiopathy, as detailed information on laboratory, clinical and outcome parameters were available for these patients (*Online Supplementary Table S2*).

We first analyzed whether the anti-spacer idiotope profiles could be linked with disease outcome. Disease outcome was previously identified in the patients at time of diagnosis by determining a score defined by Benhamou *et al.*[39] This score (either 1, 2, 3 or 4) is a risk score for early death in TTP based on three factors related to clinical and biological presentation (age, high LDH levels, and cerebral involvement). A score of ≥3 has a positive predictive value for mortality (patients at risk of 30-day mortality after

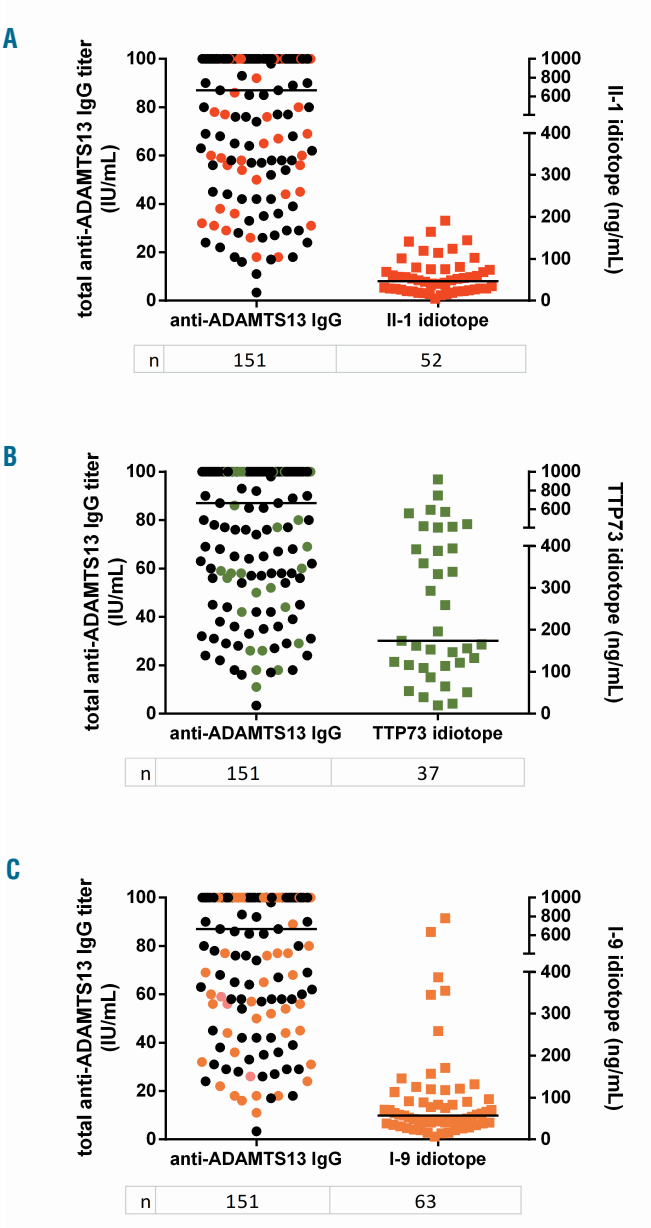


Figure 4. Total anti-ADAMTS13 IgG titers and anti-spacer II-1, TTP73 and I-9 idiotope concentrations in acute immune-mediated thrombotic thrombocytopenic purpura (iTTP) patients. (A-C) Total anti-ADAMTS13 IgG titers (IU/mL) were determined *via* TECHNOZYM® except for three samples (ID 124, 128 and 130) which were determined *via* an in house developed anti-ADAMTS13 IgG ELISA (§ *in Online Supplementary Table S1*). Anti-spacer II-1 (red square) (A), TTP73 (green square) (B), and I-9 (orange square) (C) idiotope concentrations were determined by adding patient plasma to coated murine anti-idiotypic antibody 17H9 (A), 9G12 (B) or 7D10 (C). Bound human autoantibodies were detected using a mixture of anti-human IgG_{1-4}-HRP. A dilution series of human anti-spacer autoantibodies II-1 (A), TTP73 (B) or I-9 (C) was used as a calibration curve to determine idiotope concentrations (ng/mL). Median is represented for total anti-ADAMTS13 IgG titers and II-1, TTP73 and I-9 idiotope concentrations.

treatment initiation) and a score <3 has a negative predictive value.[39] To check whether the disease outcome parameter could be linked with specific anti-spacer idiotope profiles, we performed χ^2-based analysis. However, none of the anti-spacer idiotope profiles could be linked with a score of ≥3 (χ^2, not significant) (Figure 6A). In line with this, there was no link between the anti-spacer idiotope profiles and the individual factors related to the score by Benhamou *et al.*[39] (age: ANOVA, not significant; cerebral involvement and high LDH levels: χ^2, not significant) (*Online Supplementary Figure S1*).

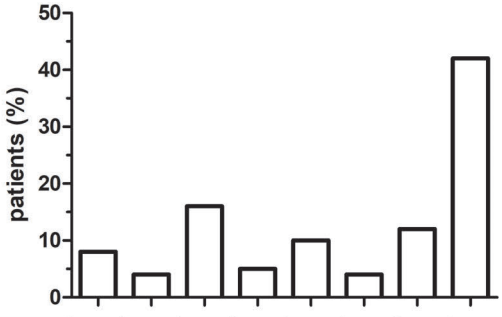
We next used the same approach to investigate whether anti-spacer idiotope profiles could be linked with the applied treatment strategy. We, therefore, compared the anti-spacer idiotope profiles in patients treated with PEX with/without rituximab and patients treated with PEX with/without rituximab and additional treatment(s) (either steroids or other immunosuppressive drugs, e.g. cyclophosphamide, bortezomib; or/and caplacizumab or/and splenectomy) (*Online Supplementary Table S2*). However, also treatment could not be linked with anti-spacer idiotope profiles (χ^2, not significant) (Figure 6B).

Discussion

In this study, we successfully generated three anti-idiotypic antibodies that specifically recognized the idiotopes of anti-spacer autoantibodies II-1, TTP73, and I-9. With this anti-idiotypic assay, we could for the first time identify the presence or absence of anti-spacer II-1, TTP73, and I-9 idiotopes in iTTP patients. In addition, grouping the patients according to the absence or presence of one, two or three of the anti-spacer idiotopes revealed an until now unknown insight into the anti-spacer II-1, TTP73 and I-9 idiotopes in these patients. Although the resulting idiotope profiles could not be linked with disease severity, our data show that anti-idiotypic antibodies are interesting tools to determine an antibody profile in patients with any autoimmune disease.

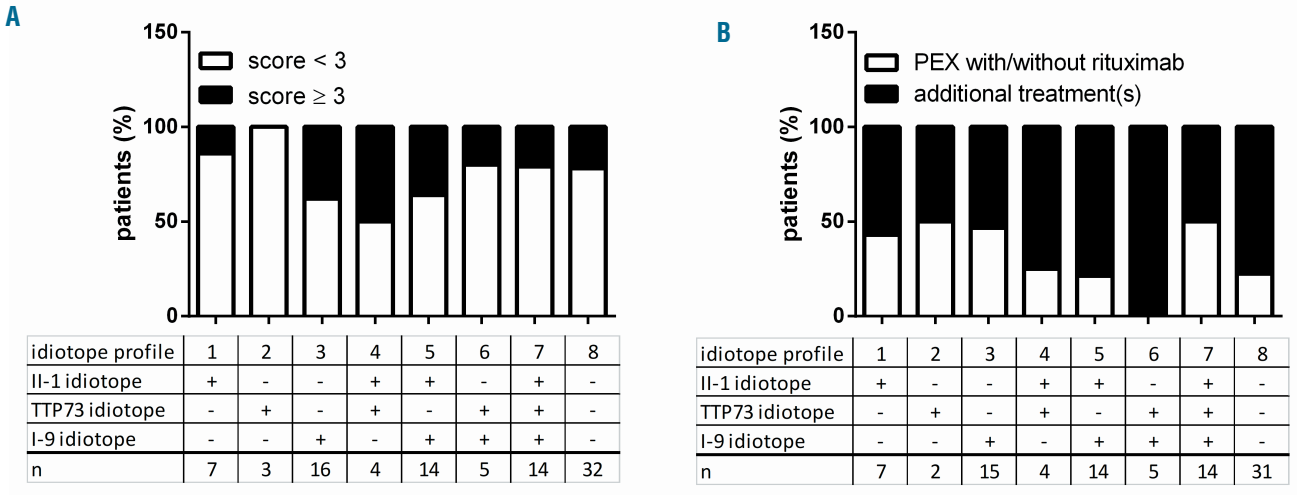
Many studies have used groups of ADAMTS13 domains to identify which ADAMTS13 domains (e.g. MDTCS, MDT, CS, T2-C2, T2-T8, C1-C2) are targeted by anti-ADAMTS13 autoantibodies in individual iTTP patients. All these studies concluded that the immune response in iTTP patients is polyclonal with an immunodominant epitope in the cysteine-rich/spacer domain.[8,10-12,29,43-45] Antibody profiling based on these data stratifies patients according to either the presence or absence of anti-ADAMTS13 antibodies against certain domain(s). Only two studies investigated the link between domain specificity of anti-ADAMTS13 antibodies and disease severity or platelet counts. Thomas *et al.*[29] stratified iTTP patients according to having either anti-MDTCS or anti-T2-C2 autoantibodies but could not identify a link with disease severity. On the other hand, Zheng *et al.*[10] reported an inverse correlation between the presence of IgG antibodies against the T2-T8 and/or C1-C2 domains and platelet counts on admission. In our study, we used anti-idiotypic antibodies to stratify iTTP patients according to the presence or absence of anti-ADAMTS13 antibodies with specific idiotopes. By using an anti-idiotypic antibody, we can, therefore, investigate whether a specific anti-ADAMTS13 idiotope is present or absent in an individual iTTP patient. Indeed, with our three anti-idiotypic antibodies, we determined the previously unknown anti-spacer II-1, TTP73, and I-9 idiotope profiles in 151 iTTP patients in acute phase. Eighteen of the 151 iTTP patients had all three anti-spacer idiotopes in their plasma, 63 patients had none of the anti-spacer idiotopes, and 70 patients had either one or a combination of two of the anti-spacer idiotopes in their plasma, showing that the presence of these three anti-spacer idiotopes is not a common feature in iTTP patients. In addition, the anti-spacer autoantibody II-1[40] used in this study is a well characterized iTTP patient autoantibody that targets the R568-F592-R660-Y661-Y665 epitope in the ADAMTS13 spacer domain[43] and is a strong inhibitor of ADAMTS13 activity.[40] Although approximately 50% of the iTTP patients have inhibitory anti-ADAMTS13 autoantibodies,[29,46] it is still not known if all these patients have a II-1 idiotope in their plasma. Using our anti-idiotypic antibody against the anti-spacer II-1 idiotope, we now provide a novel insight into the incidence of this anti-spacer II-1 idiotope in iTTP patients. Indeed, our study showed that only 34% of the patients had this anti-spacer idiotope in their plasma. A wider understanding of the diversity of inhibitory anti-ADAMTS13 autoantibodies that target the R568-F592-R660-Y661-Y665 epitope is important in view of the development of a targeted antibody therapy. In addition, anti-idiotypic antibodies allow the study of epitope spreading observed in iTTP patients by following the presence of specific idiotopes over time. An additional advantage of using anti-idiotypic antibodies for antibody profiling is that the antigen itself is not needed for the profiling assay.[21,47] Production of recombinant ADAMTS13 and its fragments in the case of iTTP is more expensive and complex than producing and purifying murine anti-idiotypic antibodies.

Finally, we investigated whether we could establish a link between these anti-spacer idiotope profiles and disease severity (disease outcome and applied treatment strategy). However, the current idiotope profiles did not allow specific profiles that are linked with disease severity to be identified. On the one hand, this could be due to the



idiotope profile	1	2	3	4	5	6	7	8
II-1 idiotope	+	-	-	+	+	-	+	-
TTP73 idiotope	-	+	-	+	-	+	+	-
I-9 idiotope	-	-	+	-	+	+	+	-
n	12	6	24	7	15	6	18	63

Figure 5. Anti-spacer idiotope profiles in acute immune-mediated thrombotic thrombocytopenic purpura (iTTP) patients. Acute iTTP patients (n=151) were stratified according to the presence (+) or absence (-) of II-1, TTP73 and I-9 idiotopes, as determined in Figure 4. n: number.



idiotope profile	1	2	3	4	5	6	7	8
II-1 idiotope	+	-	-	+	+	-	+	-
TTP73 idiotope	-	+	-	+	-	+	+	-
I-9 idiotope	-	-	+	-	+	+	+	-
n	7	3	16	4	14	5	14	32

idiotope profile	1	2	3	4	5	6	7	8
II-1 idiotope	+	-	-	+	+	-	+	-
TTP73 idiotope	-	+	-	+	-	+	+	-
I-9 idiotope	-	-	+	-	+	+	+	-
n	7	2	15	4	14	5	14	31

Figure 6. Disease outcome and treatment according to the anti-spacer idiotope profiles. Stratification of the 95 acute immune-mediated thrombotic thrombocytopenic purpura (iTTP) patients of the French Reference Center for Thrombotic Microangiopathy according to the eight idiotope profiles for (A) the scoring system of Benhamou *et al.*[39] (score < 3, white bars; score ≥ 3, black bars) and for (B) treatment with plasma exchange (PEX) with/without rituximab (white bars) or PEX with/without rituximab and additional treatment(s) (black bars). †Three patients died before treatment initiation and were excluded from the study (*Online Supplementary Table S2*).

relatively low number of patients per idiotope profile. Therefore, increasing the number of patients in each idiotope profile could show a link between certain profiles and disease severity. On the other hand, although the majority of iTTP patients do have autoantibodies against the cysteine-rich/spacer domain, autoantibodies targeting other regions within or outside the cysteine-rich/spacer domain could be important, as the immune response is polyclonal. Therefore, multiple anti-idiotypic antibodies recognizing a large number of anti-ADAMTS13 autoantibodies might be needed to identify autoantibody profiles in iTTP that predict disease outcome or that are linked with treatment. We are, therefore, currently expanding our panel of anti-idiotypic antibodies with anti-idiotypic antibodies recognizing anti-ADAMTS13 autoantibodies outside the spacer domain to identify autoantibody profiles of clinical, prognostic value.

The strength of autoantibody profiling to predict disease severity and outcome in an autoimmune disorder where autoantibodies are developed against a single self-antigen has been clearly demonstrated, for example, in myasthenia gravis. Indeed, it has been shown that the presence of autoantibodies against a specific epitope in AChR is linked with disease severity in these patients.[48,49] Therefore, the development of anti-idiotypic antibodies against anti-ADAMTS13 autoantibodies that are linked with disease severity, outcome, and relapse remains a promising approach to personalize treatment of iTTP patients.

In conclusion, we have shown that anti-idiotypic antibodies are useful to unravel anti-spacer autoantibody specificity in iTTP patients. Moreover, this approach is broadly applicable and can, therefore, be used to perform autoantibody profiling in any antibody-mediated autoimmune disease.

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