Persistence of circulating ADAMTS13-specific immune complexes in patients with acquired thrombotic thrombocytopenic purpura

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I - Supplementary Methods

Detection of free anti-ADAMTS13 antibodies by ELISA

Microtiter plates (Maxisorp, Nunc, Rochester, NY, USA) were directly coated with rADAMTS13 at 2 µg/mL in 0.05 M carbonate-bicarbonate buffer, pH 9.6 (Sigma, St. Louis, MO, USA) overnight (ON) at 4°C. The non-specific binding sites were blocked for 2 h at room temperature (RT) with phosphate-buffered saline (PBS, pH 7.4), containing 2% (w/v) bovine serum albumin (Merck, Darmstadt, Germany). Patient plasma samples and controls were serially diluted from 1:25 in casein buffer (Sigma) containing 0.1% (v/v) Tween-20 (Bio-Rad Laboratories, CA, USA) and incubated for 3 h at RT. Bound antibodies were detected using horseradish peroxidase (HRP)-conjugated goat anti-human IgG (AbD Serotec, Düsseldorf, Germany), IgA (AbD Serotec) or IgM (Sigma) antibodies. To detect IgG subclasses, HRP-conjugated mouse monoclonal anti-human IgG1 (Invitrogen, Camarillo, CA, USA), IgG2 (SouthernBiotech, Birmingham, AL, USA), IgG3 (Invitrogen) or IgG4 (Invitrogen) antibodies were used. Finally, the chromogenic TMB (3,3',5,5'-tetra-methylbenzidine) substrate (Thermo Fischer Scientific, Rockland, IL, USA) was added and the color reaction stopped by adding 2 M H_2SO_4 solution. The absorbance was read at 405 nm with a reference filter for 620 nm on a plate reader (Tecan, Crailsheim, Germany). Between each step, the plates were washed with PBS containing 0.1% (v/v) Tween-20 (PBS-T).

Data analysis. The read out (optical density, OD) of 100 tested healthy donors was used to calculate OD cut-off values for each ELISA using the nonparametric 95th percentile method. Levels of free anti-ADAMTS13 antibodies were expressed as titers. Samples above the cut-off value and the last dilution in a series that was above the calculated cut-off value were

considered positive, and the inverse of this dilution represents the corresponding antibody titer. Samples under the cut-off were evaluated as negative.

Detection of ADAMTS13-specific ICs by ELISA

An affinity-purified polyclonal anti-ADAMTS13 antibody (raised in rabbits that had been immunized with a purified recombinant full-length ADAMTS13 produced in CHO cells) was used to coat microtiter plates (Nunc) at 2 µg/mL in 0.05 M carbonate-bicarbonate buffer, pH 9.6 (Sigma) for 5 h at RT. The non-specific binding sites were blocked for 2 h at RT with 0.5% non-fat dry milk (Bio-Rad) diluted into PBS-T containing 0.6 M NaCl. Patient plasma samples and controls were serially diluted from 1:25 in blocking buffer, added to the plates and incubated ON at 4°C. The next day, the immunoglobulin fraction of the bound ICs was detected using HRP-conjugated goat anti-human IgA (Sigma) or IgM (Sigma) antibodies or HRP-conjugated mouse monoclonal anti-human IgG1 (Fitzgerald, Acton, MA, USA), IgG2 (SouthernBiotech), IgG3 (Invitrogen) or IgG4 (Invitrogen) antibodies. After incubation, the ELISA was developed as described above. Between each step, the plates were washed with PBS-T.

Data analysis. Ratios were calculated by dividing the OD values of each (n=100) healthy donor's plasma with that of the pooled normal human plasma (NHP; George King Bio-Medical, Overland Park, Kansas, USA). Ratios generated with the healthy donors were used to calculate cut-off values using the nonparametric 95th percentile method. Levels of ADAMTS13-complexed anti-ADAMTS13 antibodies were expressed as titers as described above.

Detection of ADAMTS13-specific ICs by co-immunoprecipitation

IgG-containing ICs were isolated by incubating patient plasma (150 µL) diluted 1:4 in binding buffer (20 mM sodium phosphate, 150 mM NaCl; pH 7.0) with 100 µL protein G Sepharose (GE Healthcare, Buckinghamshire, England) at RT on a rotator for 30 min. The immunoprecipitated IgG-containing material was washed and the antigen-antibody complexes eluted from the protein G Sepharose beads with sample loading buffer (Thermo Fischer Scientific) and heating at 95°C for 10 min. The resulting supernatants were loaded on a 4–12% gradient SDS polyacrylamide gel (Invitrogen) and subjected to electrophoresis under reducing conditions. The separated proteins were transferred to a polyvinyldifluoride membrane (Invitrogen), and ADAMTS13 detected with an affinity purified polyclonal rabbit anti-human ADAMTS13 IgG antibody (Baxter) in combination with an HRP-conjugated goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA) and addition of a

chemiluminescent substrate (Thermo Fischer Scientific). Immunoreactive bands were visualized with the Fusion FX7 image system (Vilber Lourmat, Eberhardzell, Germany). The relative concentration of IgG-IC (expressed in arbitrary units) was calculated by densitometry of the ADAMTS13 bands followed by image analysis using BIO-1D software (Vilber Lourmat) and rADAMTS13 as standard. The antibody used was able to detect blotted rADAMTS13 with a sensitivity of approximately 0.5 ng.

In vitro generation of ADAMTS13-specific immune complexes

ADAMTS13-specific ICs were generated in vitro by spiking 0, 1, 2, 5, 10 or 20 µg/mL of rADAMTS13 into plasma samples containing free anti-ADAMTS13 antibodies and into NHP as control. Samples were incubated for 2 h at 37°C, aliquoted and frozen at -80°C until testing.

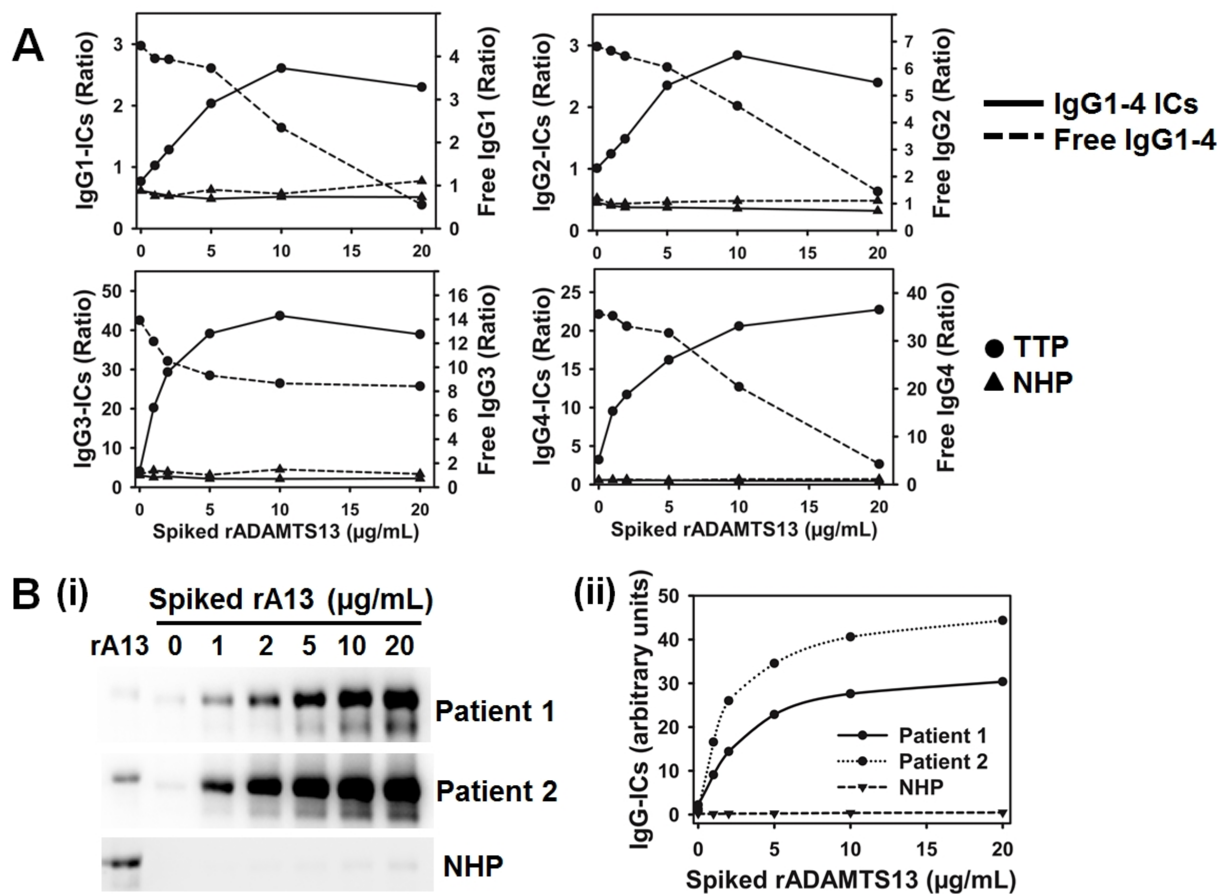
Development of ELISA-based assays to detect ADAMTS13-specific ICs

Comparative analyses of free and complexed anti-ADAMTS13 antibodies in plasma from a large cohort of patients required development of ELISA-based assays capable of detecting the immunoglobulin fraction of the ADAMTS13-specific ICs. To evaluate the specificity and sensitivity of these ELISAs, ICs were generated in vitro by spiking increasing concentrations of rADAMTS13 into plasma from two aTTP patients (containing either free IgG1 and IgG3 or IgG2 and IgG4 anti-ADAMTS13 antibodies) and into NHP as control. Supplemental Figure 1A shows that ADAMTS13-specific IgG (1-4) ICs indeed accumulated in a rADAMTS13 concentration-dependent manner until saturation; this increase was accompanied by a decrease in corresponding free antibodies. NHP showed no detectable increase in ADAMTS13-specific ICs (Supplementary Figure 1A).

Similar results were obtained when analyzing these samples using a co-IP method. The band intensity of ADAMTS13 that had been precipitated with total IgG through Protein G increased with greater amounts of spiked rADAMTS13 (Supplemental Figure 1Bi). The relative amounts of IgG-IC reached saturation for both patient samples, whereas spiking of rADAMTS13 into NHP did not yield discernible ADAMTS13-specific ICs (Supplementary Figure 1Bii).

II - Supplementary Figures

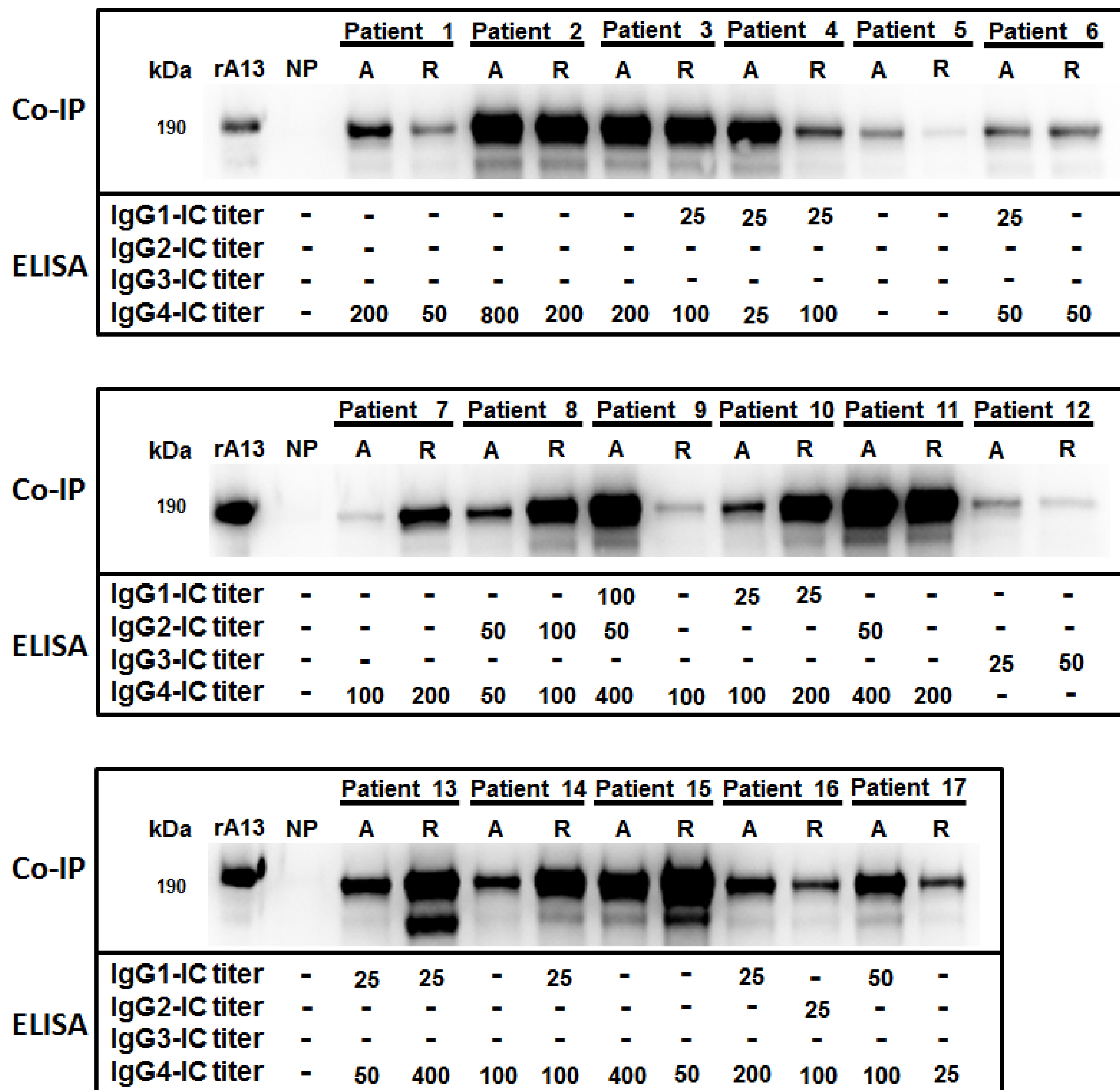
Supplementary Figure S1



Supplementary Figure S1. In vitro formation and detection of ADAMTS13-specific immune complexes (ICs).

Plasma samples from two patients with TTP containing high levels of free anti-ADAMTS13 IgG antibodies (patient 1 was used for IgG2 and IgG4, patient 2 for IgG1 and IgG3 analyses) and a NHP as control were spiked with increasing concentrations of rADAMTS13 (0, 1, 2, 5, 10 and 20 µg/mL) and incubated for 2 h at 37°C. Samples were then analyzed for the presence of ADAMTS13-specific ICs. (A) Detection of free and complexed IgG1-4 anti-ADAMTS13 antibodies by ELISA. For each assay, antibody levels of the indicated subclass were expressed as a ratio calculated by dividing the OD of the tested sample (spiked TTP-derived plasma sample or NHP) by that of the negative control (non-spiked NHP). (B) Detection of ADAMTS13-specific ICs of the IgG class by co-IP. Total IgG was isolated from plasma by Protein G and co-precipitated ADAMTS13 detected by Western blot. i) Immunoblots of the spiked samples. The position of ADAMTS13 in the gel (~190 kDa) was determined by loading 5 ng of rADAMTS13 (rA13). ii) Densitometric quantification using rADAMTS13 (5 ng) as standard.

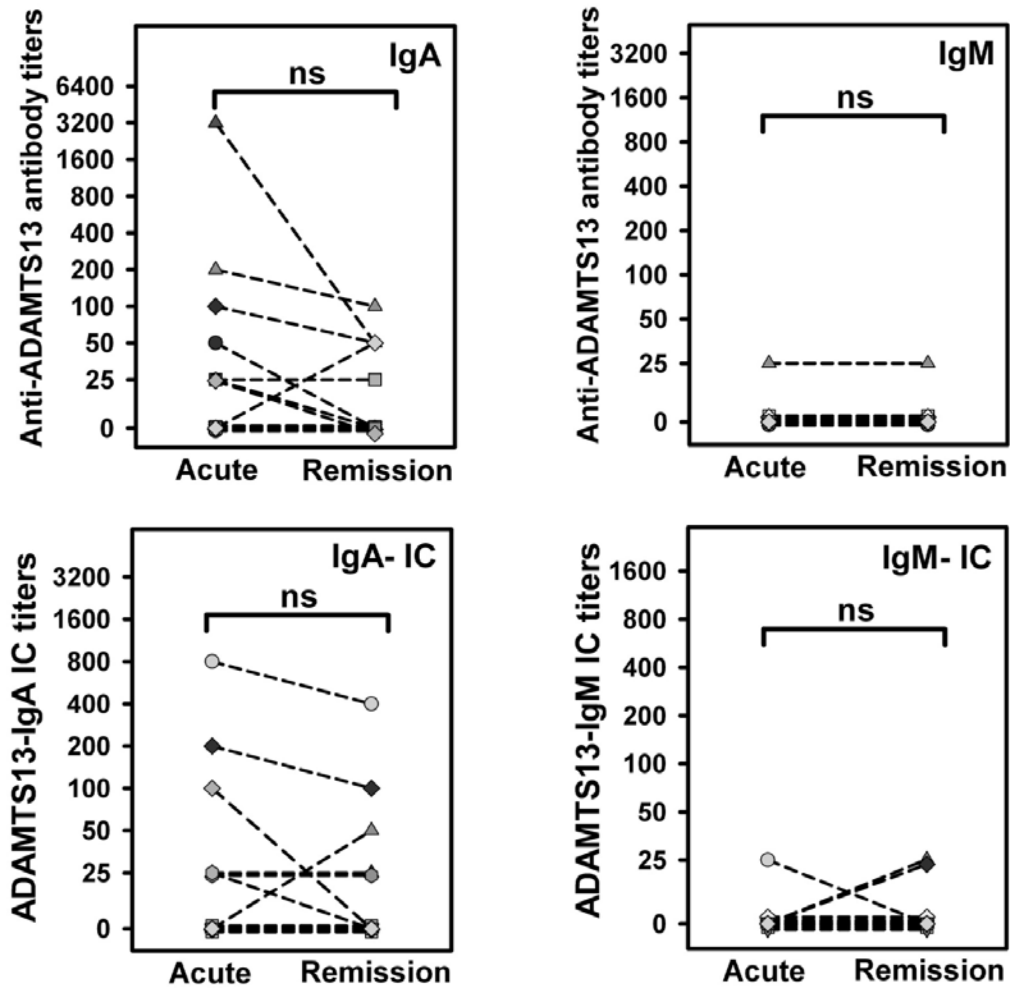
Supplementary Figure S2



Supplementary Figure S2. Comparison of detection of ADAMTS13-specific ICs in plasma samples from TTP patients by co-IP and ELISA.

Samples from 17 patients with acquired TTP taken at the acute phase (A) and in remission (R) and pooled normal human plasma (NP) as control were analyzed for the presence of ADAMTS13-specific ICs by co-IP (upper panel) and ELISA (lower panel). The immunoblot in the upper panel reveals the amount of ADAMTS13 co-precipitated with IgG antibodies. The lower panel shows the corresponding IgG1-4 ICs titers obtained by ELISA. The position of ADAMTS13 in the gel (~190 kDa) was determined by loading 5 ng of rADAMTS13 (rA13). A minus sign denotes undetectable levels of ICs.

Supplementary Figure S3



Supplementary Figure S3. Comparison of free and complexed anti-ADAMTS13 IgA and IgM antibody titers between the acute and remission phase

Plasma samples from 18 patients with aTTP taken at presentation (Acute) and during clinical remission (Remission) were analyzed. The figure shows a comparison of free and complexed anti-ADAMTS13 IgA and IgM antibody titers between the acute phase and in remission. n*s*, non-significant difference.

III - Supplementary Tables

Supplementary Table 1. IgG subclass distribution of anti-ADAMTS13 antibodies in patients with acute aTTP

IgG subclass combination	Number of patients (%)
IgG4	14 (21%)
IgG1 + IgG4	16 (23%)
IgG2 + IgG4	2 (3%)
IgG1 + IgG2 + IgG4	19 (28%)
IgG1 + IgG3 + IgG4	6 (9%)
IgG1 + IgG2 + IgG3 + IgG4	6 (9%)
IgG1	2 (3%)
IgG1 + IgG3	2 (3%)
IgG1 + IgG2 + IgG3	1 (1%)