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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 7

General Discussion

Nuno A. G. Graça

The Von Willebrand Factor (VWF)-haunted blood

In **Chapter 1** we have seen how Thrombotic Thrombocytopenic Purpura (TTP) was for many years a mysterious disease, with unknown causes, variable presentations, lack of effective treatments, and an uncomfortably high percentage of fatal outcomes. It became nonetheless a prime example of how empirical observations translated into important gains for patients, both for TTP and for other diseases as well.

Von Willebrand disease (VWD) – a disease characterized by excessive bleeding, most commonly due to VWF deficiency or dysfunction – was described for the first time in Finland by Erik von Willebrand in 1926 – ironically, almost at the same time as TTP[1]. It was found later to be dependent on VWF synthesis or quality, where ADAMTS13 processes the VWF multimers excessively, being for its larger part a hereditary syndrome[2], but can also be acquired in some medical conditions[3,4]. Today, we can consider TTP and VWD true antonyms, as they constitute the opposing mechanistic end in the ADAMTS13-VWF axis (deficient ADAMTS13 activity vs excessively high ADAMTS13 activity, respectively). Also, VWD is usually a congenital problem. But TTP is more often acquired. It is noteworthy that VWF was characterized during the 1970's and, for VWD, most of the diagnostic and treatments were developed until 2006 and came almost immediately afterwards this characterization[5], with little number of new breakthroughs done since then[4]. This constitutes a clear contrast with TTP, where most advancements came much later. This is easier to appreciate if we make a "Cosmic Calendar" for TTP. The figure of the "Cosmic Calendar" – a calendar that compresses all of history and time of the Cosmos into a single year of a common Gregorian calendar – was introduced in 1980 by the Astronomer and science popularizer Carl Sagan in his famous TV series *Cosmos – A Personal Voyage*[6]. In **Figure 1** a version of this type of figure is shown, specifically applied to the history of TTP. If TTP were described on January 1st, only 2-3 very relevant milestones could be accounted for until September. Probably also under the influence of the public *World Wide Web* which was launched close to the inception of PEX[7], it was from this time onwards that the largest number of milestones could be accounted for in this field, all within the last 3 months of this calendar. The switch from almost certain death to a high survival rate for TTP patients ushered in a panoply of questions that emerged when TTP became a more prevalent chronic disease. These questions have been progressively addressed thanks to the organization of patient registries and new information technologies[8]. The new knowledge generated by these registries came mostly from retrospective studies[9–15], but important prospective studies have also been reported[16–22]. The institution of biobanks containing patient samples that are well characterized[23] have steadily lead to potential new technologies and biomarkers to be used in the field. Such was the case of the in-house and commercial diagnosis assays[24,25], new clinical scores for diagnosis[10,11,26–29], the possibility to perform epitope mapping studies[19,30–33], new biomarkers for diagnosis and prognosis[34–40] and the

ability to test recombinant ADAMTS13 variants as potential new approaches for TTP treatment[33,41,42]. In here lies the importance of these registries: they allow science to advance and provide potential solutions that will benefit patients in the long-term. Such is also the case of a better understanding of the autoimmune process itself, a puzzle that has remained only partially comprehended to date[43].

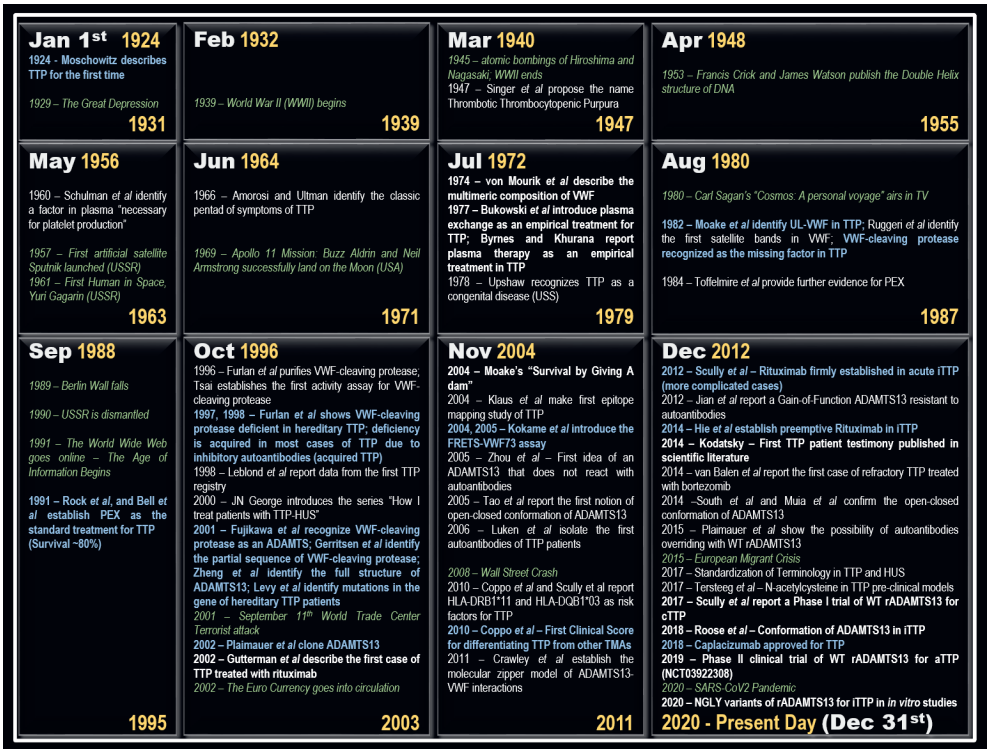

Figure 1 – A "Cosmic Calendar" for Thrombotic Thrombocytopenic Purpura (TTP) thus far.

The full history of TTP is compressed into a full Gregorian calendar year. This "year" corresponds to the 96 years that have passed since the first description of TTP published by Eli Moschowitz in 1924 (placed as the 1st January) until the present day (placed as the 31st December). Each month corresponds to 8 years. The most relevant milestone events in the history of TTP are highlighted in bold, pale-blue letters. Other events are found in regular white letters. Events with potential high impact in the field are highlighted in bold white letters (arbitrary). A few mankind landmark milestones and key events that took place during these 96 years are placed in green letters for perspective.

Of ADAMTS13 and Man

Immune TTP is an autoimmune disease, characterized by the presence of autoantibodies against ADAMTS13. These may be directly inhibitory or not[44], but

likely all of them contribute for a greater clearance of ADAMTS13 from circulation[45], leading to loss of ADAMTS13 activity. Immune complexes consisting of ADAMTS13 and these autoantibodies persist in circulation of almost all patients for years, even after remission[38]. It is worthwhile to make immediate remarks upon these facts: several patients are reported to have lower ADAMTS13 antigen levels at presentation during an acute TTP bout[46]. This collides frontally with the findings regarding ADAMTS13 immune complexes. It is tempting to conclude that a true clearance of ADAMTS13 from circulation does not exist in high extent *per se*, despite the diverse uptake mechanisms by macrophages (mannose receptor[47], CD163[48] and Fc receptors[38]). A true clearance may be the case for some patients. But is it the case for the majority? If immune complexes persist in circulation, then the antigen is present, but it is masked by the autoantibodies. The problem here lies in the tools available and generally used to study these phenomena, which rely mostly on IgG antibodies and ELISA techniques. Antibodies are proteins of a molecular weight of ~150 kDa. ADAMTS13 is extensively glycosylated[49,50] and has an apparent molecular weight of 190 kDa[51]. However, the calculated molecular weight without counting any glycans is ~150 kDa[52,53]. The relative size of these molecules should be, therefore, similar. This allows us to reason that the likelihood of having several antibodies binding to a single ADAMTS13 molecule will be dependent on where they bind, and will be influenced by prior binding of an antibody in any area of ADAMTS13. The ELISA methods used for antigen detection (and used within the studies presented here) rely on several antibody interactions spanning almost the whole extent of ADAMTS13: capturing is normally made through the metalloprotease domain and detection through the far end C-terminal TSP8-CUB1 domains. In **Chapters 3 and 4,** through differences in signal obtained with ADAMTS13 spacer domain mutations and C-terminal domain truncations, we clearly show that it is possible to detect antibodies binding in several areas of ADAMTS13 at the same time (Metalloprotease ± Spacer ± C-terminal domains). However, we notice that many patients in literature (~52.9%) have C-terminal antibodies that may be targeting the CUB domains, while fewer (~14%) have anti-metalloprotease antibodies[33]. If a patient has antibodies that bind close to or in the TSP8-CUB1 domains, or directly in the metalloprotease domain, it's likely that they will have a lower antigen level detected, because the tool being used for detection cannot access the already occupied areas. Apparently, prior binding of antibodies to ADAMTS13 does not constitute an issue with this type of ELISA, as reported by Dekimpe *et al*[54]; however, the methodology in this study did not clearly show that the patients' antibodies used for this assessment and pre-bound to ADAMTS13 were indeed targeting the areas of the far end C-terminal domains (i.e. they could be targeting the spacer domain instead, which, by our studies, clearly do not interfere, and are the most frequent and abundant in patients polyclonal mixtures)[54].

In **Chapter 2** we have discussed the available evidence regarding the

pathophysiological mechanisms leading to immune TTP onset. Being an autoimmune disease, the greatest question one may ask of immune TTP is: why does the human body produce autoantibodies against ADAMTS13? Why is there a loss of tolerance towards a self-protein? This is of course a question that has lied in the forefront of the field of autoimmunity and of immunology as a whole since the beginning and may well be one that will possibly never see a truly satisfactory (short) answer. For the context of immune TTP, as we've seen, healthy individuals may harbor non-inhibitory autoantibodies towards ADAMTS13[55,56]. It is not completely understood why these are formed, but it is tempting to think that, like for Systemic Lupus Erythemathosus (SLE)[57], these may precede clinical TTP. It is known that in certain immune-mediated inflammatory diseases like Rheumatoid Arthritis and SLE, a preclinical phase is present, identifiable through biomarkers (especially autoantibodies), and it is thought that in genetically susceptible individuals, chronic inflammatory processes allied to environmental factors trigger this loss of tolerance[58]. It is a tell-tale sign that some aspects of inflammation are found to be related to loss of tolerance to ADAMTS13: obesity is linked to a low-grade permanent inflammatory status[59] and in obese individuals non-inhibitory autoantibodies towards ADAMTS13 have been found[60]; apart from this, infections have been associated with ~41% of TTP cases[61]. The literature is full of case reports of TTP cases associated to infections with bacteria, viruses and fungi (reviewed[62]). More recently, the Chikungunya virus, and SARS-CoV2 (COVID19) have also been linked to immune TTP[63–66]. Cases of envenomation leading to immune TTP also exist[67], and even cases of vaccination – most commonly against Influenza – have been described to trigger TTP in previously healthy individuals[68–71]. The mechanisms by which these could lead to TTP remain poorly understood.

Several theories regarding how autoimmunity may be triggered have been put forward and reviewed[72]. They comprise the following: a) cryptic epitope theory; b) epitope spread theory; c) anti-idiotype theory; d) molecular mimicry theory; e) the bystander effect theory; f) dual-affinity T-cell receptors theory; g) antigenic complementarity theory and h) co-infections[72]. Among all of these, there is definite evidence that co-infections may cause TTP in some cases, as stated above. The question is: through which mechanisms? A plausible theory that fits the current knowledge on immune TTP and infectious agents (including vaccines[71]) is the molecular mimicry theory, applied already successfully with linear ADAMTS13 T-cell epitopes[73]. It is known that the HLA-DRB1*11 allele is capable of presenting the ADAMTS13 CUB2 domain core peptide FINVAPHAR[74], and this same peptide is capable of activating CD4+ T-cells of TTP patients[75]. This peptide possesses a high degree of homology with some protein peptide sequences of known pathogens of various types (bacteria, and kinetoplastid parasites like *Leishmania spp*.)[73]. A similar bioinformatics approach may be taken and applied to Influenza viruses. One bioinformatic tool that may be used for such effort is the NetMHCIIpan server[76], for example. Some peptide sequences from

Influenza were found to possess some degree of homology with parts of ADAMTS13, albeit the homologies found refer to pentapeptides[77]. However, HLA-Class II molecules are open-ended molecules and accommodate peptides variable in length (usually ~12-20 amino-acids long), through a limited number of anchor residues of a main core peptide that spans 9 amino-acids. The anchorage can be "shifted" to either side of the cleft and these side-residues modulate interactions between the HLA-peptide complex and the respective T-cell receptor[78]. The peptide-loading of HLA-class II molecules is dependent on an invariant chain (Ii) and it's CLIP (Class II-associated invariant chain peptide), and on HLA-DM molecules[79,80]. HLA-DP has a preference to present viral peptides[80]. While HLA-DR and DQ bind CLIP, some HLA-DP alleles do not, due to polymorphisms[81]. Polymorphisms also impact these molecules in another way: in DR, the greatest source of polymorphism is the beta chain. But for DQ and DP, both alpha and beta chains present polymorphisms[82]. And in the case of DP, only a few alleles are highly prevalent[83]. The polymorphisms inside and outside the HLA molecules (i.e. promoter regions) add another layer of complexity to peptide presentation control, by modulating CLIP interactions[81] or expression levels[84,85] of the HLA molecule in question. Some polymorphisms in HLA-DP indeed influence the presentation of endogenous peptides[81]. Furthermore, cytokines also influence peptide presentation[86]. And there is also HLA-DO to account for[87]. In a further entanglement, the peptides themselves may be modified (e.g. citrullination, glycosylation) eliciting different responses[88,89].

It is clear that the high affinity antibodies found in immune TTP patients required the participation of T cells at some point as these are required to undergo affinity maturation and somatic hypermutation[73]. The immune TTP response is considered to be antigen driven, because many CDR3 regions of autoantibodies from different unrelated patients are shared among them[90]. Yet, the variable heavy chain (V_H) gene of the majority of these autoantibodies is restricted to the VH1-69 germline[44,90–92] – the same germline normally employed by the immune system to direct a humoral immune response towards a highly conserved region of the hemagglutinin protein of Influenza viruses[93]. The Influenza virus is a suspect that will deserve to be further tracked in this story. But not only linear peptides may exert molecular mimicry: antigenic molecular mimicry may also be implicated – that is, there may be antigens from pathogens and other non-pathogenic microorganisms that may have 3D conformations so similar to self-antigens, that upon an immune reaction mounted towards them, the antibodies will cross-react with the self-antigen. This is well known for group A streptococcus and Rheumatic Fever[94].

In addition to T-cell epitopes, bioinformatics may also offer some answers regarding the 3D relatedness of different proteins[95–97], which may allow discovery of other antigens that resemble the 3D structure of ADAMTS13. An attempt to find such possible antigens was made using the PDBeFold server[96,97], inputting the recently crystalized sequence chain of ADAMTS13 MDTCS construct (PDB access number: 6qig)[98]. The

input process was made with the following specifications: the query process was selected to be pairwise; the chains of 6qig inputted in the query were A, B, C, D, E and F (L and H, corresponding to light and heavy chains of the mouse 3H9 antibody were taken out); the lowest acceptable match was 70%. The following options were selected (where the symbol "√" means the option was ticked, and "__" means the option was left in blank): __ match individual chains – √ match connectivity – √ best matches only – √ unique matches only – √ if no matches within limits of acceptability are found, show close ones; Precision = Normal; Sort by = Q-score; Target = Whole PDB archive. **Table 1** shows a summary of the outcome of this query.

The first row shows the entry with the best alignment, which inevitably is the "DTCS" fragment of ADAMTS13 crystalized earlier (PDB access number: 3ghm)[99]. Interestingly, we can see one of the main proteins of Influenza A retrieved in the 12th entry: H7 Hemagglutinin (highlighted in blue; PDB access number: 1ti8)[100]. If we make an alignment of all 3, we find some degree of similarity in the 3D structure of H7 Hemagglutinin of Influenza A, and the spacer domain of ADAMTS13 (**Figure 2**). Perhaps the large number of Influenza virus-associated immune TTP cases described in literature are triggered in susceptible individuals by an accidental cross-reactivity between these two antigens. This fits also the fact that most patients have anti-spacer antibodies and explains the preferential use of the VH1-69 germline, making this autoimmune process of molecular mimicry and Influenza important prime suspects. This approach currently has the weakness of lack of availability of crystalized structures of the C-terminal domains of ADAMTS13, for which many patients have autoantibodies against, and is always dependent on accurate structures available for comparison. Other normally neglected microorganisms might need to be considered too. For example, the Tobacco Mosaic Virus is a plant virus that does not replicate in human tissues, yet the immune system develops antibodies towards it in smokers[101]. In fact, humans are heavily exposed to other plant viruses, which are not immunologically neutral[102]. Could any of these microorganisms have a greater relevance in the context of autoimmunity?

A cryptic epitope theory started being tackled in immune TTP, where an open conformation of ADAMTS13 was shown to be a hallmark of acute immune TTP bouts[40]. Importantly, in immune TTP patients that are still in remission, the same open conformation precedes a relapse and is reversed by the actions of rituximab[103]. Epitope spreading has also been proved in immune TTP cases. Epitope mapping in immune TTP patients done during an acute bout and during a relapse that took place several years after a remission achieved with rituximab treatment demonstrated that different regions of the protein were targeted in the different sample timings[19].

Table 1 – Comparison of 3D structure of ADAMTS13 MDTCS (6qig) and other proteins in the PDB archive: summary of the query outcome (query made in PDBeFold public server, the first 20 results are shown).

Entry Number	Scoring Q	RMSD	Target (PDB entry)			
			Match	$\%_{ssc}$	N_{res}	Title
1	0.24	2.68	3ghm	81	369	CRYSTAL STRUCTURE OF THE EXOSITE-CONTAINING FRAGMENT OF HUMAN ADAMTS13 (FORM-1)
2	0.23	2.74	3vn4	81	375	CRYSTAL STRUCTURE OF THE EXOSITE-CONTAINING FRAGMENT OF HUMAN ADAMTS13 (P475S MUTANT)
3	0.20	2.78	3ghn	75	371	CRYSTAL STRUCTURE OF THE EXOSITE-CONTAINING FRAGMENT OF HUMAN ADAMTS13 (FORM-2)
4	0.16	2.02	2v4b	34	561	CRYSTAL STRUCTURE OF HUMAN ADAMTS-1 CATALYTIC DOMAIN AND CYSTEINE-RICH DOMAIN (APO-FORM)
5	0.15	2.12	3q2h	39	566	ADAMTS1 IN COMPLEX WITH N-HYDROXYFORMAMIDE INHIBITORS OF ADAM-TS4
6	0.15	2.12	3q2g	34	564	ADAMTS1 IN COMPLEX WITH A NOVEL N-HYDROXYFORMAMIDE INHIBITORS
7	0.15	2.06	2jih	32	570	CRYSTAL STRUCTURE OF HUMAN ADAMTS-1 CATALYTIC DOMAIN AND CYSTEINE-RICH DOMAIN (COMPLEX-FORM)
8	0.13	1.49	3b8z	38	434	HIGH RESOLUTION CRYSTAL STRUCTURE OF THE CATALYTIC DOMAIN OF ADAMTS-5 (AGGRECANASE-2)
9	0.065	1.86	3e8r	27	506	CRYSTAL STRUCTURE OF CATALYTIC DOMAIN OF TACE WITH HYDROXAMATE INHIBITOR
10	0.048	2.45	2cki	25	528	STRUCTURE OF ULILYSIN, A MEMBER OF THE PAPPALYSIN FAMILY OF METZINCIN METALLOENDOPEPTIDASES.
11	0.031	3.33	3u1r	24	456	STRUCTURE ANALYSIS OF A NEW PSYCHROPHILIC MARINE PROTEASE
12	0.025	4.62	1ti8	26	480	H7 HAEMAGGLUTININ
13	0.020	5.37	5weu	28	372	CRYSTAL STRUCTURE OF H2-DD WITH DISULFIDE-LINKED 10MER PEPTIDE
14	0.020	3.57	3wrv	28	365	CRYSTAL STRUCTURE OF NN DOMAIN OF RESISTANCE PROTEIN
15	0.019	5.43	1f4x	21	426	CRYSTAL STRUCTURE OF AN ANTI-CARBOHYDRATE ANTIBODY DIRECTED AGAINST VIBRIO CHOLERAE O1 IN COMPLEX WITH ANTIGEN
16	0.019	3.35	4icd	28	414	REGULATION OF ISOCITRATE DEHYDROGENASE BY PHOSPHORYLATION INVOLVES NO LONG-RANGE CONFORMATIONAL CHANGE IN THE FREE ENZYME
17	0.018	3.92	6nu7	22	488	STRUCTURE OF SUCROSE-6-PHOSPHATE HYDROLASE FROM LACTOBACILLUS GASSERI
18	0.018	3.38	4aj3	24	416	3D STRUCTURE OF E. COLI ISOCITRATE DEHYDROGENASE IN COMPLEX WITH ISOCITRATE, CALCIUM(II) AND NADP - THE PSEUDO-MICHAELIS COMPLEX
19	0.017	3.86	3afk	25	335	CRYSTAL STRUCTURE OF AGROCYBE AEGERITA LECTIN AAL COMPLEXED WITH THOMSEN-FRIEDENREICH ANTIGEN
20	0.017	3.84	1idd	25	414	ISOCITRATE DEHYDROGENASE Y160F MUTANT APO ENZYME

Enough evidence has been collected to conclude that the immune response against ADAMTS13 is continuous. In genetically susceptible individuals, a 2-hit scenario is plausible, where chronic inflammation (especially in the obese) leads to non-inhibitory autoantibodies that may trigger the opening of the ADAMTS13, exposing the more immunogenic spacer domain. Exposure to certain pathogens may either trigger or exacerbate the immune response towards ADAMTS13. It is yet unclear if any natural mutations in the gene of ADAMTS13 exist triggering also the opening of the conformation. It is known that spacer domain mutations may have this effect[42], and in **Chapter 6** we show that the same is possible with mutations in the complementary CUB domain areas. Once again, the main tools to investigate these effects are still antibody panels[104,105], which, upon introducing mutations into ADAMTS13, may suffer limitations if the mutation is introduced in one of their epitopes. This was indeed the case for one CUB1 mutant of ADAMTS13 (mutant D1240A) and the antibody 17G2. Nonetheless, it was possible to find clearly open-conformation mutants in R1247E and L1243A (CUB1) and in P1333A and R1336E (CUB2). ADAMTS13 possesses 14 domains, and 3 linker regions[106]. What would be the impact of mutations within the linker regions? Can mutations augment rigidity in these areas making ADAMTS13 permanently open? We also observed that several of our CUB domain mutant variants were less efficiently secreted. This is in line with previous studies of CUB mutations[107]. Other natural mutations of ADAMTS13 found in congenital TTP patients are also known to hamper ADAMTS13 secretion[108]. Since no gene sequencing is normally made in cases of immune TTP, it is unknown whether any mutations in ADAMTS13 may be behind (or concurring to) the development of autoantibodies against ADAMTS13.

One problem associated with research in autoimmunity and understanding what leads to it is the variable "time". It is clear that most cases of autoimmunity are "acquired" at some point in life – that is, neonatal autoimmunity is less common than acquired autoimmunity[109]. Scientific studies that could follow individuals known to be at higher risk (due to previous family cases[110], for example) for a very long time would be required to pinpoint when and what could lead to disease triggering. Humans' immune systems are not isolated and are not the same throughout the life span of individuals. It is permanently exposed to microorganisms and an environment that may also be drastically changed with travelling. This includes geographical, nutritional and lifestyle changes. This constant change also leads to a permanent fluidity in the way "self" perceives itself. Humans age; proteins are known to suffer age related changes. Physiology is altered. All these observations lead to the proposal of a "liquid-self" theory where time, geography and life experiences all come together to reshape the biological identity[111]. One recent example that underlines the influence of geography for TTP is a study performed on the Japanese population, where HLA-DRB1*08 was assessed as a more relevant risk factor than HLA-DRB1*11[112]. Another recent study hints towards the individual experiences of patients, as ADAMTS13 citrullination was demonstrated

to occur mostly in aged individuals with comorbidities like diabetes and hypertension, or in patients experiencing an episode of sepsis[113]. This study has the weakness of not having resorted to any acquired TTP patient plasma, but points to another possible mechanism from which immune TTP may be acquired starting from an otherwise healthy condition.

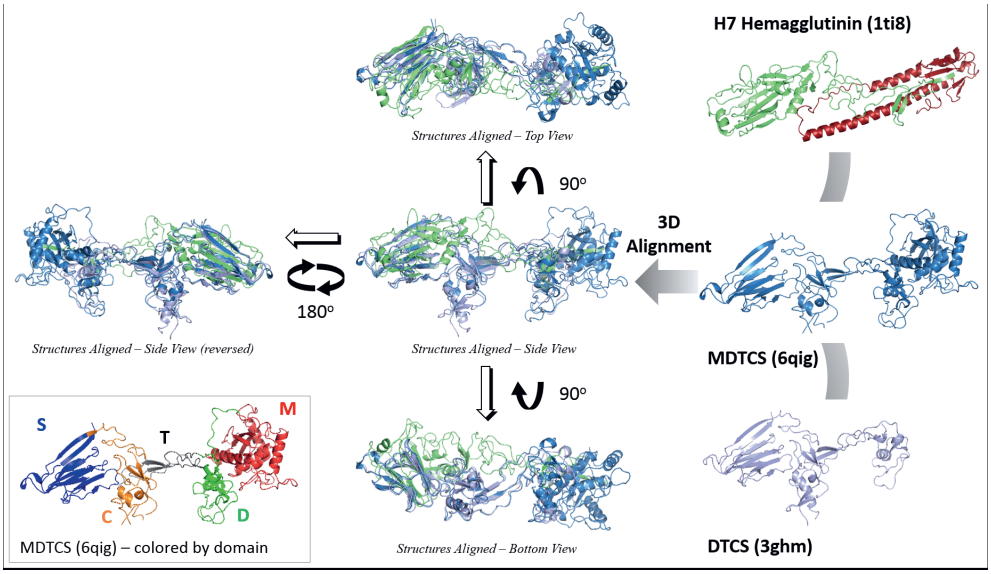

Figure 2 - A 3D alignment of the crystal structures of ADAMTS13 MDTCS domains (6qig), DTCS (3ghm) and Influenza A H7 Hemagglutinin (1ti8).

On the right handside, the 3D structures of Influenza A H7 Hemagglutinin (red and green), and ADAMTS13 MDTCS (aqua blue) and DTCS (light-blue) crystal structures are shown. These are aligned and shown in the middle and left hand side with the indicated angles. For clarity, the alpha-helices from H7 Hemagglutinin are hidden. In the bottom-left panel, the domain composition of the ADAMTS13 MDTCS and relative positions of these domains are shown: M = Metalloprotease (red); D = Disintegrin (green); T = Thrombospondin (dark grey); C = Cys-Rich (orange); S = Spacer (blue). The MDTCS and DTCS fragments superpose almost perfectly. The similarity of the 3D barrel structure of the ADAMTS13 spacer domain and parts of the H7 Hemagglutinin protein is apparent.

Studies of larger magnitude and time span are normally prohibitively prolonged and complex to perform. The most common alternative is animal research, which has allowed many advances in the field of autoimmunity, but evidently have several limitations[114–116]. In the case of immune TTP, an animal model that perfectly mimics the immune TTP disease process of humans does not exist yet[117], despite a prior description of a spontaneous acquired TTP case in a chimpanzee[118]. The closest achieved so far are models where passive polyclonal or monoclonal antibody transfers are made[119–121] or a limited number of autoantibodies are produced by the animal after liver passive

hemodynamic uptake of DNA vectors coding those autoantibodies[122].

2004-2020: A Spacer Domain Odyssey

In 2004 the first epitope mapping study of ADAMTS13 was published by Klaus *et al*[30]. Since then, several epitope mapping studies were done and published[19,31,32,44,56,90,123–128], allowing us to draw 2 main conclusions: 1) the majority of patients have autoantibodies that target the spacer domain of ADAMTS13; 2) the majority of autoantibodies within most patients' mixtures are directed towards the spacer domain (**Figure 3A**).

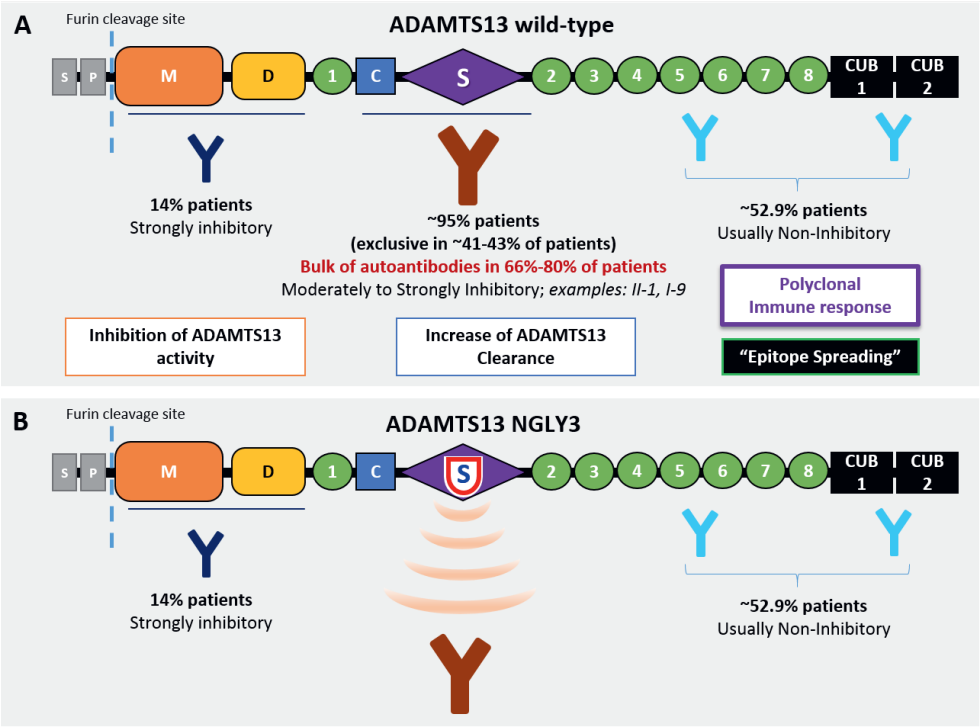

Figure 3 – For an ADAMTS13 variant with increased resistance towards immune TTP patients' autoantibodies.

A – The general characteristics of patients' autoantibody mixtures are shown. **B –** If a shield is provided to the spacer domain of ADAMTS13, resistance may be achieved against the main bulk of autoantibodies in the immune TTP patients' mixtures.
Domain structure of ADAMTS13 in single letter and number codes.
From left to right: S – Secretion Peptide; P – Pro-peptide; M – Metalloprotease; D – Desintegrin; 1 – Thrombospondin (TSP) repeat 1; C – Cys-Rich; S – Spacer; 2-8 – TSP repeats 2-8; CUB-1 and CUB-2 – complement subcomponent C1r/C1s, embryonic sea urchin protein Uegf, bone morphogenetic protein 1 domain.

Three questions were addressed in **Chapters 3 and 4**: 1) is the spacer domain of ADAMTS13 targeted in a single place, or are there several hotspots present? 2) Most epitope mapping studies have relied on classic alanine scans to pinpoint the spacer domain exosite-3 as the main target of the autoantibodies mixtures. If alanine mutations work to abolish autoantibodies' interactions with ADAMTS13, is it possible to make other mutations that are more effective than alanine scans? 3) If yes, how do these impact activity of ADAMTS13?

In **Chapter 3** we found that the spacer domain does not have a single epitope as a hotspot of autoantibody binding, but 3 hotspots: regions spanning amino-acids 588-592, 602-610 and 657-666. Of these 3, 2 regions overlap partially the canonical exosite-3 residues R568/F592/R660/Y661/Y665. Only R568 is not represented. Importantly, the region spanning amino-acid residues 602-610 does not overlap. This is therefore a new region previously not reported and may potentially have relevance for pathogenicity.

In **Chapter 4**, working around exosite-3 we show that it is possible to find more effective mutations than alanine scans. In fact, the less conservative the mutations inserted, the more effective is the resistance towards autoantibody binding. This may find other translational applications to fields where protein-protein interactions have high relevance. However, as the effectiveness towards binding resistance increases, the effect towards ADAMTS13 activity is more detrimental. We show that one mutant variant – AAAAA – is capable of escaping almost all autoantibodies from most patients screened (9% median reactivity), but had a significant loss of activity in a FRETS-VWF73 assay and VWF-multimer assay. It is relevant to state that FRETS-VWF73 is the standard method for assessing ADAMTS13 in plasma samples for diagnostic purposes, and this AAAAA mutant retained an activity level of ~18%. Another highly successful mutant – RARAA – retained higher levels of activity on FRETS-VWF73 (~35%), and even displayed a visible residual activity in a VWF-multimer assay[33]. How would the Chapter 3 ADAMTS13 spacer domain partial swaps with ADAMTS1 spacer domain affect activity of those mutants? We did not assess these because that was not the goal of the study. In retrospective, those mutants are not prime candidates for further pre-clinical development because they run the risk of specificity loss as previously demonstrated when mixing ADAMTS13 and ADAMTS5 exosites[129]. However, they would likely serve the purpose of understanding if there is any correlation between autoantibody target region and their inhibitory potency, provided that the relevant mutants would retain such activity.

In 2005 an idea was put forward: using ADAMTS13 variants that don't bind autoantibodies and are not inhibited by them as a replacement therapy for immune TTP[130]. This idea was left "brewing" in the field until 2010-2012, when the spacer exosite-3 was resolved to residue level and a gain-of-function variant was discovered[42,127,128]. Since then, all efforts were centered in the recombinant wild-type ADAMTS13: it was shown that by using the recombinant wild-type ADAMTS13 it is possible to override patient

autoantibodies *in vitro*, restoring activity to relevant levels with precision, based on initial autoantibody titers[41]. This was also possible to do *in vivo* using an animal model with passive transfer of antibodies[121]. These successes were followed by encouraging results of a phase I clinical trial to evaluate the pharmacokinetics and immunogenicity profile of a single dose of the recombinant wild-type ADAMTS13 in congenital TTP patients[131]. This recombinant wild-type ADAMTS13 demonstrated similar pharmacokinetics as for fresh frozen plasma infusions, and within the time span of the study no antibodies against the product were detected[131]. The study stressed that these findings must still be replicated in multiple dose schedules. At the moment, new clinical trials are recruiting patients to study the efficacy and safety profile of multiple dosing regimens of this recombinant wild-type ADAMTS13 in congenital TTP patients (*NCT03393975*, Phase III) and the efficacy of the same ADAMTS13 in treatment of acquired TTP patients in addition to plasma exchange (PEX) and standard of care (*NCT03922308*, Phase II, SOAR-HI study). If the reality regarding congenital TTP patients is relatively straightforward, the scenario for acquired TTP patients (in particular immune TTP patients) will be completely different: the presence of autoantibodies that promote clearance and/or are directly inhibitory will likely pose important issues for pharmacokinetics and regimens, as well as dosing schedules that will need to be adapted individually for higher success rates. Important gains may be attained with reduction of PEX cycles, however, the doses necessary to override the autoantibodies that are permanently produced may be very high. Consequently, this will likely make for a prohibitively high-cost treatment at a time when caplacizumab costs are already a concern. Being able to escape the main bulk of inhibitory antibodies in most patients would confer obvious advantages, at least in theory (**Figure 3B**). Reasonable speculations based on our data[33] can be made: 1) autoantibody overriding should be attained with lower doses; 2) higher doses should be even more effective in clinical recovery; 3) it may also allow for some advantages in the context of medical emergencies, as an easily injectable and safer replacement therapy in cases where PEX is not readily available. An important observation is that in congenital TTP patients, a higher residual ADAMTS13 activity is correlated with less severe disease[132]. In fact, experience with congenital TTP patients indicate that activity levels of 5%-10% are sufficient to prevent thrombotic microangiopathy[133]. In acquired TTP, ADAMTS13 activity levels of ~ 20% appear to be sufficient for a response[134]. Overriding the autoantibodies will likely not require achieving activity levels much higher than this for a response to be elicited.

In the context of very high doses, autoantibody override and activity recovery intuitively becomes a matter of having enough free molecules after autoantibody saturation. The number of free molecules and the baseline intrinsic activity of those molecules will both dictate the final activity recovered. Let us consider a more-or-less abstract example: if we were to deliver a high enough dose of ADAMTS13 for an initial concentration of, say, 5 ug/mL to a patient whose autoantibodies neutralize 1 ug/mL (the

normal concentration of ADAMTS13 in circulation), then 4 ug/mL are left for activity. If neutralization were equal for all mutant variants of ADAMTS13, in theory we would have 400% activity with a wild-type ADAMTS13 under these conditions, and "only" 140% with a RARAA ADAMTS13, for example. The normal range of ADAMTS13 activity is 50-150%[135]. Having 140% is therefore "normal", but 400% is not and could even be detrimental (similar to an acquired VWD, perhaps[3]). However, since these two variants bind autoantibodies differently, this is clearly not the case. Care must be taken not to overdose the patients, which in any case, is not the goal[121]. But let us consider this difference between the variants binding: the RARAA mutant binds only 27% of what the wild-type normally binds[33]. Then, instead of neutralizing 1 ug/mL, perhaps only 0.27 ug/mL would be neutralized, leaving not 4, but 4.73 ug/mL available, and consequently ~165% activity. The autoantibodies and ADAMTS13 are in permanent production, and it is highly likely that the quantity of autoantibodies present inhibit more than 1 ug/mL. Again, on a molecular weight basis, both IgG's and ADAMTS13 are similar in size if we leave out the N-glycans. And immune TTP patients can have total IgG titers that range from 2x to 32x those of healthy individuals[55]. Besides this, several rounds of PEX are normally required for a response[136–138], and the autoantibody titers are not lowered automatically with PEX alone[138]. Let us imagine then that the wild-type ADAMTS13 is neutralized much further, leaving only, say, 0.5 ug/mL free, for example. This would be acceptable in the sense that it would leave a theoretical normal activity of 50%. However, in this case 4.5 ug/mL (accounting for 90% of the initial dose) would be lost on purpose, specifically to saturate these antibodies. For RARAA, which binds only 27% of what the wild-type binds, 27% of the inhibited 4.5 ug/mL would be the neutralized dose: 1.22 ug/mL. That would leave 3.78 ug/mL still available with concurrent theoretical activity of ~132%. Then, in theory, for half the dose (2.5 ug/mL), ~1.90 ug/mL would be left, leading to a theoretical activity of ~66% – still higher than for double the dose of the wild-type! These are obviously blunt calculations: the function ruling these mathematical relationships is not linear, and will depend on where the patients antibodies bind[41,121]. But they allow us to appreciate that having an ADAMTS13 mutant variant that escapes the autoantibody response without a significant loss of activity is evidently preferable.

A Suit of Armor around ADAMTS13

In **Chapter 5** we present an innovative way to confer antibody resistance to ADAMTS13. We provided a shield to several regions of the spacer domain, in the form of an artificially introduced N-glycan. The regions were all within the initial 3 main hotspots found in **Chapter 3**. The N-glycan inserted at K608 (K608N) is located in the region spanning residues 602-610 and it yielded resistance towards patients autoantibodies without affecting directly the activity of ADAMTS13 (variant NGLY3).

Insertion of artificial N-glycans into ADAMTS13 was attempted in the past.

However, this targeted the Cys-Rich domain and was mainly focused on studying protein activity[139]. Artificial N-glycans abolish factor VIII interactions with an antibody[140]. Additionally, glycans appear to be important for tumor cells, as increased sialylation and increased glycan complexity at the surface of tumor cells promotes disruption of cell-cell interactions with Natural Killer cells, preventing their actions[141]. This suggests that artificial N-glycans may potentially be used to promote disruption of protein-protein interactions through steric hindrance. We attempted this strategy in ADAMTS13 areas usually targeted by autoantibodies and found success with the ADAMTS13 NGLY3 variant (**Figure 3B**). Interestingly, no ADAMTS13 activity was lost with the modification made on this variant. It presented a strong resistance to binding of patient autoantibodies in ELISA format compared to the wild-type and a control mutant, K608A. In the presence of patient autoantibodies some activity was lost for NGLY3, nevertheless to a much lower extent when compared to the recombinant wild-type ADAMTS13. This likely reflects the polyclonal nature of the patients' autoantibody mixture. Almost the entire span of ADAMTS13 can be targeted by autoantibodies (**Figure 3A**), making it difficult to escape all autoantibodies at once. In spite of that, the fact that the majority of autoantibodies within the mixture can be escaped already demonstrates that this strategy may be viable and advantageous. On a different level, as further knowledge for the epitopes of ADAMTS13 are accumulated, it may be possible to add further artificial N-glycans on other areas (or shift the natural N-glycans existing in ADAMTS13) in order to further improve resistance of ADAMTS13 to autoantibodies. On top of that, unnecessary domains may be removed without significant activity loss (removing yet another layer of autoantibody interactions)[142], and combinations with surgical mutations may be used for further refined improvements. Dressing ADAMTS13 in a suit of armor becomes an attractive strategy for further pre-clinical and clinical development of ADAMTS13 as a replacement therapy for immune TTP.

Finally, immunogenicity of these new variants will need to be monitored closely. A few remarks regarding this point are necessary. First, the main life-saving treatment employed is PEX. This exposes the patients to the very same antigen towards which they have an immune response already ongoing. We can therefore consider this to be equivalent to immune system boosting. However, corticosteroids and other immunosuppressants (i.e. rituximab) are also administered simultaneously to stop autoantibody production. These variants will likely face barriers in making a full replacement of the life-saving treatment PEX. But they will not take these immunosuppressants out of the equation either. This may reduce their immunogenicity risk to a minimum. In any case, this risk will have to be weighed against their potential benefit.

Future Perspectives

Several questions persist in the field of TTP. On one hand, it is still not fully understood why humans develop autoimmunity against ADAMTS13. Studies that can take advantage of biobanks will be the way forward and may aid in understanding better what are the genetic risks associated with TTP. For example, the ADAMTS13 peptidomes of Class II HLA-DR[74] and HLA-DQ[143] have been studied and are known. But the peptidome of HLA-DP is not known yet. Can HLA-DP – a set of class II HLA molecules specialized in presenting viral peptides[80,83] – present ADAMTS13 peptides that cross react with T-cell receptors which are directed against viral (or other pathogens) peptides? If ADAMTS13 can be citrullinated[113], can it be found in immune TTP patients' plasma? And does it become more immunogenic[89]? On the other hand, pre-clinical studies will be required with adequate animal models to validate new ADAMTS13 variants that escape patient autoantibodies for further clinical development. Can these new variants override autoantibodies and restore activity at lower doses compared to wild-type ADAMTS13[41,121]? The wild-type ADAMTS13 is actively being studied as an adjunctive treatment in acquired TTP patients (*NCT03922308*, SOAR-HI study). The impact of already existing autoantibodies on ADAMTS13 pharmacokinetics will become clearer in the upcoming years[144]. The initial vision of Joel Moake on "Survival by giving A dam"[145] may become a reality approximately two decades later after his vision was put forward. Can recombinant ADAMTS13 increase further short-term survival and reduce refractory disease rates? And will it be able to impact on the long-term relevant outcomes for patients? These must keep being evaluated in stage IV clinical trials (i.e. pharmacovigilance) to assess if such an impact is made[146].

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