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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 1

The "Nautical Chart" of Thrombotic Thrombocytopenic Purpura – a General Introduction

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Summary

The 100th Anniversary of the first description of Thrombotic Thrombocytopenic Purpura (TTP) as a disease by the hand of Dr. Eli Moschowitz is approaching. For many decades TTP remained mostly a mysterious fatal condition, where diagnosis was often post-mortem. A pentad of symptoms was identified, a pattern that later revealed to be fallible. Sporadic (and, in retrospective, prescient) observations lead to empiric interventions that paved the way for the first impactful breakthrough in TTP, almost 70 years after its first description. The main body of knowledge within the field was gathered in the latest 3 decades: the general mechanisms of disease have been described; the diagnosis was refined; and new treatments and biomarkers that provided major improvements on prognosis and management have been introduced. All of these technologies available at the clinicians' disposal require expertise to be used effectively, can be confusing for non-experts, have been in constant change lately, and will likely see further changes and improvements in the upcoming decades. The field of TTP is a "sea of information", its tides being sometimes tricky to sail, with currents that can steer a ship and its captain off course – especially the less experienced in these waters. Nautical charts provide an invaluable hand to sailors less experienced in certain fields. This Chapter seizes an opportunity to provide such a chart for TTP, introducing a brief historic overview on TTP, what it is and what it is not, how it is diagnosed, and how it is currently treated and managed. These will be helpful to set the stage for the scope and contents of this thesis.

A Brief History of Thrombotic Thrombocytopenic Purpura (TTP)

The year of 1924 marks the first description of a case of TTP, by the hand of Dr. Eli Moschowitz. K. Z., a 16 year-old girl died after being hospitalized for 1 week due to an acute illness with high fever, pallor, arm pain, petechiae, paralysis of the left arm and leg, and a pre-terminal coma. Upon autopsy, multiple "hyaline" thrombi were found in many vessels of the heart muscle, the congested spleen, and in the kidneys. A second description of the case was made in the following year. The possible cause was initially thought to be poisoning, and an important observation was made: 4 other similar cases seen by a fellow doctor (Dr. Max Lederer, in Brooklyn), recovered after blood transfusion[1]. The disease cause and right treatment remained elusive, though, and 11 cases accumulated over time in medical literature. In 1947, Singer *et al*, described another case, where an 11-year-old girl died with a similar story 2 weeks after an upper respiratory tract infection. The findings upon autopsy were similar, and 3 of them stood out: hyaline thrombi, a severe thrombocytopenia and petechiae. This is the origin of the name "Thrombotic Thrombocytopenic Purpura", highlighting the presumed pathogenesis behind this disease [2].

As cases accumulated more in medical literature it was possible to detect their main patterns. A pentad of symptoms was described by Amorosi and Ultman in 1966 based on the review of 255 cases: fever, haemolytic anemia with abundant schistocytes, thrombocytopenia (by consumption), purpura or other bleeding symptoms, neurologic signs (transient or permanent) and renal disease. A differential diagnosis was first established as other diseases had signs and symptoms that partially overlap with TTP. With a dismal prognosis where the majority (72%) of patients would die within 3 months, a progressive disease course was identified for which no effective medical treatment was recognized. The etiology and pathophysiology was still only partially understood at the time. Suggested causes included drugs, toxins, viral infections, and autoimmunity, which required more conclusive evidence[3].

It wasn't until the late 70's however that TTP would for the first time be considered a congenital disease, as it was pinpointed that an important factor existing in plasma was deficient in these patients. This is owed to important case reports by Upshaw in 1978[4] and a similar earlier report by Schulman in 1960[5], leading to the alternative name Upshaw-Schulman syndrome. A TTP patient was followed for several years by Dr. Upshaw. This patient had had many episodes with concurrent hospitalizations and always displayed an encouraging response to blood or fresh frozen plasma transfusions and antibiotics within only 48h[4]. Other reports of this empirical treatment – whether as plasmapheresis/plasma exchange (PEX) or as plasma infusions – were published near these years, adding further (empirical) evidence for the existence of this unknown yet missing factor[6,7]. Simultaneously these reports added further discussion about the

possibility of the disease being related to autoimmunity, because patients with systemic lupus erythematosus (SLE) often developed TTP, and because glucocorticoids had been used alone in some cases that achieved remission[6,7]. By 1984, plasma therapy had become a popular empirical treatment but without any optimal regimen, also due to the disease's puzzling character, which was clear that could take upon many forms initially[8]. A major finding regarding its pathogenesis came in during this time: Joel Moake *et al*, found that the unknown agent missing in the blood of TTP patients was necessary for the maintenance of a normal-sized Von Willebrand Factor (VWF), an important blood clotting factor. In TTP patients, this agent (which became known as VWF-cleaving protease) was missing, and this caused the appearance of "Unusually-Large" (or Ultra-Large) VWF multimers (UL-VWF) in plasma, explaining the thrombocytopenia commonly observed in these patients[9].

The year 1991 marks one of the greatest breakthroughs in the field of TTP: PEX was shown to be superior to plasma infusions for successful remission of the majority of TTP patients, becoming a real game changer in survival and firmly establishing an effective first line treatment, although with major heterogeneity between patients: some of the unspecific symptoms present in the initial pentad were shown to be often absent, while some were present almost always, and some patients required more PEX cycles than others to recover. Until this point, survival was systematically very low (10-20% of patients), but from this point onwards it switched to 80-90% of patients. However, PEX was clearly not a cure *per se*: a relapse pattern became more visible[10,11].

From 1997 onwards, a true explosion in the knowledge of TTP ensued with most of the major discoveries having been made within the last 20 years, in many cases thanks to the establishment of local or national TTP registries and thrombotic microangiopathy (TMA) reference centers (Canada; Oklahoma, USA; UK; France; Japan; Switzerland and lately Australia)[12–18]. An overview of the myriad of achievements during the years 1997-2010 contrasts heavily with the preceding 7 decades: Furlan *et al*, established the first sensitive (but rather cumbersome) diagnostic assay to assess the VWF-cleaving protease's activity[19], discovered the presence of acquired inhibitors (autoantibodies) in most TTP patients' plasma[20] and clearly distinguished the general mechanisms of two forms of TTP (congenital TTP, now also known as the classic name Upshaw-Schulman Syndrome, and acquired TTP) while simultaneously setting them apart from other closely resembling TMAs for which treatments are completely different (namely Haemolytic Uremic Syndrome, HUS)[21] (1997-1998); the VWF-cleaving protease was discovered to be a new member of the ADAMTS family[22], had its structure elucidated and identified as ADAMTS13[23,24], was cloned[25], and found to be missing in the plasma of congenital TTP patients due to hereditary mutations in its gene[26] (2001-2002), providing a rationale for the recombinant ADAMTS13 to be used as treatment at least in congenital TTP cases[27] (2004); the first epitope mapping studies of acquired TTP patients' autoantibodies were performed, showing a clear predominance of ADAMTS13's spacer domain as

the prime target[28] (2004); a more simple ADAMTS13 activity assay was developed[29,30] (2004-2005); the importance of several C-terminal domains for ADAMTS13 activity in more physiologic conditions was found[31], as well as the first hint for a conformational flexibility of this protease[32] (2005) – fully elucidated nearly a decade later by the studies of South *et al*[33], and Muia *et al*[34]; the autoantibodies against ADAMTS13 revealed to also promote its clearance[35], and to be a factor for prognosis[36]; the patients' immune response was shown to be polyclonal, and their first autoantibodies were cloned[37] (2006); multiple contact points between ADAMTS13 and VWF were found, contributing for its specificity[38,39] (2008-2009) and a molecular-zipper model was defined[40] (2011); and finally, the major epitope target in ADAMTS13's spacer domain was identified and resolved to residue level[41], while it became clearer that almost all of ADAMTS13 can be targeted by the polyclonal response, in unique combinations for each patient[42] (2010).

However, despite the enormous progress that was made during this time, a true impact on treatment and disease long-term outcomes was not made, as reported by Kremer-Hovinga *et al*[43]. While survival seemed to improve in the short term with PEX, relapses were still frequent (40% risk within the first 7.5 years of remission), and issues with quality of life and morbidity remained to be addressed. Importantly, overall long-term mortality remained unchanged for the 2 decades after establishment of PEX as frontline treatment[43].

Since the early-to-mid-2000s until the present day, a series of further insights were gained for understanding and battling this disease. The most relevant cases are: some HLA alleles were shown to be risk factors for acquired TTP (DRB1*11 and DQB1*03) or protective factors (DRB1*04)[44,45] (2010); several animal models were developed showing that complete lack of ADAMTS13 may not be enough for TTP symptomatology to be triggered in other species (a second-hit is necessary)[46] (reviewed in 2013); the distribution of congenital TTP cases that manifest only during adulthood were found to be almost exclusively manifesting during pregancy[47] (2012); and new treatments were introduced successfully with profound impacts for relapse prevention and disease management – namely rituximab (used as a preemptive[48] and with suggestive evidence of having advantages as a first-line adjunctive treatment for acquired TTP patients[49]) (2002-2018), and, more recently, caplacizumab (as an adjunctive treatment in TTP) (2016-2020)[50–53].

Owing to the complex terminology used in the field of TTP throughout its history, official terminology guidelines were introduced [54] (2017), for which the most important modification was the introduction of the term "immune TTP" (iTTP), now used in cases where there is proof of the involvement of the immune system (i.e. presence of detectable autoantibodies), while the term "acquired TTP" became a more general descriptive term that includes patients who developed TTP in any circumstance (idiopathic and non-idiopathic contexts), applicable to both iTTP patients and other specific situations. Other terms important to understand the clinical work-flow are also

defined in these guidelines (see **Box 1**).

The field has seen much research made during the last decades; consequently, several new players came to stir the already turbulent waters of the "sea of information" that is TTP. It is useful for any newcomer to have proper guidance in "navigating" this sea. A "Nautical Chart" that can easily compile the most relevant diagnostic and treatment aspects of TTP is therefore very useful. Algorithm-like charts directed to TTP management began to appear in medical literature by the year 2000[55], and have been a subject of updates by expert clinicians in this field throughout time[56–63]. These charts are essential for the practicing clinicians and newcomers alike, because they provide a quick overview of what is the current landscape of management. However, the ones that exist a) are often lacking several aspects that deserve an inclusion and a highlight regarding both the diagnosis and the initial and long-term management of a TTP patient – although it should be recognized that the most recent charts and literature reviews provide some degree of complementarity among each other; b) are rather dispersed in literature, making it more difficult to integrate and compute all the information available; and c) to the candidate's personal view, none of them allows one to have a firm grasp of what are the most important unmet medical needs in the field unless there is already some clinical experience in dealing with TTP cases, and this is a factor that should drive and push research forward in all fronts, at the most basic biological and at the clinical translational levels. Steering a ship (whether we speak about management of a patient's condition, or where to focus research on) with such integrated insights allows one to find a quicker way towards the right path. Therefore, an opportunity for further contribution at this level is seized in this chapter, setting the stage for the scope of this thesis.

What TTP is… and what it is not

Thrombotic Microangiopathic Anemias (TMAs) are characterized by the common triad of severe thrombocytopenia with disseminated microthrombi, microangiopathic hemolytic anemia (MAHA) and organ injury. The main existing TMAs are TTP and HUS, but there are others (**Table 1**). TTP and HUS may occur at any age (early childhood and adolescence, or during adulthood), with sudden or gradual onset, may be hereditary or acquired, may occur in non-idiopathic or in idiopathic circumstances – that is in situations where a potential trigger or associated condition can be identified or not, respectively – and usually present themselves as a progressive syndrome with high fatality if untreated, in most cases with periods of recurrence spaced by periods of complete remission[64,65]. TMA's are considered rare conditions (incidence of 10/million people/year). Adult onset TTP has an estimated incidence of ~3-4 patients/million people/year[15,66,67], and an estimated prevalence that is dependent on the region analyzed, varying from 3/million in Japan, to 13/million in France, and 18/million in the region of Oklahoma (USA). However it accounts for up to one-quarter of all diagnosed TMA

cases, with a median age of onset of ~41-45 years, a women-to-men ratio of 2:1, with approximately half of all cases being of idiopathic origin. Among all adulthood TTP cases, ~75% are of autoimmune nature, 22% are acquired with unknown causes, while only 3% are of congenital origin (the latter with practically all cases being associated with pregnancy; however, only 34% of pregnant women who develop TTP are congenital patients, still a rather large fraction of this sub-population)[68]. Childhood- and adolescent TTP comprises cases of a first episode before 18 years of age, being representative of only 9% of the whole TTP patient population. Within this sub-population, ~33% of cases are congenital, while the rest are acquired. Among the acquired cases of childhood-onset TTP, there is also a nearly equal balance between idiopathic and non-idiopathic cases, with about 82% of all acquired cases being autoimmune. The ratio girls-to-boys is 2.5:1[69] (**Figure 1A**). People of African-Caribbean descent (or black ethnicity) are overrepresented in TTP registries[67,70,71], yet they have significantly better survival outcomes than white patients, despite similar clinical presentation[71].

ADAMTS13 activity is the most specific biomarker for TTP, differentiating this TMA from other primary and secondary TMAs[65]. In TTP, ADAMTS13 activity is impaired (<10%, or <10 IU/dL), leading to accumulation of UL-VWF multimers (**Figure 1B**). This is either because of a) a hereditary set of (autosomal recessive) mutations that may directly impair activity, synthesis, or secretion of the protease[26,72,73] (these have clusters identified in several regions of the globe[74–76], comprising ~5% of all cases globally), or b) due to an acquired impairment (~95% of all cases), which is frequently due to developed autoantibodies against ADAMTS13 (~80% of all acquired cases) (**Figure 1C**). ADAMTS13 activity is important for cleavage of VWF. VWF is a large multimeric protein synthesized in various sizes and stored in Weibel-Palade bodies of endothelial cells, as well as α-granules of platelets. UL-VWF exists within these storage granules, and upon exocytosis is cleaved in smaller multimers by ADAMTS13. Thus, UL-VWF multimers in plasma are only found in non-physiologic conditions[77,78]. Weibel-Palade bodies' secretion is finely tuned[79], occurring in both normal conditions and in certain pathologic conditions upon stimuli that activate the endothelium[80]. Upon secretion, part of the VWF multimers remain anchored to the endothelium, and the remainder will travel in circulation in a globular form. Upon reaching the microcirculation, the shear stress generated by blood flow in these areas is enough to extend the molecule, revealing the cleavage sites in the A2 domain of VWF for ADAMTS13 to cleave the multimers into smaller sizes. The multimers that remained attached are more sensitive to the forces exerted by blood flow, as the anchorage allows the shear forces to act more effectively through extension from the points of attachment in the direction of the flow. This also makes the cleavage sites of VWF's A2 domain accessible to ADAMTS13[81]. The larger the multimers' size the more pro-thrombotic they are (**Figure 1B**). These aspects are important as they help to understand the most typical clinical findings in TMAs, in particular TTP. However, other TMAs' mechanisms are not related with

ADAMTS13 loss of activity. In infection-associated-HUS (IA-HUS), for example, there is endothelium activation triggered by a toxin produced by pathogenic bacteria, namely *Escherichia coli* O157:H7, with a consequent exaggerated release of VWF multimers, and bloody diarrhea, which is managed with supportive treatment (e.g. hydration, peritoneal dialysis)[82]; in contrast, complement-mediated-HUS (CM-HUS) may be hereditary or acquired and is caused by uncontrolled complement factor activation in the complement system, leading to endothelial damage with similar consequences, but managed with directed therapies (e.g. eculizumab)[65].

Table 1 – The general clinical spectrum of Thrombotic Microangiopathies (TMAs)

Criteria for a TMA diagnosis	
· Thrombocytopenia (platelet counts < 150 x10^9/L) · Anemia (Hemoglobin < 10 g/dL) (Hemolytic Nature) · Organ failure of varying degree (caused by systemic thrombosis in smaller vessels)	
Important TMAs	
· Thrombotic Thrombocytopenic Purpura (TTP) · Hemolytic Uremic Syndrome (HUS) · Autoimmune Haemolysis/Evans Syndrome · Disseminated Intravascular Coagulation	· Pregnancy Associated o HELLP (Haemolysis, Elevated Liver enzymes, Low Platelets) o Eclampsia · Catastrophic Anti-Phospholipid Syndrome
TTP **vs**	**HUS**
· **ADAMTS13 activity < 10%** · Severe Thrombocytopenia ≤ 30 x 10^9/L · Schistocytes > 1.0% (usually ≥ 2.0%) · Elevated Lactate Dehydrogenase (usually LDH ≥ 200 U/L) · Central Nervous System symptoms are common · Fever (>37.5 °C) · Renal Impairment is usually mild o Creatinine ≤ 2.26 mg/mL (usually < 1.5 mg/mL) · Reduced Hematocrit (can be < 20%) · Elevated Reticulocytes (> 1.5%) · Haptoglobin: undetectable · Nucleated Red Blood Cells usually present · Cardiac Involvement common (predictive for death) o Troponin-T > 0.05 µg/L or Troponin-I > 0.1 µg/L · Anti-Nuclear Antibodies positive · Normal INR, PTT and fibrinogen · Coombs Test usually negative (positive in previously diagnosed autoimmune diseases)	· **ADAMTS13 activity ≥ 10%** · Renal Involvement is normally severe o Creatinine >2.26 mg/mL (usually ≥1.5 mg/mL) o Marked Proteinuria common · Infection-Associated-HUS (IA-HUS*): o Diarrhea present (usually bloody) or o Stool Culture positive for *Escherichia coli* O157:H7 or o PCR positive for Shiga-Toxin · Complement-Mediated-HUS (CM-HUS**): o Complement Activation o Complement Gene Tests show mutations in Alternative Pathway proteins *Previously called typical-HUS **Previously called atypical-HUS
Conditions commonly associated with TTP (non-idiopathic TTP)	
· Hematopoietic Stem-Cell Transplantation (HSCT) · Pregnancy/Post-Partum · Drugs (ticlopidine, quinine, cancer-chemotherapeutic agents, simvastatin, calcineurin inhibitors; possibly clopidogrel) · Systemic Lupus Erythematosus (SLE) and other autoimmune diseases	· Infections o Viral: HIV, Influenza, SARS-CoV2, HSV, CMV, Chikungunya, Adenovirus o Bacterial: Meningococcus, Pneumococcus o Fungal: Aspergillus · Malignancies · Malignant Hypertension · Rare cases of envenomation from animal encounters (Scorpion stings, vipers, bee stings)

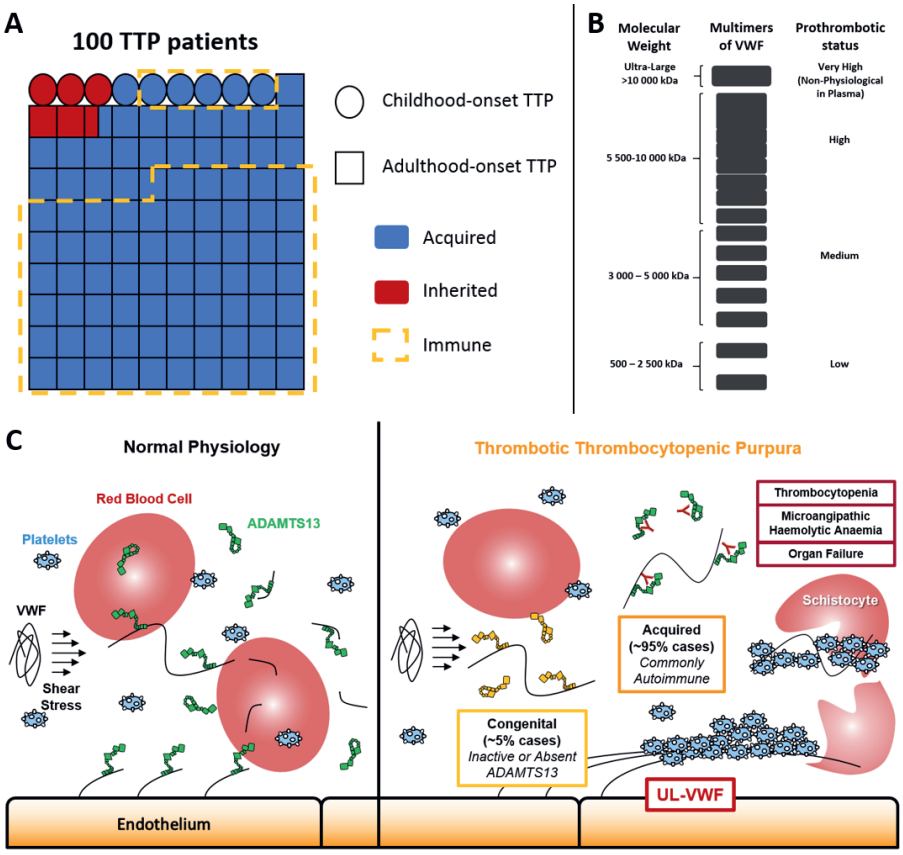


Figure 1 – Thrombotic Thrombocytopenic Purpura (TTP) for people in a hurry.

A. A brief overview of a population of 100 TTP patients and the likely distribution of the different types of TTP is shown. **B.** The multimeric size-distribution of VWF. **C.** Comparison between normal physiologic conditions of blood flow, and TTP. The basic mechanisms of disease are shown, as well as the characteristic signs and symptoms of disease. In TTP, ADAMTS13 activity < 10%.

Diagnosing TTP

The dismal prognosis of TTP if not correctly and promptly treated make it fundamental to achieve the quickest diagnosis possible. However, the general signs and symptoms normally observed in TMAs are quite heterogeneous and overlapping between each other. Distinguishing the type of TMA is essential to direct therapy and avoid further organ damage and mortality[83,84]. A TMA diagnosis should be always differential – that is, the underlying cause of TMA should be investigated. In the case of TTP, together with a severe thrombocytopenia and MAHA, it is very common to find also

high numbers of schistocytes (>1%) in the blood smear, with elevation of reticulocyte counts, nucleated red-blood cells, elevated levels of Lactate Dehydrogenase (LDH), and signs of organ failure to varying degree, with possible involvement of the kidneys (usually mild, with creatinine < 1.5 mg/dL), central nervous system involvement (common), and cardiac or gastrointestinal system involvement[85]; especially cardiac involvement (defined by higher troponin I or T levels) at presentation is a predictive risk factor for death in TTP[86,87] (**Table 1**).

The defining biomarker for TTP is a low ADAMTS13 activity (<10%, or <10 IU/dL). Several assays for activity measurement exist, none of which is perfect due to several reasons (reviewed)[88]. The most common test employed today is the FRETS-VWF73-based test, which employs the FRETS-VWF73 substrate – a 73-amino acid peptide from the A2 domain of VWF, containing the scissile bond to be cleaved by ADAMTS13 (Y1605-M1606) and residues spanning from D1596 to R1668 (VWF73), modified with fluorophore and quencher moieties that will allow for Fluorescence-Resonance Energy Transfer (FRET) upon cleavage by ADAMTS13 and correct wavelength excitation[30,89–92]. It requires a citrated plasma sample taken before treatment initiation, because EDTA and heparin samples will lead to a faulty assessment of ADAMTS13 activity, and standard treatment strategies followed will bias the outcome (although, in case treatment is initiated by mistake before sampling, an early sample may still be informative)[93]. This assay is more independent on ADAMTS13 exosite interactions with VWF (i.e. more dependent on metalloprotease activity), and thus has potential to give ADAMTS13 >10% in some cases of congenital TTP[93–95]; an alternative assay with the full-length VWF substrate should be used in cases where this is more likely to occur and the clinical judgement requires faster confirmation (e.g. collagen-binding assay)[92]. The need for a real-time diagnosis collides with the fact that ADAMTS13 tests are not always directly available in all healthcare facilities due to the rarity of the disease, necessary equipment and skill, and the intrinsic costs of the assay. Therefore, the test may have to be outsourced to specialized laboratories[93]. Even in facilities where these tests are available, the possible batching of the samples to analyze in order to reduce costs may constitute an additional time lag[96].

The Differential Diagnosis of TTP

The initial diagnosis of TTP is thus, a clinical diagnosis. To assist the clinician in predicting the ADAMTS13 activity level, scoring systems based on clinical findings have been developed[97–101]. Among the most popular are the PLASMIC Score[100–102] and the scoring system set out by the French TMA Reference Center (CNR-MAT)[16]. No system has been shown to be superior to another, which means that at this point, the clinical experience and awareness of TTP being a possible diagnosis is essential to

prevent misdiagnosis and delayed treatment[103,104]. Renal involvement has been shown to be less frequent in TTP cases while neurologic involvement is more frequent contrasting with other TMAs[16,102]. Before a definitive/confirmatory diagnosis is made, treatment should have already initiated, with blood sampling for ADAMTS13 activity made prior to treatment start[62].

After the definitive/confirmatory diagnosis is made (i.e. ADAMTS13 ≤10% confirms TTP), the diagnosis does not end there: a clinical investigation should be pursued to find the causative factor behind the low ADAMTS13 activity. This means understanding whether we are in the presence of an immune TTP case or if the loss of ADAMTS13 activity is due to a different mechanism. An ELISA assay should be used to assess presence of inhibitors (i.e. autoantibodies) against ADAMTS13 using commercial kits[105]. If autoantibodies are absent, we may be in the presence of an acquired TTP with a different mechanism, or it could be a congenital TTP case. The clinical context (age, comorbidities) and outcome to the already initiated treatment will provide important clues. For example: if a clinical response (a sustained recovery of platelet counts > $150x10^9$/L, at least for 2 days) and even a clinical remission (a clinical response sustained together with normalization of all parameters of end-organ damage > 30 days of treatment) are achieved but biological remission (ADAMTS13>10%) is not observed, and inhibitors are persistently undetectable, we are likely facing a congenital TTP case. The pathway to the full, complete diagnosis, is intimately linked with treatment and this will all be happening more-or-less in real time, with the patient's life at stake. To help "steer and keep the ship afloat", **Figure 2** presents the overall "Nautical Chart" to TTP diagnosis, treatment and management.

Different scenarios may be presented: the initial TTP diagnosis is regarding a situation where the patient is experiencing a bout of disease for the first time (usually in the context of a medical emergency – acute TTP). However, relapses may occur in previously diagnosed TTP patients in the context of TTP management (chronic TTP). Whenever there is reason for suspicion of TTP within the context of a medical emergency (based on clinical parameters), treatment should be initiated as early as possible, even before the results of ADAMTS13 activity are known[58,60,106–108]. In the case of relapses, current management and follow-up standards focus on relapse prevention, which makes it rare for relapses to present as a medical emergency[109] (see next section).

Obstetrical TTP constitutes a special population to be considered in both acute TTP and chronic TTP, as at least half of acute TTP cases affect women of childbearing age[110]. Pregnancy has been associated with a wide spectrum of TMAs ranging from TTP to HUS to HELLP syndrome (Haemolysis, Elevated Liver enzymes, Low Platelets), the latter frequently associated with the most common pregnancy complication, preeclampsia[111]. Up to 34% of pregnancy-associated TTP cases are of congenital TTP[68], a figure that can rise to 68% if only first pregnancies are considered[110]. ADAMTS13 is the most useful biomarker to distinguish between these syndromes[111], although each

syndrome will have more characteristic clinical features that guide clinical judgement (extensively reviewed[112,113]). Decreased ADAMTS13 activity and antigen levels are natural during pregnancy[114], and are facilitated by: 1) the increased secretion of VWF antigen which occurs during a normal pregnancy (prominently during the second and third trimesters[115]); 2) hormonal changes[116] and 3) increased levels of inflammation (particularly present in the case of preeclampsia)[117–119], all of which contribute for progressively reduced ADAMTS13 antigen availability and activity. First time obstetrical TTP occurs normally during the second and third trimesters, and in some cases post-partum, but it is unusual during the first trimester[114]. If not managed promptly, fetal outcome rates may be dramatic due to intra-uterine fetal death (miscarriage and spontaneous abortions) or early infant death (accounting to 40%)[114]. In another scenario, a female TTP patient of childbearing age undergoing an otherwise untroubled remission period may decide to become pregnant during this time (more common in acquired TTP patients). This is to be considered during follow-up medical management, as the risk of TTP relapse during pregnancy is high if no preemptive measures are taken (range between 60-90% in the past[120]). Pregnancy is not contra-indicated in TTP[121], and prevention of relapses can be done[122].

Another special population to consider are HIV positive patients. TTP may be the first presentation of AIDS, or present itself due to low CD4+ counts following non-compliance with anti-retroviral treatment. Regional prevalence of HIV infection influences the frequency of HIV infection in TTP patients[123]. Screening for HIV is relevant as remission is dependent on (re-)institution of Highly Active Antiretroviral Therapy (HAART)[57,106,124,125].

Treatment and management of the TTP patient

The frontline treatment to employ in a patient strongly suspected for TTP diagnosis (congenital or acquired) is plasma exchange (PEX), to be performed as 1.5x plasma volume once daily for the first days followed by 1.0x plasma volume once daily, until a sustained clinical remission is achieved[10,11]. PEX is preferable to plasma infusions, but in case this modality of plasma therapy is not available within a reasonable amount of time (as per clinical judgement), plasma infusions should be used until PEX becomes available[126]. This is also valid to HIV+ patients, who benefit from this therapy (especially patients with a severe ADAMTS13 activity loss (<5%); HIV+ patients with detectable levels of ADAMTS13 (>5%) normally have more advanced HIV infections with severe immune deficiency and have worse responses to plasma therapy; in either case, HAART should be instituted)[106,124,125]. Once confirmatory diagnosis is made, PEX should continue until a sustained clinical and biological response is achieved. Platelet counts should be performed regularly (daily basis) initially and after clinical response to monitor progress.

Box 1 – Glossary for Thrombotic Thrombocytopenic Purpura (TTP)

This box contains important definitions to keep in mind when discussing TTP cases or how new treatments, tests, and biomarkers may impact the current and future clinical practice. In the bottom, a very compressed map represents the usual flow of clinical practice in TTP.

TTP bout: An episode of acute TTP.

Clinical response: This is defined as sustained normalization of platelet counts (above the lower limit of the established reference range, e.g. > $150x10^9$/L) and of LDH (< 1.5 upper limit of normal) after plasma exchange.

Clinical remission: This is defined as a sustained clinical response after cessation of plasma exchange, maintained for > 30 days. Clinical response and clinical remission are associated with stabilization of parameters if end-organ damage is severe or there is an improvement in function with normalization of laboratory parameters. However, clinical remission may occur without biological remission.

Biological remission: This is defined as a sustained recovery of ADAMTS13 activity to levels above 10%.

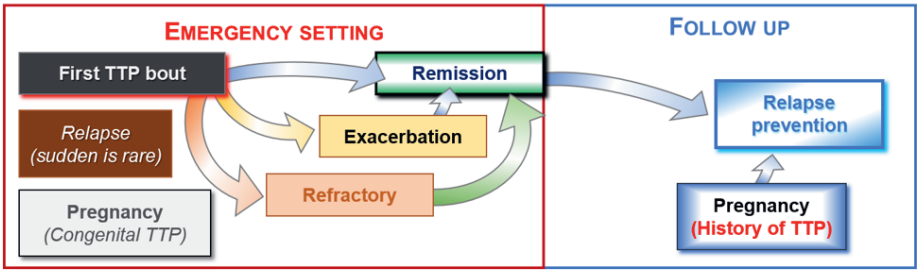
Exacerbation: This is defined as a reduction in platelet counts to below the lower limit of the established reference range (e.g.<$150x10^9$ /L), an increased LDH level, and the need to restart plasma exchange within 30 days of the last plasma exchange after a clinical response was obtained.

Relapse: This is defined as a fall in platelet count to below the lower limit of the established reference range (e.g <$150x10^9$/L), with or without clinical symptoms, >30 days after stopping of plasma exchange for an acute TTP episode, requiring re-initiation of therapy. This is usually associated with a new increase in the LDH level and preceded by a progressive decrease of ADAMTS13 activity.

Refractory TTP: This is defined as persistent thrombocytopenia, lack of a sustained platelet count increment or platelet counts of <$50x10^9$/L and a persistently raised LDH level (>1.5 upper limit of normal) despite five plasma exchanges and steroid treatment. This would be defined as 'severe' if the platelet count remained at < 30 x 10^9/L. The clinical condition may be more stable at this point.

The flow of clinical practice in a nutshell

TTP bouts that occur for the first time (including during pregnancy, where congenital TTP is more prevalent than usual) normally occur in an emergency setting, requiring hospitalization of the patient in intensive care units. The main goal will be leading the patient through to remission (clinical and biological). Some patients that are on route to a remission may suddenly worsen for any reason (exacerbation) requiring further attention. In some cases, patients fail to respond: these are refractory to frontline treatment, requiring either intensification of frontline or alternative treatments. These patients may achieve remission, and after full recovery all must be followed to prevent relapses (in the case of women of childbearing age, pregnancy planning should receive focus).



Given the prominent number of immune TTP cases, there is a rationale to administer corticosteroids together with PEX frontline, despite the low level of evidence for this practice being superior to PEX alone. High-dose methylprednisolone (10 mg/kg/day for 3 days and then 2.5 mg/kg/day) was shown to be more efficacious than low dose methylprednisolone[127]. Together with the safe profile of these drugs[128], the data suggest these should be standard frontline therapy.

Especially in immune TTP cases, rituximab, an anti-CD20 antibody should be considered as an adjunctive therapy frontline (4x 375 mg/m^2/week in 4 weeks or within 2-3 weeks), for 2 reasons: a) it improves the short-term probability of remission and the

long-term probability of relapse-free survival (within the first year after remission) when used frontline[48,49], with limited adverse effects reported so far[129,130] and b) it normally takes ~14-days for the clinical effects to manifest, a factor that must be considered in the context of intensive care. It does however warrant a rationale for administration (i.e. an immune TTP diagnosis) and ruling out potential contra-indications (such as underlying Hepatitis B and C, or HIV infections)[48,129].

Finally, Caplacizumab has been recently approved for acute TTP treatment as an adjunct to PEX and immunomodulation, being capable of reducing time for response, with concurrent reduction in hospitalization times, at the expense of an added risk for manageable bleeding events[50–53]. However, clinical experience with this agent is still quite limited, and the added cost should be considered[131].

In cases of exacerbation and refractory patients, PEX should be increased to twice daily 1.5x plasma volume, and rituximab introduced. Other treatments may be considered on a case by case basis: there is no consensus on how to treat these patients[60].

Once a sustained remission is achieved (regardless of congenital or acquired TTP) and the patient recovers, the management proceeds with a regular follow up focused on relapse prevention where ADAMTS13 activity is measured in adequate time intervals, being tightened if required. If ADAMTS13 start decreasing (normally happens in a progressive manner), preemptive rituximab should be considered, especially in immune cases, being enough to recover normal ADAMTS13 activity levels in most cases[132,133]. Congenital TTP patients require regular plasma infusions to be maintained relapse-free (10-15 mL/kg every 2-3 weeks)[134]. Of special note, within previously diagnosed TTP patients who wish to get pregnant, pregnancy outcomes are usually dependent of ADAMTS13 baseline activity values, and it has been recognized that close monitoring alone leads to poorer outcomes[135,136]. Planning is important: pro-active relapse prevention measures should be taken, with monthly assessments of ADAMTS13 levels and prophylactic monthly or weekly PEX (ADAMTS13 <10%), extending to post-partum. Importantly, unlike with HELLP and eclampsia, fetal excision does not correct the condition[110].

Figure 2 attempts to provide general guidance, however it does not replace clinical expertise. Contacts with specialized reference centers and expert clinicians are encouraged. In France for example, the activities of Telemedicine are an integral part of the healthcare infrastructure in TTP management[137].

Figure 2 (Next Page) – The "Nautical Chart" for TTP Diagnosis, Treatment and Management.

Medical emergencies where a TMA is apparent and, according to the clinical context, TTP is suspected, are to be followed by immediate treatment, even in the absence of ADAMTS13 activity. Diagnosis investigation should attempt to differentiate in full the disease mechanism. After recovery, the patient proceeds to Medical Follow up. Special populations are considered. Telemedicine is a resource that should be encouraged. Clinicians should be aware of the existence of Reference Centers in their country, or in other countries, and the possibility to contact these centers. Further details in text.
PEX = Plasma Exchange; Plasma therapy = plasma infusions or PEX

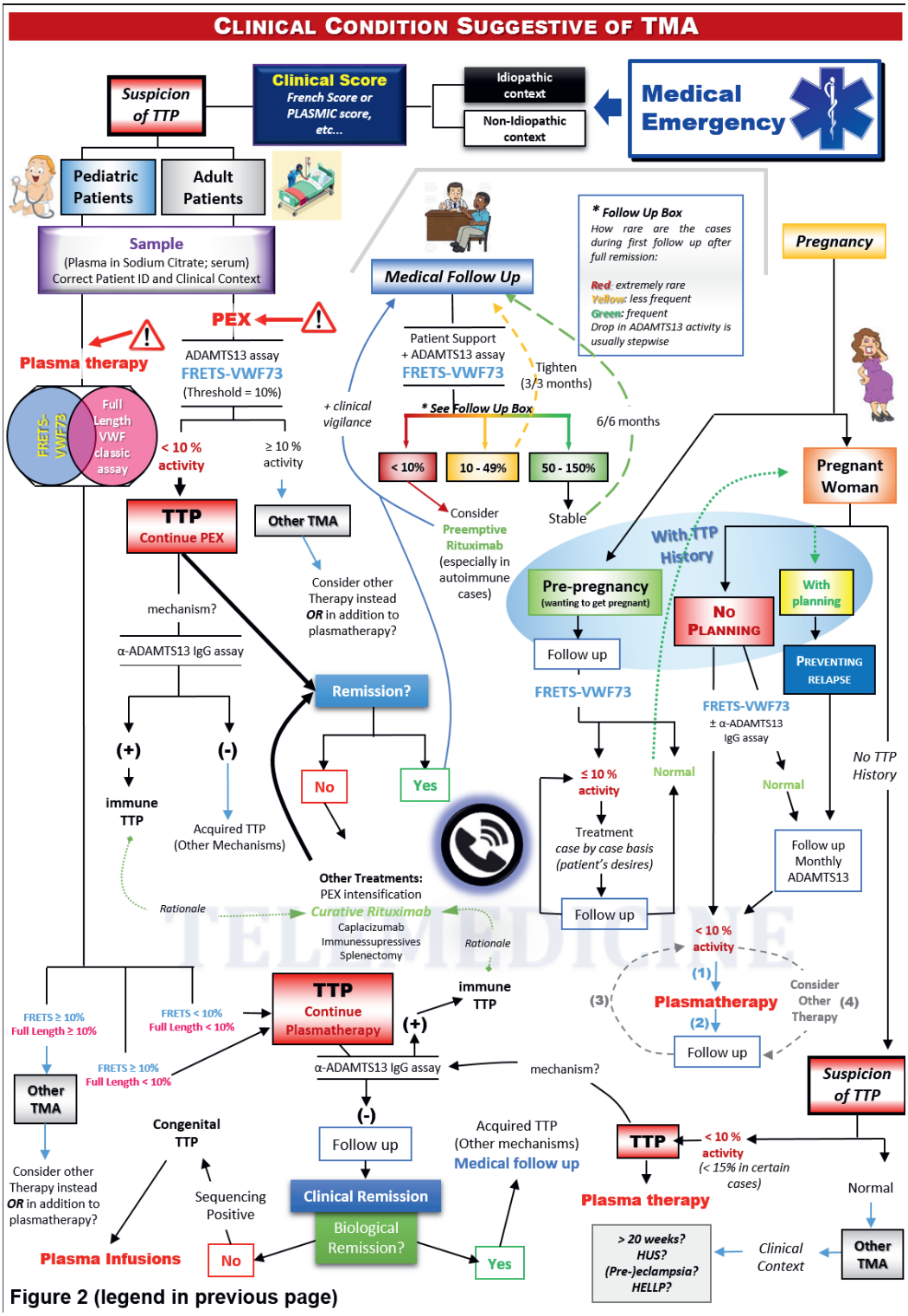


Figure 2 (legend in previous page)

Unmet medical (and patient) needs

There are still several unmet needs that require attention from the research community and warrant pre-clinical and clinical studies. These can be categorized in 4 areas, although some may overlap with more than one area: 1) Basic Research; 2) Diagnosis; 3) Treatment and 4) Management. They are:

- A better understanding of the etiology of acquired TTP (in particular, immune TTP) to allow development of more effective (possibly personalized) treatments; this is correlated with the attempt to understand the reasons why autoimmunity exists[138];
- There is a need for a fast and correct diagnosis. This must be tackled in two fronts:
    - Increasing physicians' awareness (particularly general practitioners): despite its rarity, TTP is the most deadly form of TMA; physicians should consider TTP a possibility more often. TTP treatment should be privileged more often. Inexperienced physicians need extra-formation and need to know who to contact in case help is required (reference centers, expert clinicians) and to know what information should be given and why[137,139].
        - ADAMTS13 activity assay:
            - expertise is still centralized: available commercial kits present false negatives more often than in-house methods[91,93];
            - non-automatized testing still takes several days[93];
            - sample batching for economic restrictions further delays the test[96];
            - it is still only a confirmatory diagnosis procedure[97,102,140,141];
            - new test kits are being developed to allow a more real-time diagnosis and early intervention; however, these tests haven't yet shown a better outcome than centralized in-house methods or clinical scores[142–144];
            - plasma pools are not always standardized everywhere (however, there was a recent introduction of the first international plasma standard by the World Health Organization[145]);
            - the range of a 'normal' ADAMTS13 activity is very broad among healthy individuals (50-150%); diagnosis and follow-up are not guided by a basal ADAMTS13 physiological activity level of the patient – i.e. there is no 'before' and 'after' TTP ADAMTS13 activity, and this may cause diagnostic issues for relapse prevention and prognosis;
- More reliable markers are needed for clinical diagnosis:

- combining creatinine levels with platelet counts and anti-nuclear antibodies has 48.1% specificity; 98.8% sensitivity; negative predictive value 93.3%, positive predictive value 85%[97]; other clinical score scales are being used[99–102] and developed[146,147], but neither has been directly compared and shown to be superior to the others;
- borderline values in several cases and the broad clinical spectrum do not allow clear distinction between TTP and other TMAs (e.g. CM-HUS);

- Treatments are effective but not optimal:
  - PEX is not standardized: treatment varies on a location basis (tapering PEX? Stop suddenly? Include other products like 4% albumin?[56,60] What plasma product to use?[148]) and on a case-by-case basis – some patients recover fast, others take many sessions[56], requiring in some cases over 600 healthy plasma donors to achieve remission[10,11], and consuming on average 40 L of plasma per TTP bout in the United Kingdom[148]; the logistics are complex, specialized equipment and personnel are needed, a fast central venous access is required[149] and prolonged catheterization of the patient as well as adverse reactions to plasma products and added risks imbue frontline therapy with many complications that undermine current treatments[150–152] (although their frequency has been reduced throughout the years)[64];
  - Rituximab is an off-label drug: it's use in TTP patients must be justified by immunity; on average rituximab takes ~2 weeks to start working to achieve remission and relapse prevention, and there is limited data regarding long-term safety of its use[48];
  - Treatments require clinical supervision upon administration. There is a need for treatments with quicker effects and more simple regimens, ideally not needing specialized human resources for administration, and giving also more freedom to patient mobility;
  - most data collected comes from retrospective studies; no or few randomized clinical trials have yet been made; this is due to ethical concerns that are difficult to overrule and difficulty in patient recruitment (rare disease)[109,153];
  - Caplacizumab is very recent, compared to rituximab and PEX: its relative efficacy is not entirely known yet, and treatment costs are a concern[141];
  - Other alternative treatments, like bortezomib, and adjunctive treatments such as N-acetylcysteine have very limited experience and their relative efficacies are also still unknown and under study[109,141];
  - It is not known how the immune-genetic background of the patient affects treatment efficacy;
  - Pregnancy remains a population in need for safe TTP treatments; rituximab has been used before pregnancy as a preemptive treatment and women

are advised to wait 1 year after administration before conceiving; some have become pregnant before this without any major adverse effects reported to date[110];

- Patient information and awareness is required:
  - o patients need to understand the disease (Layman's terms communication, scientific and medical jargon to be avoided) and understand the importance of medical follow up (e.g. some patients miss their consultations; pregnancy planning in women of childbearing age is important);
  - o patients must understand the need of having recorded data (for patient own welfare and for scientific knowledge)[154].
- As with any rare disease, treatments should also work to improve the relevant clinical outcomes for patients and address patient concerns[155]: a patient's perspective was published showing that for patients, current treatments are cumbersome and patients fear the added risk of high volumes of PEX usually required[156]; TTP is known to be followed by several sequelae in the central nervous system[157] with chronic fatigue, headache, memory impairment, reduced work productivity, depression, anxiety[158–160] and post-traumatic stress disorder[161]; it is clear that current treatments do not prevent these long-term outcomes; these are also more difficult to study due to the long time span required to collect the information and analyze it.

A concise set of claims based on real-life events and examples was also published in scientific literature recently compiling most of these problems[139].

Scope of this thesis

This thesis proposes to be not a mere scientific treatise and/or a compendium of published (or soon to be published) scientific articles, but a contribution to the attempt of solving societal problems, namely some of the unmet medical and patient needs stated in the previous section.

In **Chapter 1** a more or less thorough review of the current state-of-the-art in clinical practice in the TTP field was presented, which addresses the need to contribute to increased physicians awareness of this disease entity and their formation. It may be possible that this also contributes (although expectedly to a much more limited extent) to patients' understanding of the disease.

In **Chapter 2**, a review dedicated to the background pathophysiology of TTP in terms of genetic risk factors (such as HLA alleles), is presented, complementing Chapter 1.

In **Chapter 3** we have an original research scientific study with a detailed epitope

mapping of the spacer domain of ADAMTS13 beyond the classic exosite-3, showing that 3 regions of the spacer domain are important epitopes in a high number of patients. These are the regions of the amino acid residues 588-592, 602-610 and 657-666.

In **Chapter 4**, the residues that compose the spacer exosite-3 epitope are explored in depth for their contribution to a) autoantibody resistance and b) activity of recombinant ADAMTS13 variants. There is an inverse correlation between activity and autoantibody resistance which is dependent on the degree of similarity of amino-acid side-chain structure. The higher this similarity, the higher the activity of the ADAMTS13 variant, but the lower is it's the resistance towards patients' autoantibodies binding. The inverse is also true: the lower this similarity, the lower the ADAMTS13 variant activity will be, but the higher will be the resistance towards autoantibodies binding.

In **Chapter 5**, a modification that allows the "best of both worlds" seen in Chapter 4, is presented: by adding an artificial N-glycan motif to one of the 3 spacer regions found on Chapter 3, it was possible to find an ADAMTS13 variant that shows higher resistance towards patient autoantibodies without any intrinsic activity loss. This is an important first step towards making Joel Moake's old concept of "Survival by giving a dam"[27] a reality for iTTP patients.

In **Chapter 6**, a structural study of ADAMTS13 is presented. The capacity of the protein to alter its conformation status is explored to understand which intrinsic factors contribute for the maintenance of an ADAMTS13 closed conformation.

In **Chapter 7**, a general discussion of the findings presented in this thesis is made, with emphasis on potential impact in the field, and future directions to be taken.

Finally, in the **Appendix**, besides the regular content traditionally reserved for this part, a testimony is given in the form of a joint written interview (published in the journal *Hemasphere*; see Related Publications). This regards the type of training programme followed by the current PhD candidate throughout 60% of the total time that was required to fulfil this PhD (followed jointly with other PhD candidates). Considering its content, it is believed to deserve an honorable mention here, and a place inside this thesis.

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