Interaction Between the Metalloprotease ADAMTS-13 and the Proteins of the Alternative Pathway of the Complement System

Thesis

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for the degree of

Doctor of Philosophy

Discipline of Life and Biomolecular Sciences

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30\textsuperscript{th} September 2016
ABSTRACT

Von Willebrand factor (VWF), a multimeric protein that has a central role in hemostasis, has been shown to interact with complement components. However results are contrasting and inconclusive.

By studying 20 patients with congenital thrombotic thrombocytopenic purpura (cTTP) who can not cleave VWF multimers due to genetic ADAMTS13 deficiency, we investigated the mechanism through which VWF modulates complement, and its pathophysiological implications for human diseases.

Using assays of ex-vivo serum-induced C3 and C5b-9 deposits on endothelial cells we documented that in cTTP complement is activated via the alternative pathway on the cell surface. This abnormality was corrected by restoring ADAMTS13 activity in cTTP serum, which prevented VWF multimer accumulation on endothelial cells, or by an anti-VWF antibody. This study documented: 1) ADAMTS13 is able to interact with FB, but how this interaction could modulate complement activation remains still unknown; 2) VWF does not affect regulatory complement activity of FH for inactivation of C3b or dissociation of C3 convertase, in contrast to what reported in literature; 3) VWF interacts with C3b through its A2 domain and initiates a previously unrecognized Calcium-dependent mechanism of alternative pathway activation, with assembly of active C3 and C5 convertases and formation of the terminal complement products, C5a and C5b-9. Finally, this work documented that in condition of ADAMTS13 deficiency, VWF-mediated formation of terminal complement products, particularly C5a, alters the endothelial anti-thrombogenic properties and induces microvascular thrombosis in a perfusion system.

Altogether, the results demonstrated that VWF provides a platform for the activation of the alternative pathway of complement, which profoundly alters the phenotype of microvascular endothelial cells. These findings link hemostasis-thrombosis with the alternative pathway of complement and open new therapeutic perspectives in cTTP and in general in thrombotic and inflammatory disorders associated with endothelium perturbation, VWF release and complement activation.
To my Family
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<td>ADAMTS13</td>
<td>A Disintegrin-like And Metalloprotease with TromboSpondin type 1 domain, number 13</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine DiPhosphate</td>
</tr>
<tr>
<td>AP</td>
<td>Alternative Pathway</td>
</tr>
<tr>
<td>aHUS</td>
<td>Atypical Haemolytic Uremic Syndrome</td>
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<tr>
<td>ANA</td>
<td>ANAphylatoxin domain</td>
</tr>
<tr>
<td>ANOVA</td>
<td>ANAlysis of VAriance</td>
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<tr>
<td>Ba</td>
<td>The smallest cleavage fragment released from FB</td>
</tr>
<tr>
<td>Bb</td>
<td>The biggest cleavage fragment released from FB, which forms C3 convertase</td>
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<td>C3</td>
<td>Complement C3</td>
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<td>C3a</td>
<td>Anaphylatoxin, the smallest cleavage fragment released from C3</td>
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<td>C3b</td>
<td>The biggest cleavage fragment released from C3, which forms C3 convertase</td>
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<td>C5a</td>
<td>Anaphylatoxin, the smallest cleavage fragment released from C5</td>
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<td>cTTP</td>
<td>Congenital form of TTP</td>
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<td>CP</td>
<td>Classical Pathway</td>
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<td>CUB</td>
<td>A structural motif of approximately 110 residues found in complement component C1r/C1s, Uegf and Bone morphogenic protein 1</td>
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<tr>
<td>DAF</td>
<td>Decay Accelerating Factor</td>
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<tr>
<td>DAPI</td>
<td>DiAmidinoPhenylIndole, fluorescent stain for DNA</td>
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<td>DEAP-HUS</td>
<td>Deficiency of CFHR plasma proteins and Autoantibody Positive form of Haemolytic Uremic Syndrome</td>
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<td>DGKE</td>
<td>DiacylGlicerol Kinase Epsilon</td>
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<td>DTT</td>
<td>DiThioThreitol</td>
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<tr>
<td>EDTA</td>
<td>EthyleneDiamineTetraacetic Acid</td>
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<td>EGTA</td>
<td>EthyleneGlycolTetraacetic Acid</td>
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<td>ESRD</td>
<td>End-Stage Renal Disease</td>
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<td>ESRF</td>
<td>End-Stage Renal Failure</td>
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<tr>
<td>FB</td>
<td>Factor B</td>
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<td>Haemolytic anemia, Elevated Liver enzymes, Low Platelets syndrome</td>
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<td>Human Microvascular Endothelial Cell line 1</td>
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<td>Hematocrit</td>
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<td>ICS</td>
<td>Intracellular Complement System</td>
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<td>LDH</td>
<td>Lactate Dehydrogenase</td>
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<td>LFB-VWF</td>
<td>Plasma VWF from LFB Biotechnologies</td>
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<td>MAC</td>
<td>Membrane Attack Complex</td>
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<td>Minor Allele Frequency</td>
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<td>rADAMTS13</td>
<td>Recombinant ADAMTS13</td>
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<td>Rvwf</td>
<td>Recombinant VWF</td>
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<td>rVWF-A1 or - A2 or -A3</td>
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<td>TAFIa</td>
<td>activated form of Thrombin-Activatable Fibrinolysis Inhibitor</td>
</tr>
<tr>
<td>TED</td>
<td>ThioEster Domain</td>
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<tr>
<td>THBD</td>
<td>Thrombomodulin</td>
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<tr>
<td>TMA</td>
<td>Thrombotic MicroAngiopathy</td>
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<tr>
<td>TSP1</td>
<td>ThromboSpondin-like 1</td>
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<tr>
<td>TTP</td>
<td>Thrombotic Thrombocytopenic Purpura</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
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<tr>
<td>ULVWF</td>
<td>Ultra Large Von Willebrand Factor</td>
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<tr>
<td>VWF</td>
<td>Von Willebrand Factor</td>
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<td>VWF-CP</td>
<td>VWF Cleaving Protease</td>
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<td>WB</td>
<td>Western Blot</td>
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<td>WPBs</td>
<td>Weibel-Palade Bodies</td>
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ABSTRACT
Von Willebrand factor (VWF), a multimeric protein that has a central role in hemostasis, has been shown to interact with complement components. However results are contrasting and inconclusive.

By studying 20 patients with congenital thrombotic thrombocytopenic purpura (cTTP) who can not cleave VWF multimers due to genetic ADAMTS13 deficiency, we investigated the mechanism through which VWF modulates complement, and its pathophysiological implications for human diseases.

Using assays of ex-vivo serum-induced C3 and C5b-9 deposits on endothelial cells we documented that in cTTP complement is activated via the alternative pathway on the cell surface. This abnormality was corrected by restoring ADAMTS13 activity in cTTP serum, which prevented VWF multimer accumulation on endothelial cells, or by an anti-VWF antibody. This study documented: 1) ADAMTS13 is able to interact with FB, but how this interaction could modulate complement activation remains still unknown; 2) VWF does not affect regulatory complement activity of FH for inactivation of C3b or dissociation of C3 convertase, in contrast to what reported in literature; 3) VWF interacts with C3b through its A2 domain and initiates a previously unrecognized Calcium-dependent mechanism of alternative pathway activation, with assembly of active C3 and C5 convertases and formation of the terminal complement products, C5a and C5b-9. Finally, this work documented that in condition of ADAMTS13 deficiency, VWF-mediated formation of terminal complement products, particularly C5a, alters the endothelial anti-thrombogenic properties and induces microvascular thrombosis in a perfusion system.

Altogether, the results demonstrated that VWF provides a platform for the activation of the alternative pathway of complement, which profoundly alters the phenotype of
microvascular endothelial cells. These findings link hemostasis-thrombosis with the alternative pathway of complement and open new therapeutic perspectives in cTTP and in general in thrombotic and inflammatory disorders associated with endothelium perturbation, VWF release and complement activation.
1. INTRODUCTION
**1. INTRODUCTION**

**Thrombotic microangiopathies**

The term “thrombotic microangiopathy” defines a lesion of the microvasculature characterized by detachment and swelling of the endothelial cells from the basement membrane, deposition of amorphous material in the subendothelial space, and intraluminal platelet aggregation leading to microthrombosis with partial or complete obstruction of the vessel lumina (Ruggenenti et al. 2001).

Microvascular thrombosis is the mechanical obstruction of the vessels of microscopic bore (as arterioles and capillaries), caused by the accumulation of platelets, tissue factor and fibrin on the disrupted endothelium, happening when regulatory mechanisms are overwhelmed (Furie and Furie 2008). This process hampers blood perfusion, producing fragmented erythrocytes, called “schistocytes” or “helmet cells” and generating microangiopathic haemolytic anemia (MAHA) related to the destruction of the red blood cells (Tsai 2013).

Thrombotic microangiopathies (TMA) manifest clinically with a spectrum of multisystemic rare diseases characterized pathologically by occlusive microvascular thrombosis and clinically by profound thrombocytopenia, MAHA, and variable signs and symptoms of organ ischemia (Moake 2002; Zheng and Sadler 2008). TMA is diagnosed in patients experiencing one or more episodes of MAHA and thrombocytopenia (platelet count less than 150000/mm³, hematocrit < 30%, haemoglobin < 10 g/dl, Lactate Dehydrogenase - LDH > 460 IU/l, undetectable serum Haptoglobin, red cell fragmentation in the peripheral blood smear) with fever. These symptoms reflect consumption and disruption of platelets and erythrocytes in the microvasculature of kidney, brain, and other organs that leads to their consequent tissue dysfunctions (Ruggenenti et al. 2001; Zheng and Sadler 2008; Zipfel et al. 2011).
TMA are considered a group of diseases with overlapping clinical characteristics and heterogeneous etiology, highlighting the multicausal nature of microvascular thrombotic disease. The two most important forms of TMA are thrombotic thrombocytopenic purpura (TTP) and haemolytic uremic syndrome (HUS) (Moake 2002; Tsai 2013).

1.1 Thrombotic thrombocytopenic purpura

The term TTP was first introduced in 1924 by Eli Moschcowitz who described a 16 year-old female patient with abrupt onset of petechiae, acute febrile attack, haemolytic anemia, bleeding, renal failure, neurologic involvement and death within two weeks. Autopsy revealed widespread hyaline thrombi in terminal arterioles and capillaries of heart and kidney (Moschcowitz 2003). Other similar identified TTP cases were reported in a paper that was published in Blood in 1947 (Singer et al. 1947). The disease was clinically established in 1966 as a pentad of symptoms: thrombocytopenia, haemolytic anemia, renal dysfunction, neurological involvement and fever (Moschcowitz 2003). In the subsequent decades, thanks to the successful treatment of different TTP-like episodes, the disease was named the Upshaw-Schulman syndrome (Schulman et al. 1960; Upshaw 1978), and up to 1980 almost 500 cases have been reported and deeply described in the literature (Cuttner 1980; Kennedy et al. 1980). Nowadays the term “thrombotic thrombocytopenic purpura” is used to describe microangiopathic haemolytic anemia and thrombocytopenia occurring in adults without an apparent alternative cause, with or without neurologic or renal abnormalities (George 2006; Scully et al. 2012). Neurological symptoms can be seen in more than 90% of patients during the entire course of the disease, while renal impairment has been reported in around 25% of patients, although severe renal insufficiency is rare (Ruggenenti et al.
2001). In the majority of the cases TTP manifests as a single, sporadic acute episode, which can cause death if it is not treated. The 20 - 50 % of patients that survive from the acute bout develop the chronic form of the disease with a relapse within 6 months (George 2000; Sadler 2008).

### 1.1.1 Classification

TTP has been considered one of the most intriguing and mysterious disorders for years, and its pathogenesis remained disregarded until the end of 19\textsuperscript{th} century. In 1982, Moake described patients with chronic relapsing TTP with circulating “unusually large” (UL) multimers of von Willebrand Factor (VWF), a multimeric glycoprotein that plays an essential role in platelet-mediated primary hemostasis (Moake et al. 1982). Moake proposed that his patients lacked a VWF depolymerase, which later was named VWF-cleaving protease (VWF-CP) (Furlan et al. 1996; Tsai 1996) and identified as a new member of the super family ADAMTS (A Disintegrin-like And Metalloprotease with a ThromboSpondin type1) designated as ADAMTS13 (Gerritsen et al. 2001; Soejima et al. 2001). Since then, TTP diagnosis remained clinical but the measurement of ADAMTS13 activity within patient plasma could help to confirm the diagnosis and monitor the course of the disease.

Today, two forms of acute TTP with severe ADAMTS13 deficiency (<5% of that in normal human plasma) are distinguished:

- Congenital severe ADAMTS13 deficiency is found in hereditary TTP, also called Upshaw–Schulman syndrome, and it is the result of compound heterozygous or homozygous mutations in the ADAMTS13 gene (Levy et al. 2001; Kokame et al. 2002; Matsumoto et al. 2004; Veyradier et al. 2004; Donadelli et al. 2006; Schneppenheim et
al. 2006; Kokame et al. 2008). Severe ADAMTS13 deficiency is present both during acute and remission phases of the disease, in the absence of anti-ADAMTS13 autoantibodies. Congenital TTP (cTTP) cases are extremely rare (1 case per million person years) and represent approximately 10% of all TTP cases (Galbusera et al. 2009). Based on reported congenital TTP case reports, 48% of patients develop TTP in the neonatal period, 31% during childhood (2 months to 18 years) and 21% during adulthood (Lotta et al. 2010). The clinical severity of disease episodes varies between patients. Some have asymptomatic episodes of thrombocytopenia and anaemia, whereas others have multi-organ failure (Schneppenheim et al. 2003).

- **Acquired idiopathic TTP** is an immune-mediated, non-familial form of TTP that most likely accounts for the majority of cases (from 60% to 90%) reported to date as acute idiopathic or sporadic TTP. In acquired TTP, severe ADAMTS13 deficiency is the result of circulating anti-ADAMTS13 autoantibodies either inhibiting ADAMTS13 activity (Furlan et al. 1998; Tsai and Lian 1998; Shelat et al. 2006) or enhancing ADAMTS13 clearance (Scheiflinger et al. 2003; Rieger et al. 2005). Of note, only about two thirds of patients clinically diagnosed with acute idiopathic TTP have been found to be severely deficient in ADAMTS13 activity (Lammle et al. 2008; Sadler 2008), with some patients having normal or moderately reduced ADAMTS13 activity (Zheng et al. 2004; Bettoni et al. 2012). The ADAMTS13 cysteine-rich and spacer domains have been shown to be the primary autoimmune target in patients with TTP (Klaus et al. 2004; Luken et al. 2005), but the propeptide and thrombospondin domains could also be targeted by anti-ADAMTS13 autoantibodies (Luken et al. 2006). It has been reported that the characterization of anti-ADAMTS13 antibodies (IG isotype, titre and inhibitory effect) could have a prognostic value for the prediction of relapse and the progression of the disease (Ferrari et al. 2007). Evidence of the pathogenetic role of TTP-associated
anti-ADAMTS13 autoantibodies is derived by finding that they usually disappear from the circulation when remission is achieved by effective treatment and this occurs in parallel with the normalization of ADAMTS13 activity. The transient nature of the acute idiopathic TTP raises the possibility that the antibodies to the protease represent a deranged response to certain triggering events (Tsai and Lian 1998).

A particular diagnosis of acquired TTP is defined as secondary TTP, and it comprises about 40% of all cases of TTP. The term secondary TTP has been proposed for cases with an associated clinical condition, to distinguish them from the classical idiopathic form of TTP. Oxidative injury, free radical formation, a reduced production of prostacyclin, impaired fibrinolysis, the presence in the plasma of platelet-aggregating agents and an abnormal processing of VWF multimers have been proposed to be pathogenically relevant for the development of secondary TTP (Furlan and Lammle 2001). Secondary TTP is generally associated with cancer, hematopoietic stem cell or solid organ transplantation, preeclampsia, systemic infections, drug toxicity, or other predisposing conditions. Typically, secondary TTP is not associated with severe ADAMTS13 deficiency and rarely responds to the standard therapy of plasma infusion (Sadler 2006). The mechanism of secondary TTP is poorly understood, and a probable etiology may involve endothelial damage.

1.1.2 Incidence and risk factors

TTP is a rare disease with an incidence of approximately 2-10 cases per million person years, with a female predominance (female: male ratio, 3:2 to 5:2), it most frequently occurs in the fourth decade of the life. Recently, greater awareness and perhaps
improved diagnostic facilities have given the impression that the incidence is increasing (Terrell et al. 2005; Galbusera et al. 2009; Kappler et al. 2014).

TTP sometimes occurs in association with pregnancy, autoimmune disease, allogeneic bone marrow transplantation, infection, and drugs including quinine, cyclosporin, calcineurin inhibitor, mitomycin, ticlopidine and clopidogrel (Elliott et al. 2003). Above all, pregnancy is a prominent risk factor for the development of TTP. Pregnancy associated TTP is reported to represent 5-10% of all adult TTP cases and is estimated to occur in 1 out of 198,000 pregnancies (Moatti-Cohen et al. 2012). However, pregnancy can trigger TTP in women who have congenital or acquired ADAMTS13 deficiency (George 2003). Autoimmune diseases of various kinds have been described in association with severe ADAMTS13 deficiency and thrombotic microangiopathy. Patients with idiopathic and severe ADAMTS13 deficiency (<5%) often have manifestations of systemic lupus erythematosus, polyarthritis, extramembranous glomerulonephritis, or autoimmune thyroiditis (Coppo et al. 2004). The incidence of TTP following hematopoietic progenitor cell transplantation varies considerably, ranging from 0% to 74% probably due to confounding complications associated with the transplant (George et al. 2004). Infection is recognized to be a triggering causal factor for acute TTP as it can precipitate an episode in patients with other predisposing causal factors such as low ADAMTS13 activity level (Morgand et al. 2014). Of the wide variety of drugs observed to cause immune TTP, quinine antibiotics are the first described and best-studied probably due to their broad spectrum of action in patients following chemotherapy (Cheah et al. 2009). Ticlopidine, a potent antiplatelet agent, causes thrombotic microangiopathy with a frequency of a 1 case per 1600 to 5000 patients treated, often after 2 to 12 weeks of drug use, usually by inducing the formation of autoantibodies to ADAMTS13 (Tsai et al. 2000). Clopidogrel, another related
antiplatelet drug, has replaced ticlopidine in combination with aspirin, and has been associated with TTP with an incidence of 1.2 to 27.8 per million (Pisoni et al. 2001). Lastly, different studies reported episodes of human immunodeficiency virus (HIV)-associated TTP, defined as a subgroup of secondary TTP and considering the virus as an identified precipitant of the disease (Hart et al. 2011).

1.1.3 Therapy

In 1960 Schulman et al demonstrated for the first time the potential value of plasma therapy in TTP, reporting a case of an 8-year old girl with repeated episodes of thrombocytopenia and haemolytic anaemia who was treated with plasma infusions (Schulman et al. 1960). During the next 11 years, Upshaw successfully treated 32 TTP-like episodes of thrombocytopenia and microangiopathic haemolysis with plasma infusions. Upshaw suggested that the efficacy of plasma therapy for TTP could be explained as it replaces a single plasma factor that was deficient in the blood of TTP patients (Upshaw 1978).

Up to now, plasma therapy is still conventionally considered the gold standard for the treatment of all forms of TTP (Shepard and Bukowski 1987; George 2000; Kremer Hovinga and Meyer 2008). Therapeutic plasma administration transformed the historically fatal prognosis of TTP (10% survival), leading to the current overall survival rates of 90%. Patients with congenital ADAMTS13 deficiency are treated during acute episodes by infusion of fresh frozen plasma containing donor ADAMTS13 to compensate for their deficiency. Prophylactic treatment with plasma infusion every 2-3 weeks is used to prevent relapses (Barbot et al. 2001; Kentouche et al. 2002). In patients with acquired TTP, plasma exchange (PEX) has been the standard of care since
the early 1990s, when a randomized clinical trial established that PEX is more effective than plasma infusion in the treatment of the disease (Rock et al. 1991). PEX is effective because it has the additional benefit of removing the anti-ADAMTS13 inhibitory antibodies and inflammatory cytokines from the patient’s blood. Moreover, high-dose corticosteroids are frequently used as an adjunct therapy not only to reduce major complications risks of PEX, such as infection, thrombosis or haemorrhages, but also to provide a more durable response (Kremer Hovinga and Meyer 2008; Som et al. 2012). However, there is no indication for administering steroids to patients with congenital TTP. In recurrent or refractory TTP, which occurs in 20-30% of cases, treatment options include PEX with splenectomy or others immunosuppressive therapies, but the results are variable and often inconclusive (Yomtovian et al. 2004). The anti-CD20 chimeric monoclonal antibody rituximab (Smith 2003) has been used successfully as an additional treatment or pre-emptive therapy in patients with chronic TTP, obtaining a progressive disappearance of inhibitors with a subsequent increase of ADAMTS13 protease activity and maintenance of a remission disease-free state (Chemnitz et al. 2002; Tsai and Shulman 2003; Bresin et al. 2009).

Novel successful approaches to treatment of hereditary TTP include infusions of recombinant ADAMTS13 (Plaimauer et al. 2011), and plasma cryosupernatant (Moake et al. 1985) or solvent-detergent treated plasma (Kentouche et al. 2002) as a source of the exogenous protease in single patients. The intermediate-purity plasma-derived Factor VIII concentrate (FVIII) Koate (Kedrion Biopharma) contains the highest amount of ADAMTS13 activity yet reported and has been used effectively in treating congenital TTP (Naik and Mahoney 2013) as well as refractory TTP in combination with rituximab, corticosteroids and plasma therapy (Pandey et al. 2015). The anti-thrombotic efficacy of anti-VWF agents, such as ARC1779 (Diener et al.
1. INTRODUCTION

2009; Spiel et al. 2009) or the nanobody ALX-0681 (Callewaert et al. 2012) and Caplacizumab (Peyvandi et al. 2016), has been proved in several preclinical and clinical studies, although with some limitations (Cataland et al. 2012). Other promising agents including N-acetylcysteine, bortezomib, and inhibitors of the glycoprotein-Ib/IX-VWF axis are under evaluation (Coppo and Froissart 2015). N-acetylcysteine, commonly used to reduce the viscosity of mucous secretion in respiratory disorders, has been shown to reduce VWF multimers in mouse and baboon models (Tersteeg et al. 2017) and was successfully used in conjunction with plasma exchange, corticosteroids and rituximab in a patient with refractory acquired TTP (Li et al. 2014).

1.1.4 Pathogenetic mechanisms

VWF is a multimeric plasma glycoprotein synthesized in endothelial cells and megakaryocytes. It mediates the initial platelet adhesion to the subendothelium of the damaged vessel wall at high shear rates. From the storage organelles (Weibel-Palade bodies) of the activated endothelial cells, VWF is secreted in the form of unusually large VWF multimers (ULVWF), which are haemostatically active. In conditions of endothelial damage and shear stress, VWF undergoes a conformational transition from a globular state to an extended chain conformation, with the exposure of intra-molecular globular domains (Siedlecki et al. 1996). At these sites, encountered in arterioles and capillaries, the large VWF filaments apparently unfold (Zhang et al. 2009) and establish multiple interactions with receptors such as glycoprotein Ib/IX and IIb/IIIa on the platelet surface (Federici et al. 1989). The numerous interactions of repeating binding sites on extended filaments of VWF may dramatically increase the stability of the bonds between platelet and VWF, and thus lead to platelet agglutination and vessel repair.
In normal conditions, ADAMTS13 regulates VWF-platelet aggregation by cleavage extended filaments of VWF into small multimers (Furlan et al. 1998; Tsai and Lian 1998; Soejima et al. 2001). Only the highly polymeric forms of VWF are haemostatically active whereas the circulating VWF in normal plasma shows only weak binding affinity for platelets (Moake et al. 1986). (Fig. 1-1)

Patients with TTP generally have a congenital or acquired severe deficiency in the enzyme ADAMTS13. This deficiency leads to the accumulation of ULVWF multimers within the circulation and on endothelial cell surface (Moake et al. 1982), which in turn develops into an uncontrolled formation of systemic platelet-rich thrombi into the microvasculature of TTP patients, without endothelial injury. The pathophysiological hallmarks of acute TTP bouts are VWF-platelet-rich thrombi occluding the microvasculature (Asada et al. 1985). In consequence, the cause of the haemolytic anaemia is the mechanical fragmentation of erythrocytes during flow through partially occluded microvessels, while thrombocytopenia is the likely result of platelet consumption in the microcirculation (Tsai 2013). This pathogenetic mechanism results in a progressive obstruction in microcirculation of some organs like brain, kidney, pancreas, heart and spleen with defects caused in organ functionality (Ruggenenti et al. 2001). (Fig. 1-1)
1. INTRODUCTION

1.1.5 VON WILLEBRAND FACTOR

In 1926 Dr. Erik Adolff von Willebrand suggested for the first time the existence of VWF from his description of the inherited bleeding disorder “pseudo haemophilia”. This condition was later renamed von Willebrand disease (VWD), which, in 1957, was confirmed to be determined by the lack of a plasma factor (Nilsson et al. 1957). The first immunological detection of what is presently known as VWF was done in 1971 and initially named FVIII-related antigen (Zimmerman et al. 1971). In the 1980s the protein was clearly characterized by various cloning experiments (Sadler et al. 1985).

![Diagram](image)

Figure 1-1. Pathogenesis of TTP caused by ADAMTS13 deficiency.
A. Multimeric VWF adheres to damaged endothelial cells or to connective tissue exposed in the inquired vessel wall. Platelets adhere to VWF through their membrane receptors. B. In normal flowing conditions ULVWF multimers are stretched and cleaved by ADAMTS13, limiting platelet adhesion. C. In TTP, where ADAMTS13 is deficient, VWF-dependent platelet accumulation continues, eventually causing microvascular thrombosis, hemolytic anemia and thrombocytopenia.
1.1.5.1 Molecular structure

The VWF gene (VWF) is located on chromosome 12 and contains 52 exons with 178 kb of DNA encoding an 8.9 kb mRNA (Ginsburg et al. 1985). The translated product precursor protein is initially synthesized as a monomer, which is also referred to as pre-pro-VWF, consisting of 2813 amino acids with a molecular weight of 380 kDa (Goodeve 2010). Pre-pro-VWF contains five distinct types of repeated domains as depicted in Fig. 1-2, which mediates interactions with other molecules:

- **Signal (SS) and Pro-peptide**, important for the intracellular trafficking and maturation of the protein (Titani et al. 1986; Haberichter et al. 2000).

- **D domains**, contain binding sites for factor VIII and heparin (Foster et al. 1987; Fujimura et al. 1987) and are important for self-multimerization process (Haberichter et al. 2000).

- **A domains**, The A1 domain contains a binding site for platelet GPIbα in the GPIb-IX-V complex and for collagen VI (Mohri et al. 1988). It can also bind to heparin-like molecules (Fujimura et al. 1987) and to sulfatides (Christophe et al. 1991), playing a potentially important role in the immobilisation of soluble VWF onto complex extracellular matrices (Ruggeri 1997). Of note, it has been reported that A1 domain negatively regulates ADAMTS13 activity on VWF (Nishio et al. 2004). The binding and the cleavage sites for ADAMTS13 are located in the A2 domain (Furlan et al. 1996; Tsai 1996). The A3 domain includes the binding sites for collagen I and III (Pareti et al. 1987) and it is proposed to serve as a docking site for ADAMTS13 (Dong et al. 2003).

- **C domains**, which include the binding site for the platelet integrin receptor αIIbβ3 (GPIIb/IIa) (Ruggeri et al. 1982) and, with C-terminal CK domain, are essential for the self-dimerization process of the protein (Katsumi et al. 2000).
During its translation, a series of events occur. First, in the endoplasmatic reticulum, pre-pro-VWF subunits engage into a homodimeric structure via disulfide bridge formation in the C-terminal domains (Katsumi et al. 2000). Subsequently, the VWF dimers are transported to the Golgi complex, where self-multimerization of pro-peptides leads to the production of differentially sized VWF multimers, that contain as many as 60 subunits (Haberichter et al. 2000). After that, the pro-peptide is separated from the mature subunit via proteolytic processing by Furin, which then assists in the trafficking of mature multimeric VWF (van de Ven et al. 1990). A second important event in the maturing of the VWF protein is its extensive glycosylation with both O- and N-linked glycans (Titani et al. 1986).

The production of VWF is restricted to megakaryocytes (Sporn et al. 1985) and endothelial cells (Wagner et al. 1982). In both cell types, mature VWF is targeted to storage organelles: α-granules in platelets and Weibel-Palade bodies (WPBs) in endothelial cells. Release of VWF multimers into the bloodstream from α-granules requires activation of platelets by a variety of agents including histamine (Hamilton and Sims 1987), thrombin (Levine et al. 1982), fibrin (Ribes et al. 1987), calcium ionophore A23187 (Loesberg et al. 1983), the vasopressin analogue 1-desamino-8-D-arginine vasopressin (DDAVP) (Mannucci et al. 1975) and phorbol myristate acetate (Loesberg et al. 1983). In contrast, VWF multimers are released from endothelial cells in both a constitutive and regulated fashion in response to a variety of physiological stimuli such as...
as thrombin, histamine, fibrin, and the terminal complement complex C5b-9 (Wagner 1990; Nightingale and Cutler 2013). The half-life of endogenous VWF released into circulation is highly individual-dependent and may vary between 6 and 26 h, with particular reference to blood group ABO(H) structures (Borchiellini et al. 1996; Gallinaro et al. 2008). Circulating VWF multimers have a plasma concentration of approximately 10 µg/ml and range in size from 500 KDa to 20,000 KDa (Mayadas and Wagner 1991).

### 1.1.5.2 VWF functions and interactions

VWF is a glycoprotein secreted into bloodstream as a large polymeric form but exists in normal plasma as a series of multimers with progressively smaller sizes. In normal physiological conditions, the predominant source of circulating VWF comes from endothelial cells (Wagner 1990). There are three types of VWF in the human body: soluble plasma VWF, basement membrane VWF (extracellular matrix VWF) and cellular VWF. Only the larger extracellular multimers of VWF are effective in the control of haemostasis and their biological effectiveness depends directly on the size of the concatamers. The primary function of VWF is to support platelet adhesion at sites of microvascular injury, where it binds to type I, III and VI collagen and other components of the exposed vessel wall, becoming rapidly unfolded and activated by high levels of shear stress at blood-vessel wall boundary (Ruggeri 1999). Another important role for VWF is to mediate platelet-platelet interactions through two membrane receptors: GPIb and GPIIb-GPIIIa complexes (Federici et al. 1989). Notably, unusually large VWF multimers derived from endothelial cells are more efficient in platelet interactions than the largest VWF forms present in normal plasma (Moake et al. 1986). Shear-induced
interactions of platelets in the presence of VWF are essential to prevent excessive bleeding, as demonstrated by platelet agglutination abnormalities in VWD patients, who have a quantitative deficiency of VWF (Nilsson et al. 1957; Trossaert et al. 2011). Apart from platelet plug formation, VWF also acts as a “molecular bus” since it circulates in the bloodstream in association with other proteins (Lenting et al. 2012). The first and best characterized example of the activity as a circulating carrier is VWF’s ability to associate with FVIII (Weiss et al. 1977). Factor VIII (FVIII) is a cofactor of the intrinsic clotting cascade, and its deficiency manifests as haemophilia A. VWF is considered as the consort of FVIII, since in its absence, FVIII is rapidly removed from the circulation, resulting in the pathological progression (Tuddenham et al. 1982). Other proteins that are associated with VWF in the circulation include ADAMTS13, factor H, osteoprotegerin (Shahbazi et al. 2007), and galectin-1 and 2 (Saint-Lu et al. 2012). With regard to ADAMTS13, it has been speculated that binding to VWF would facilitate immediate action of the protease to cleave the active platelet-binding VWF conformation at the site of injury (Feys et al. 2009; Crawley et al. 2011). Recent reports have also identified VWF as an additional protein partner for FH, the major negative regulator of the alternative pathway of the complement cascade. Nevertheless, the physiological meaning of this interaction has not yet been clearly clarified (Feng et al. 2013; Rayes et al. 2014). Concerning the other proteins associated with VWF, their mutual effects on each other’s functions has been only sparsely investigated here. Aside from its well known haemostatic potential, VWF has been becoming involved in several pathologic processes beyond coagulation: there is evidence of VWF activity in inflammation processes since its deficit provokes impaired P-selectin surface expression and subsequent defects in leukocytes recruitment in the early stage of inflammation (Denis et al. 2001); recent researches have reported that endothelial VWF deficiency
can promote angiogenesis (Starke et al. 2011); in vitro studies have revealed that VWF directly stimulates smooth muscle cell proliferation (Qin et al. 2003) and preliminary studies have suggested that VWF reduced metastasis by inducing cell death of tumour cells (Terraube et al. 2007).

1.1.6 ADAMTS13

In 1982 Moake and co-workers provided the first description of a “VWF-depolymerase” that normally cleaves ULVWF to prevent intravascular platelets aggregation and thrombosis that characterize TTP (Moake et al. 1982). In 1996, a candidate VWF-cleavage protease (VWF-CP) was identified in human plasma by Tsai (Tsai 1996) and independently by Furlan (Furlan et al. 1996), and defined as a metal-dependent protease that requires divalent cations for its activity. In 2001 the VWF-CP was finally purified, cloned and characterized, and identified as the 13rd member of the super family ADAMTS (Fujikawa et al. 2001; Levy et al. 2001; Zheng et al. 2001).

1.1.6.1 Molecular structure

The ADAMTS13 gene (ADAMTS13) contains 29 exons spanning approximately 37 Kb on chromosome 9q34 (Levy et al. 2001; Soejima et al. 2001; Zheng et al. 2001), and encodes a 4.7 Kb transcript. ADAMTS13 is primarily expressed in the hepatic stellate cells (Zhou et al. 2005), but it is produced also in placenta, skeletal muscle, certain tumor cell lines, platelets (Suzuki et al. 2004), podocytes (Manea et al. 2007) and endothelial cells (Turner et al. 2006; Wang et al. 2016). The concentration of ADAMTS13 in plasma of healthy volunteers is approximately 1 µg/ml (Gerritsen et al. 2001) and its half-life is 2-3 days (Furlan et al. 1999). Up to now, there is no clear
1. INTRODUCTION

evidence for alteration in transcriptional activity of ADAMTS13 that accounts for diminished function of the protease in plasma (Claus et al. 2005).

The full length ADAMTS13 transcript encodes a precursor protein of 1427 aminoacid residues. The protein undergoes extensive glycosylation and other post-translation modifications that greatly influence its secretion (Ricketts et al. 2007; Zhou and Tsai 2009). Consistently with the ADAMTS superfamily, ADAMTS13 exhibits a multi-domain structure (Zheng et al. 2001) (Fig. 1-3):

- **Signal and propeptide domains**, which are only responsible for secretion and preservation of the integrity of the newly synthesized protease (Majerus et al. 2003).

- **Metalloprotease domain**, which contains zinc-coordinating and calcium-binding residues that control the cleavage activity of the protein (Di Stasio et al. 2008). Of note, this domain alone does not have the ability to specifically bind and cleave VWF. The other non-catalytic domains are necessary for substrate recognition (Gao et al. 2012).

- **Disintegrin-like domain**, functionally coupled with the metalloprotease domain (Ai et al. 2005). It is named for the primary sequence similarity to the snake venom disintegrins and it contains important exosites for VWF binding (Akiyama et al. 2009).

- **Thrombospondin (TSP) repeat modules**, which are very well conserved among ADAMTS family proteases. TSP repeats contain the glycosylation sites and cooperate in substrate recognition (Gao et al. 2012) also with disulfide bonds of VWF (Yeh et al. 2010). Although the TSP-1 repeat between the Dis- and Cys rich- domains is well conserved within the ADAMTS proteases, the arrangement and number of TSP-1 modules following the spacer domain varies.

- **Cysteine-rich domain**, structurally similar to the Disintegrin-domain. It appears to be important for VWF binding and cleavage (Soejima et al. 2003; Klaus et al. 2004).
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- **Spacer domain**, which has the highest binding affinity for the A2 site of VWF (Jin et al. 2010; Pos et al. 2010). The spacer and the Cys-Rich domains function closely with and similarly to one and other (Soejima et al. 2003; Luken et al. 2005).

- **CUB domain**, characteristic for ADAMTS13. The two CUB domains in the C-terminus of the protein have been shown to mediate binding with VWF for the initial docking to the substrate (Zhang et al. 2007; Zanardelli et al. 2009). However, in the presence of shear stress, the CUB1 peptide seems to inhibit proteolysis of VWF (Tao et al. 2005).

After cleavage of the signal peptide and propeptide, mature ADAMTS13 is formed consisting of 1353 amino acids with a calculated molecular weight of 145 KDa, and an observed mass from 150 to 190KDa due to its extensive glycosylation (Zheng et al. 2001).

![Figure 1-3. ADAMTS13 domain structure.](attachment:image)

Cartoon representing domain organization of ADAMTS13. S, signal peptide; P, propeptide; M, metalloprotease domain; D, Disintegrin-like domain; T1-8, Thrombospondin repeat modules; C, Cysteine-rich domain; S, Spacer domain; CUB1-2, CUB domains.

The entire crystal structure of ADAMTS13 has not been obtained yet. The crystal structure of the region encompassing the disintegrin-like, TSP-1, cysteine-rich and spacer domain (DTCS) of ADAMTS13 has been solved (Akiyama et al. 2009), and the metalloprotease domain modeled based upon the crystal structure of ADAMTS1, 4 and 5 (Gerhardt et al. 2007; Mosyak et al. 2008), since it is well conserved within the ADAMTS family. Thus, the ADAMTS13-MDTCS (metalloprotease>spacer domain) model is currently available.
1. INTRODUCTION

1.1.6.2 ADAMTS13-VWF interaction

VWF is the only known ADAMTS13 substrate to date. The proteolytic cleavage of unusually large multimers of VWF by ADAMTS13 is essential for maintaining the delicate balance between haemostasis and coagulation. The remarkable substrate specificity is largely achieved by the extensive interactions between VWF and non-catalytic domains of ADAMTS13 (Majerus et al. 2005; Gao et al. 2008; Akiyama et al. 2009). Structures remote from the cleavage site (Y1605-M1606) into VWF are also known to influence proteolysis (Nishio et al. 2004; Zanardelli et al. 2006). The multiple interactions that exist between VWF and ADAMTS13 are illustrated in Fig. 1-4 (Zanardelli et al. 2009; Crawley et al. 2011). Under normal physiological conditions, VWF circulates in plasma in a “ball-of-yarn” conformation where the A2 domain is hidden within a hydrophobic core comprised of vicinal Cys residues located at the C terminus of the domain (Luken et al. 2010). According to multi-domain binding model, firstly ADAMTS13 and VWF make contact with a modest affinity through their C-terminal domains, which are constitutively exposed. As a consequence of changes in local shear stress conditions and the growing of a haemostatic plug, VWF unravels. Conformational changes in VWF lead to the exposure and higher affinity binding of exosites present in N-terminal region of VWF and ADAMTS13: the spacer domain of the protease recognizes VWF residues Glu1660-Arg1668 (Gao et al. 2008); the ADAMTS13 Disintegrin-like domain interacts with a complementary exosite on VWF involving Asp1614 in a critical binding that helps orientating the scissile bond in the active center of ADAMTS13 (de Groot et al. 2009); VWF1596-1604 N-terminal sequence contains a structural important determinant that makes VWF Leu1603 and Val1604 residues as docking sites for a complementary subsite in ADAMTS13.
involving Leu198, Leu232, and Leu274 (Xiang et al. 2011). Together, these interactions position the scissile bond in the catalytic cleft of ADAMTS13. At this stage, additional binding interactions occurring between adjacent domains finally lead to VWF cleavage. Once cleavage has taken place, there is a consequent reduction in affinity of protease and substrate, enabling ADAMTS13 to recycle (Crawley et al. 2011). Of note, ADAMTS13 does not require a fixed spatial relationship between the scissile bond and auxiliary binding sites on the substrate. The mobility of protease domains suggests that a spectrum of ADAMTS13 conformations exist, with different possibilities of interaction modes with VWF molecules, which also present a wide range of conformations under shear-stress conditions in circulation (Gao et al. 2008; Akiyama et al. 2009).
Crawley and co-workers in 2011 proposed that the cleavage of ADAMTS13 occurs in 7 distinct steps, as follows: ADAMTS13 can reversibly bind to globular VWF (step 1). When shear stress induces the unfolding of VWF, additional exosite binding sites on VWF are revealed (step 2). The ADAMTS13 spacer domain recognises residues Glu1660-Arg1668 (step 3) revealed when the vicinal Cys disulphide bind plug is extracted from the hydrophobic core of the A2 domain. This increases the affinity of ADAMTS13 for VWF. An ADAMTS13 disintegrin-like exosite involving Arg349 recognises a complementary site of VWF compromising VWF1614 in a critical but low affinity interaction (step 4). Thereafter an essential contact is made between VWF Leu1603 and a complementary S3 subsite in ADAMTS13 involving Leu198, Leu232 and Leu274 (step 5). Together these interactions bring the Tyr1605-Met1606 scissile bond over the ADAMTS13 active site. This allows the P1 (1605) and P1’ (1606) residues to engage their respective S1 and S1’ subsite pockets involving Leu151/Val195 (S1) and Asp252-Pro256 (S1’) respectively (step 6). Once cleavage has taken place (step 7) there is a reduction in the affinity between ADAMTS13 and VWF, enabling ADAMTS13 to recycle. Image modified from (Crawley et al. 2011).
1. INTRODUCTION

1.1.6.3 ADAMTS13 functions

The primary and unique activity of ADAMTS13 consists of regulating the size of circulating VWF multimers by cleavage of the unusually large concatamers into smaller ones, thereby determining their haemostatic (and proinflammatory) potential (Tsai 2006). ADAMTS13 cleaves VWF as soon as it is released from endothelial cells, resulting in the shedding of ULVWF from the endothelial cell surface and in fragmentation of VWF strings, preventing platelet adhesion (Dong et al. 2003). Although ADAMTS13 is constitutively active, VWF is not cleaved without substantial changes in its secondary structure to expose the cryptic cleavage site to the surface. Indeed, ADAMTS13-VWF complexes can be detected in normal human plasma suggesting that ADAMTS13 can bind native VWF without cleaving it (Feys et al. 2009). Structural studies have provided evidence that ADAMTS13 contains several VWF-binding exosites that might function independently to interact with the substrate (Gao et al. 2008; Feys et al. 2009). According to the proposed multi-domain binding model, substrate recognition takes place in 7 distinct steps, as described in details in the section above and in Fig. 1-4. Substrate cleavage only occurs when VWF is in a sheared elongated conformation that provides high affinity binding between N-terminal domains of VWF and the complementary sites on ADAMTS13 (Crawley et al. 2011).

ADAMTS13 cleaves VWF precisely at the scissile bond Tyr1605 - Met 1606 on its central A2 domain (Furlan et al. 1996; Tsai 1996). VWF Tyr1605 has an aromatic side chain, which is a determinant of its adjustment in the S1 pocket that accommodates ADAMTS13 Leu151 and Val195 amino acids (Xiang et al. 2011). VWF Met1606 interacts with ADAMTS13 residues Asp252 - Pro256 that forms a specific compartment called S1’ pocket (de Groot et al. 2010). It is proposed that VWF Leu1603, Tyr1605,
and Asp1614 contact ADAMTS13 Leu198, Val195, and Arg349, respectively, ensuring that the scissile bond is brought into position over the active centre of the protease for cleavage to occur (Xiang et al. 2011). In common with the other proteases of ADAMs family, the metalloproteinase domain of ADAMTS13 has a putative zinc ion catalytic site, one predicted calcium ion-binding site and conserved Asp284 and Met249 that support the active site of the protein (Zheng et al. 2001). The role of divalent metal ions in ADAMTS13 activity is not fully understood. However, some studies reported that cations other than zinc can support enzyme activity, and might mediate protein structure (Anderson et al. 2006). Of note, VWF does not have metal ion-dependent adhesion sites (MIDAS); so that it is unable to bind divalent ions contained in ADAMTS13.

VWF proteolytic activity of ADAMTS13 ranges from 40% to 170% in normal plasma in children and adults, including the neonatal period, but it may be lower in newborn and premature infants up to 6 months of age (Schmugge et al. 2004). Decreased ADAMTS13 activity levels have also been reported in healthy individuals older than 65 years and pregnant woman during the last two trimesters of pregnancy (Mannucci et al. 2001). In addition, ethnic differences have been reported; for example, Chinese individuals seem to possess lower ADAMTS13 antigen levels than Caucasians, although this result remains to be corroborated with ADAMTS13 activity levels (Feys et al. 2006).

Cysteine residues in the VWF C-domains are in thiol forms exposed on the surface of the quaternary structure of VWF multimers, and capable of forming disulfide bonds upon exposure to high shear stress (Choi et al. 2007). Yeh and co-workers demonstrated that some of these thiols are targeted and reduced by ADAMTS13, providing evidence that ADAMTS13 possess an additional reducing activity on its substrate VWF. Of note, the disulfide bond reducing activity does not disrupt both VWF multimer structures and
proteolytic cleavage by ADAMTS13 (Yeh et al. 2010). However, the physiological significance of this ADAMTS13 activity remains to be further investigated, but it may be speculated that it could prevent elongation of VWF multimers via shear-induced VWF thiol-disulfide exchange.

1.1.6.4 Regulation of ADAMTS13 activity on VWF

There have been no physiological inhibitors of ADAMTS13 identified to date. ADAMTS13 is secreted as an active protease (Majerus et al. 2003), and it is slowly and continuously released into the circulation, providing constant cleavage of long anchored VWF strings secreted from the neighboring endothelial cells (Dong et al. 2003; Turner et al. 2009) and of circulating sheared ULVWF (Tsai et al. 1994; Tsai 2006). Upon secretion, ADAMTS13 is essentially regulated at substrate level (Mannucci et al. 2004; Feys et al. 2009), and it could be “consumed” as a consequence of a compensating phenomenon of accumulating agglutinating forms of VWF (Mannucci et al. 2001). However, several endogenous factors have been shown to affect the interaction between ADAMTS13 and VWF and modulate the cleavage activity of the protease, both positively and negatively, by various mechanisms.

Inflammatory cytokines such as interleukin-6, released during the early stage of systemic inflammation, may stimulate the ULVWF release and inhibit its cleavage, resulting in the accumulation of hyper-reactive multimers in plasma and on the surface of endothelial cells, which in turn induce platelet aggregation and adhesion on the vascular endothelium (Bernardo et al. 2004). Similarly to anti-ADAMTS13 autoantibodies, antimicrobial human neutrophil peptides 1-3, released from neutrophils following infection or inflammation, may inhibit residual plasma ADAMTS13 activity.
by interfering with its interaction with VWF. Moreover, it has been reported that high concentrations of serum haemoglobin, probably resulting from in vitro haemolysis, inhibit ADAMTS13 activity, without a clear clinical association with TTP (Studt et al. 2005). Of note, in all these conditions, ADAMTS13 inhibition results as a consequence of an inflammatory activation and culminates in the amplification of a haemostatic and prothrombotic state of endothelium, promoting intravascular coagulation.

Proteases of the coagulation cascade provide a useful feedback mechanism that turns off the intravascular haemostatic process. Following the initiation of coagulation cascade, the active form of thrombin and plasmin inhibit ADAMTS13 activity by directly cleaving the protease at the site of vascular injury, promoting thrombus growth. In the late phase of coagulation, on the surface of the adjacent uninjured endothelium, thrombin is recruited by thrombomodulin, and consequently thrombin-dependent ADAMTS13 proteolysis would be inhibited, restoring homeostasis (Crawley et al. 2005).

Other potential plasma inhibitors including FH (Feng et al. 2013); (Rayes et al. 2014) and heparin (Nishio et al. 2004) may modulate ADAMTS13 activity through direct binding to VWF.

Inorganic divalent cations, which exert a functional and structural effect on both ADAMTS13 and VWF, have been shown to affect the activity of the protease. At concentrations exceeding physiological levels, Zn$^{2+}$ and Ca$^{2+}$ stabilize the folded conformation of VWF impeding its unfolding, consequently protecting it from cleavage (Anderson et al. 2006; Lynch et al. 2014). High concentrations of Mg$^{2+}$, instead, can enhance VWF cleavage under flow (Dong et al. 2008).
1.2 **Haemolytic uraemic syndrome**

Clinically defined by thrombocytopenia, nonimmune microangiopathic haemolytic anaemia, and acute kidney impairment, haemolytic uraemic syndrome (HUS) is a microvascular occlusive disorder that belongs to the category of diseases known as TMA (Ruggenenti et al. 2001). Nowadays, HUS represents the most common preventable cause of acute renal failure in children.

The term “haemolytic uraemic syndrome” was initially described by von Gasser and colleagues in 1955 in a clinical report of five children with haemolysis, thrombocytopenia and acute renal failure who died with renal cortical necrosis (Gasser et al. 1955). Following this, it was recognized that for a small number of patients the disease was familial or recurrent (Kaplan et al. 1975), and the clinical course of HUS children with a prodrome of diarrhoea, was different from those without diarrhoea (Dolislager and Tune 1978). However, it was not until 1983 that the link was made between Shiga toxin-producing organisms, diarrhoea and HUS (Karmali et al. 1983; Riley et al. 1983). This finding also confirmed the differentiation between diarrhoea-associated and non-diarrhoeal forms of HUS. The thought of diarrhoea-negative HUS as a genetic disease started with the identification of mutations in the \( CFH \) gene (Richards et al. 2001; Caprioli et al. 2003), which codes for the central inhibitor of the complement alternative pathway, factor H. Later on, mutations in additional complement genes were identified in HUS patients (Bu et al. 2012).

The bloody diarrhoea, which occurs in about 90% of cases of HUS, is generally the sign indicating that enteric \( E. \, coli \) infection is occurring. Post-diarrhoeal HUS patients have a low haemoglobin level and thrombocytopenia (low platelet count), may manifest
pallor and fatigue, and may present in different stages of acute renal impairment (Elliott and Robins-Browne 2005; Garg et al. 2009).

The initial presentation of diarrhoea-negative HUS, typically as a severe onset, includes haemolytic anaemia (low haemoglobin, decreased haptoglobin, increased lactate dehydrogenase, and positive schistocytes) and thrombocytopenia without preceding diarrhoea. Some patients show complement activation in the form of low C3 plasma levels and a decreased plasma C3/C3d ratio. Disease manifestation is often preceded by an infection, reported as a triggering event. Some patients experience one episode of kidney failure only, recover, and experience no disease recurrence, while others have multiple relapses or the progression to end-stage renal disease (ESRD) (Noris et al. 2010).

### 1.2.1 Classification

Twenty-two years ago today, Karmali and colleagues found a toxin, named verotoxin, lethal to cultured African green monkey (Vero) kidney cells in stools of children with post-diarrhoeal HUS. This toxic property was attributed to *E. coli* of various serotypes and the toxin was later on called Shiga-like toxin (Stx). Since that, following the recognition of clinical patterns of HUS, cases should be assigned to one of the subclasses of the disease based on the presence or the absence of diarrhoea and/or detection of Stx-producing bacteria and their products in stool cultures (Ariceta et al. 2009). These two major subtypes of HUS are the following.

- **Typical HUS**, also defined as post-diarrhoea or diarrhoea-positive HUS (D+HUS) is the most frequent form of HUS (90 %), and it is associated with infections by Shiga like toxin (Stx) producing bacteria (STEC), such as enterohaemorrhagic *Escherichia Coli*
(EHEC) or *Shigella dysenteriae* and it is more appropriately referred as STEC HUS. The most frequently isolated *E. coli* serotype in cases of D+HUS is *E. coli* 0157:H7 (Tarr 1995). Stx is divided into two major subtypes, Stx1 and Stx2, which have approximately 50% homology each other. STEC HUS is acquired as a food-borne illness or from contaminated water. It affects predominantly children, except in epidemics when it may occur in individual with a wider range of ages (Tarr et al. 2005). Rare cases of typical HUS are triggered by neuraminidase producing *Streptococcus pneumoniae*. These forms are classified as pneumococcal-HUS or neuraminidase-associated HUS (Copelovitch and Kaplan 2010).

Patients with the typical STEC HUS present haemolytic anaemia, thrombocytopenia and acute kidney injury with almost 40-50% of cases requiring dialysis (Garg et al. 2009). Usually they recover, and renal function returns to normal.

- The atypical HUS (aHUS) has been historically used to describe rare cases of the disease (less than 10%) in which infections by Stx producing bacteria or *S. Pneumoniae* or other secondary causes can be excluded. aHUS is also improperly defined as diarrhoea negative HUS (D-HUS) (indeed about 30% of patients present with diarrhoea), and can be hereditary, affecting members of the same family several years apart (Kaplan et al. 1975), or sporadic (Noris et al. 2010). In 1998, Warwicker et al. (Warwicker et al. 1998) studied three families with HUS and he firstly established linkage in the affected individuals to the complement cluster gene on human chromosome 1q32, which encodes for several regulatory proteins of the complement system, the major non-specific defense mechanism of the innate immune system. During the last two decades, genetic or acquired defects leading to the dysregulation of complement cascade have been discovered in about 60% of patients with aHUS (Noris and Remuzzi 2009; Noris et al. 2010). Up to now, different HUS-associated mutations
or polymorphisms were identified in complement genes encoding for: Factor H (FH) (Caprioli et al. 2003), Factor I (FI) (Fremeaux-Bacchi et al. 2004), Membrane Cofactor Protein (MCP) (Noris et al. 2003; Fremeaux-Bacchi et al. 2006), Component 3 (C3) (Fremeaux-Bacchi et al. 2008), Factor B (FB) (Goicoechea de Jorge et al. 2007), complement Factor H Related 5 (FHR5) (Monteferrante et al. 2007), complement Factor H Related 1 and 3 (FHR1, FHR3) (Zipfel et al. 2007), Thrombomodulin (THBD) (Delvaeye et al. 2009), and diacylglycerol kinase epsilon (DGKE) (Lemaire et al. 2013).

In about 10% of juvenile patients with aHUS, the disease correlates with the deficiency for complement Factor H Related proteins (CFHRs) in combination with the presence of anti-FH autoantibodies. This form represents a unique subtype of aHUS called DEAP-HUS [Deficiency of CFHR plasma proteins and Autoantibody Positive form of Haemolytic Uremic Syndrome] (Skerka et al. 2009; Zipfel et al. 2010). Also autoantibodies to FI have been recently reported in patients carrying FH mutations, but their role remains still unclear (Kavanagh et al. 2012). Acknowledged theories assume the need of multiple hits by gain-of-function mutations in complement activators, loss-of-function mutations in complement inhibitors, and/or presence of autoantibodies for complete penetrance of HUS (Esparza-Gordillo et al. 2006; Kavanagh and Goodship 2011). Similarly, it has been proposed that the “complotype”, defined as the repertoire of inherited abnormalities in complement genes, likely confers a predisposition to developing aHUS, rather than directly causing the disease (de Cordoba et al. 2012). Conditions that trigger complement activation may precipitate an acute event in those patients with the predisposing genetic background (Caprioli et al. 2006; Noris et al. 2010). aHUS can be recurrent and patients can develop ESRD.

- There are many other forms of HUS, due to a variety of causes, which are called secondary aHUS. These forms are associated with different conditions, including viral
(human immunodeficiency virus and H1N1 influenza A) and other bacterial infections, parasites such as *Plasmodium falciparum* (Kute et al. 2013) or *S. pneumonia* (Brandt et al. 2002), cancer chemotherapy (Lesesne et al. 1989) and several categories of drugs (Dlott et al. 2004), bone marrow or solid organ transplantation (Bonser et al. 1984; Ostronoff et al. 2007), malignant hypertension (Shibagaki and Fujita 2005), autoimmune disorders such as systemic lupus erythematosus (Zhao et al. 2011), HELLP (Haemolytic anemia, Elevated Liver enzymes, Low Platelet) syndrome (Fang et al. 2008), glomerulopathies (Manenti et al. 2013), or, in children, methyl malonic aciduria with homocystinuria, cbIC type, a rare hereditary defect of cobalamine metabolism (Sharma et al. 2007). As TTP, also HUS is pregnancy-associated (George 2003; Noris and Remuzzi 2005; Fakhouri et al. 2010), since during pregnancy increased procoagulants factors concentrations may act as predisposing agents. However, many of the above conditions often act as a trigger of the disease in individuals with a genetic background leading to complement dysregulation. Triggering and underlying clinical condition have been reported in up to 70% of patients with complement gene mutations, showing that both genetic predisposition and a precipitating event are required for the development of the disease (Caprioli et al. 2006; Noris et al. 2010).

### 1.2.2 Incidence

The annual incidence of STEC HUS is overall about 0.2-1/100,000 persons/year, with a peak of 5 to 6 per 100,000/year in children under the age of 5 years (Noris and Remuzzi 2009). Interestingly, there is some geographic variation in incidence both internationally (Elliott et al. 2001; Lynn et al. 2005) and within countries (Tozzi et al. 2003). In 2011, several European countries, particularly Northern Germany, experienced one of the
largest STEC HUS outbreaks ever reported (Frank et al. 2011). The annual incidence of aHUS is thought to be about 2 per million for adults (Constantinescu et al. 2004) and 3.3 per million in children younger than 18 years (Ohanian et al. 2011). Onset during childhood (<18 years) appears slightly more frequent than during adulthood (approximately 60 and 40% of cases, respectively) (Noris et al. 2010). Both sexes are equally affected in childhood, while in adults the incidence and prevalence is greater in females (Sullivan et al. 2010). A familial occurrence is observed in approximately 20% of aHUS patients and genetic predisposition can be inherited in an autosomal dominant or autosomal recessive manner by a pathogenic variant(s) in a single gene, or, rarely, in a multi-gene panel (Noris and Remuzzi 2009).

1.2.3 Diagnostic measures for aHUS

Patients suspected of having aHUS should be initially screened by measuring complement C3, C4, FB, FH, MCP, FI, FHRs and autoantibodies against FH. Classically, serum C3, C4 and FB levels are determined by nephelometry, using a standard procedure and commercially available diagnostic kits (Siemens Healthcare Diagnostics or Beckman Coulter). For the assessment of plasma levels of complement components FB, FH, FI and MCP a variety of different complement assays are being undertaken but they remain within the specialized diagnostic laboratories. FH, FI and FB levels are measured by immunochemical methods (Oppermann et al. 1990; Dragon-Durey et al. 2004) or by functional analysis (Lesavre et al. 1979; Goicoechea de Jorge et al. 2007; Martinez-Barricarte et al. 2010; Marinozzi et al. 2014). Anti-FH or anti-FI autoantibody screening is currently performed by home-made ELISA assays (Dragon-Durey et al. 2005; Kavanagh et al. 2012). Membrane expression of MCP is usually
1. INTRODUCTION

analyzed using granulocytes or peripheral blood mononuclear cells (PBMC) in a flow cytometry analysis (FACS) (Kolev and Kemper 2014). Functional FH complement regulatory defects can be detected in the patients’ sera with a FH-dependent haemolytic assay (Sanchez-Corral et al. 2004). Assays like the CH50, AP50 and the Wieslab complement system are used to assess the functional capacity of the different complement pathways. C5a and SC5b-9 have been used as surrogate biomarkers of complement activation with some limitations since their circulating levels remain normal in almost 40-60% of patients during the acute phase. A useful alternative assay was recently proposed for the assessment of complement activation and the monitoring of the therapy in aHUS patients. In this test, sera from aHUS patients compared to healthy subjects caused more C3 and C5b-9 deposition on ADP-activated endothelial cells (Noris et al. 2014).

All these studies highlight the lack of the “perfect assay” to test and monitor complement activation in aHUS. In the absence of international standards, the normal ranges and units vary from laboratory to laboratory. In addition, functional assays of specific AP proteins from patient plasma are generally not very sensitive and may not provide accurate information. Therefore the assessment of complement protein levels or functions in plasma is insufficient and genetic analyses are necessary (Noris and Remuzzi 2009). However, the underlying genetic and functional alterations remain unknown in approximately half of aHUS patients. Based on the current understanding of the pathophysiology of aHUS, several candidate genes can be defined based on the role of their encoded proteins as regulators of the complement activation. These candidate proteins include properdin, which promotes complement activation, CR1, which has overlapping functions of FH and MCP, and heparin, which modulates FH localization on endothelial cells (Jokiranta et al. 2007).
1.2.4 Therapy

Clinically, HUS can consist of variable combinations of microangiopathic haemolytic anaemia, thrombocytopenia, renal failure, pulmonary oedema, systemic arterial hypertension, and neurologic abnormalities (Ruggenenti et al. 2001).

Patients in all phases of HUS should be monitored carefully and continuously for signs and fluid overload, because their renal and vascular statuses are in flux, and volume overload or depletion could exacerbate injury. Diuretics, sometimes given during early HUS, rarely avert anuria, and thrombus development may be facilitated (Tarr et al. 2005). Vasodilators are the preferred agents for the treatment of hypertension (Corrigan and Boineau 2001).

HUS patient can become profoundly and rapidly anaemic. Administration of erythrocyte and platelets usually produces a transient reversal of the anaemia and thrombocytopenia. However blood product transfusions often will cause an exacerbation of HUS as a result of rapid worsening of haemolysis and thrombosis; consequently blood product transfusions are reserved for patients with life-threatening bleeding or markedly symptomatic anemia (Camp-Sorrell 2008).

Since in most cases, STEC HUS is associated with enterohaemorrhagic colitis, bowel rest is important in the management of this disease. Anti-motility agents should be avoided since they may prolong the persistency of Stx-producing bacteria in the intestinal lumen and therefore increase patient exposure to its toxin. The use of antibiotics should be restricted to the very limited number of patients presenting with bacteremia since in children with gastroenteritis they may increase the risk of HUS by 17 folds (Chiurchiu et al. 2003). Moreover, several antimicrobial drugs are potent inducers of the expression of Stxs, therefore they should be avoided.
1. INTRODUCTION

The failure of Synsorb-Pk, an oral agent that binds Shiga toxins, to ameliorate the course of typical HUS, unresolved the debate in consideration of antitoxin treatment for STEC HUS, primarily caused by enteric bacterial infections. Anyway, among newer treatments with development of Stx-neutralizing monoclonal antibodies (SHIGATEC, NCT0152199) is now the most advanced (Bitzan 2009). Many other approaches, such as corticosteroids (Perez et al. 1998), aspirin (O'Regan et al. 1980), heparin and urokinase/streptokinase (Diekmann 1980), have been unsuccessful in HUS, and are not commonly used today.

As in the management of other TMAs, plasma exchange and plasma infusion were considered first-line therapies in aHUS, but the reported clinical response varies from complete remission to no response and immediate end-stage renal failure (ESRF), depending on the underlying genetic or acquired defect (Caprioli et al. 2006; Ariceta et al. 2009). There is no theoretical justification for plasma therapies in STEC HUS, since there is no evidence that TMA originates from some circulating factors that can be removed or replaced by plasma exchange. Because elevated levels of circulating immune complexes may play a role in aHUS, plasma treatment could theoretically be beneficial to remove these complexes (Pisoni et al. 2001). Controversial results are reported in response to plasma infusion, since mutant circulating molecules or autoantibodies are not removed from blood circulation (Ariceta et al. 2009; Davin et al. 2009; Noris et al. 2010). Patients with autoantibodies, such as DEAP-HUS patients, receive plasma therapy in combination with immunosuppressive agents (steroids, azathioprine or even anti-CD20 rituximab, such as in the case of acquired TTP) in order to reduce the titer of antibodies (Dragon-Durey et al. 2009); disease recurrence following discontinuation of plasma treatment has been reported (Lee et al. 2009).
Patients with aHUS tend to require more aggressive and prolonged treatment and have a worse renal outcome and higher mortality than patients with STEC HUS. They may also have a prolonged disease course and/or recurrent episodes of HUS. The clinical outcome of aHUS is unfavorable: up to 50% of cases progress to ESRF and 10-15% may die during the acute phase of the disease (Ruggenenti et al. 2001). The majority of patients with FH, FI, C3 and THBD mutations or anti-FH antibodies lost renal function or died during the presenting episode or progressed to ESRD as a consequence of relapses (Noris et al. 2010). Indications for dialysis in HUS are similar to those in other forms of acute renal failure: hyperkalaemia (high potassium concentration), high serum urea concentrations; persistent acidosis; hypertension; anuria; and volume overload. The choice between haemodialysis and peritoneal dialysis varies among specialists and centers (Corrigan and Boineau 2001).

Kidney transplant should be considered as an effective and safe treatment for those patients, who progress to ESRD, even if organ graft generally fails to control the syndrome. Kidney transplantation in HUS patients is severely compromised by the risk of recurrence (Loirat and Niaudet 2003), especially in patients with gene mutations encoding for complement plasma proteins synthesized predominantly by the liver, such as FH and FI rather than C3 and FB (Bresin et al. 2006). Liver transplantation corrects these complement abnormalities preventing disease recurrences but had a high mortality rate (Saland et al. 2009; Saland et al. 2009). Plasma prophylaxis has also been proposed as a strategy to prevent relapses after single kidney transplantation in aHUS associated with FH mutations (Hirt-Minkowski et al. 2009). Combined liver-kidney transplantation should be recommended for selected patients with ESRF and FH or FI abnormalities, as a way both to restore renal function and to prevent disease reappearance (Saland et al. 2009). In contrast, outcome of kidney graft alone is favorable in patients with mutations
in gene encoding for MCP, a transmembrane protein highly expressed in the kidney (Noris et al. 2010).

Since complement abnormalities are underlying causes of pathophysiology of aHUS (Noris and Remuzzi 2009), and that uncontrolled complement activation may contribute to microangiopathic lesions of STEC HUS (Morigi et al. 2011; Noris et al. 2012), in the last decade complement inhibitors have been proposed as new therapeutic treatments for the cure of the disease. The first successful example of complement inhibitor is eculizumab (Soliris), a humanized monoclonal antibody that blocks complement activity by inhibiting the cleavage of complement C5, thereby preventing the generation of the inflammatory peptide C5a and the cytotoxic membrane-attack complex C5b-9 (Thomas et al. 1996). Eculizumab has been approved for the treatment of paroxysmal nocturnal haemoglobinuria, a disease characterized by complement mediated haemolysis due to deficiency in glycosylphosphatidylinositol-anchored complement regulatory proteins CD55 and CD59 (Hillmen et al. 2004). Its cost is extremely high and is associated with a risk of meningococcal sepsis/meningitis (Struijk et al. 2013). Different case reports and clinical trials reported that eculizumab induces remission of aHUS (Gruppo and Rother 2009; Nurnberger et al. 2009; Legendre et al. 2013; Cofiell et al. 2015) and represents a valid first-line alternative treatment of acute episodes (Zuber et al. 2012) and also in prevention of recurrences after kidney graft (Chatelet et al. 2009). These encouraging results prompted nephrologists to use eculizumab therapy in HUS in the STEC O104:H4 outbreak in Germany, but whether eculizumab is a useful adjunct to treating the most severe forms of STEC HUS need to be clarified by prospective randomized, controlled trials (Kielstein et al. 2012).

Many other complement inhibitors are currently in development (Ricklin and Lambris 2013). Monoclonal antibodies that target FD, FB, C3b, properdin and mannose
associated serine proteases (MASPs) have been tested with potential application in aHUS. Small molecules have been also developed to block C3, FB and FD activities or C3a and C5a signaling at their receptors, with the advantage to be administered subcutaneously or orally. However, because the complement system is an important part of the immune response, the major risk of all therapeutic complement inhibitors remains infections.

1.2.5 Pathogenetic mechanisms

1.2.5.1 In typical HUS

Stx-producing bacteria, ingested in contaminated food, colonize the mucosa of the intestine causing the destruction of brush border villi. Stx, produced by the adherent microorganism and elaborated in the gut lumen, is responsible for the early stage watery diarrhoea of the disease. Stx is also adsorbed into the circulation and carried throughout the body. Internalized in a wide range of endothelial cells by its globotriacyl ceramide (Gb3) receptors, and activated through the Golgi apparatus, Stx exerts its virulent intracellular activity. For many years, it has been assumed that the only relevant biologic activity of Stxs was to induce cell death by modifying the ribosome and blocking protein synthesis (Melton-Celsa 2014), especially in human proximal and distal tubular epithelial cells. However, it has been shown that Stxs upregulate mRNA expression and protein levels of inflammatory cytokines including TNFα, interleukins, and other chemokines, which promote transmigration of leukocytes (Morigi et al. 1995), and initiate intravascular coagulation (Grabowski 2002; Lee et al. 2013). The formation of microthrombi causes in turn impaired oxygenation and end-organ damage. Systemic effects of Stxs include platelets consumption, haemolysed red blood cells, and damage
to the kidney, brain, pancreas, and heart, and other organs in which Gb3 is expressed (Elliott and Robins-Browne 2005). Altered complement activation products levels have been found in patients with STEC HUS (Stahl et al. 2011; Arvidsson et al. 2015), but whether those changes are part of the etiology of the disease or occur as a consequence is not clear. In a recent study Stx-induced complement activation, via P-selectin and the alternative pathway of complement system, was identified as a key mechanism of microvascular thrombosis in STEC HUS (Morigi et al. 2011). Moreover, in a mouse model of HUS, obtained by co-injection of Stx2 and lipopolysaccharides, upregulation of P-selectin is associated with C3 deposits and platelets clumps (Morigi et al. 2011). Altogether those findings suggest a role of complement in the pathogenesis of the STEC HUS.

1.2.5.2 In atypical HUS

The endothelium protects itself from complement attack by interacting with soluble complement regulators, such as FH and FI, that inhibit the fluid phase complement activation, and by expressing surface molecules with complement inhibitor activity. The expression of the membrane-bound complement regulators DAF (decay accelerating factor), MCP and CD59 protect the surface of endothelial cells. Reduced control of complement activation (due to loss-of-function mutations in complement regulators or gain-of-function mutations in complement activating proteins) is thought to be responsible for the loss of endothelial cell integrity, the activation of pro-coagulation pathways and development of the TMA (Noris and Remuzzi 2009). The kidney is the organ that is especially vulnerable to complement-mediated damage, as on the one hand it filters substantial amount of blood under high pressure and on the other hand only a
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A single layer, flat endothelium separates the glomerular structures from the bloodstream (Tryggvason and Wartiovaara 2005). In aHUS, endothelial cell damage and platelet dysfunction represent primary events that lead to microvascular lesions particularly in the kidney: endothelial swelling with retraction of the cells and exposure of the basal lamina, microthrombi formation that occlude arterioles and capillaries in the kidney (Stuhlinger et al. 1974; Warwicker et al. 1998).

Persistent and remarkably reduced serum levels and glomerular deposition of the component 3 (C3) of complement system have been reported since 1974 in both familial and sporadic forms of aHUS (Stuhlinger et al. 1974; Noris et al. 1999), confirming an inherited defect causing dysregulation of the complement cascade. Moreover, low serum C3 level in a aHUS patient who has a normal complement component 4 (C4) level indicates selective activation of the alternative pathway of complement system (Noris et al. 1999).

After viral or bacterial infection or endothelial insult, complement is normally activated and C3b is formed. In the presence of normal FH, C3b is rapidly inactivated to inactive C3b (iC3b) by FI (co-factor activity). In addition, FH binds to polyanionic proteoglycans that are present on endothelial cell surface and in the subendothelial matrix, where, because of its high affinity for C3b, it entraps fluid-phase C3b, thus, preventing its deposition on host surfaces and its binding with FB to form the alternative pathway C3 convertase complex (C3bBb), which amplifies the complement cascade. In addition FH dissociates the C3convertase (Rodriguez de Cordoba et al. 2004). The subendothelial matrix lacks endogenous complement regulators, thus, it is completely dependent on FH to control complement activation (Zipfel et al. 2006). In the same way as FH, MCP inactivates C3b deposited on endothelial cells by favoring its cleavage to iC3b by FI, but it only protects those cells on which it is expressed.
THBD, exposed on endothelial surface, enhances FI-mediated inactivation of C3b in the presence of FH and promotes activation of the thrombin-activatable fibrinolysis inhibitor (TAFI into TAFIa), which degrades anaphylatoxins C3a and C5a (Conway 2012). (Fig. 1-5A)

In the presence of events that enhance alternative pathway activation, carriers of complement mutations undergo excess C3b formation and deposition on vascular endothelium. An uncontrolled level of C3 convertase leads to more C3b molecules and to more C5 convertase, initiating the formation of the membrane-attack complex (C5b-9), a multimeric structure that forms pores in the membranes of target cells, causing cell activation and lysis. The proteolysis of C3 and C5 by convertases causes the release of the chemotactic anaphylatoxins C3a and C5a that bind to receptors on inflammatory cells and attract them toward the endothelial layer. Complement-mediated endothelial injury creates a prothrombotic state through exposure of adhesion molecules such as subendothelial collagen, VWF, P-selectin and fibrinogen (Tedesco et al. 1997; Morigi et al. 2011) (Fig. 1-5B). Moreover, it has been shown that mutant FH has defective binding to platelets, allowing C3 and C9 deposition and platelet activation (Stahl et al. 2008). In addition, cell injury may reduce the surface expression of regulatory proteins, and stimulate local production of complement proteins by renal cells (Farrar et al. 2006). After endothelial damage, cell detachment ensues and exposes basement membranes. In these conditions, platelets from the microcirculation adhere and aggregate to the exposed matrix leading to thrombus formation. Mutations in CFH, CFI, CFHRs, MCP and THBD genes or FH autoantibodies found in aHUS patients lead to systemic FH, FI or MCP deficiency, resulting in the loss of regulation of complement activation.
Functional analysis of the C3 or FB mutants identified in the cohorts of aHUS has demonstrated either increased generation or enhanced stability of the alternative pathway C3 convertase. While mutations in *DGKE* gene, encoding for a protein involved in phosphoinositol metabolism, have been detected in patients presenting with aHUS under the age of 1 year, the link with complement remains poorly defined (Lemaire et al. 2013); DGKE appears to be critical to the normal function of podocytes (Ozaltin et al. 2013), and for endothelial cell function and survival (Bruneau et al. 2015).
A Normal Endothelial Cell

B Endothelial Cell with Dysfunctional Complement Regulation

Figure 1-5. Model for the mechanisms leading from impaired regulation of the alternative pathway to thrombotic microangiopathy.
A. In a normal endothelial cell, complement factor H (FH) binds to the endothelial surface and to C3b and together with membrane cofactor protein (MCP) acts as a cofactor for cleavage of C3b, which is mediated by complement factor I (FI), a process that prevents its interaction with factor B (FB). FH also dissociates the C3 convertase of the alternative pathway (C3b). Thrombomodulin (THBD) enhances FI-mediated inactivation of C3b in the presence of FH and promotes activation of the thrombin-activatable fibrinolysis inhibitor (TAFI into TAFIa), which degrades C3a and C5a. B. In patients with loss-of-function mutations in complement regulatory genes (FH, FI, MCP, and THBD [the gene encoding thrombomodulin]) C3b is not degraded efficiently and forms the C3 and C5 convertases of the alternative pathway. Mutated THBD cannot inactivate C5a and C3a because of reduced formation of TAFIa. C5b initiates the assembly of the membrane-attack complex (MAC), leading to cell injury and activation, with expression of adhesion molecules, such as P-selectin, which together with C3a and C5a recruit and activate leukocytes. Subsequent cell detachment results in a prothrombotic state. Aggregated platelets release procoagulant platelet-derived microparticles (PMP), which facilitate the assembly of clotting enzymes. A similar situation applies to patients with gain-of-function mutations in CFB and C3. Mutant FB forms a “superconvertase” that is resistant to dissociation by FH. Mutant C3b does not bind FH and MCP and is resistant to degradation by FI. GAG denotes glycosaminoglycans.
1.2.6 The Complement System

The complement system is an important component of the adaptive and innate immune system and consists of more than 40 plasma and membrane-associated proteins. Nowadays it is more and more evident that the complement system acts both as a first line of defense against pathogens and as a guardian of the host homeostasis (Ricklin et al. 2010). When complement is activated, a cascade reaction is initiated, leading to the cleavage of inert plasmatic complement components that generate bioactive fragments with pro-inflammatory, chemoattractant and cell damaging functions.

The most important roles of complement system are the recognition of pathogens, the activation and chemotaxis of leucocytes, and the induction of the non-self cell lysis by incorporation of the terminal membrane attack complex (C5b-9, MAC). The complement cascade can be activated by three different pathways, classical, lectin and alternative (Fig. 1-6). The classical pathway (CP) is activated by immune complexes or apoptotic cells after specific recognition of a target structure by C1q. The alternative pathway (AP) is permanently active and may attack any surface that is not specifically protected. In the lectin pathway (LP), mannose-binding lectin (MBL) or ficolins recognize patterns of carbohydrates such as N-acetylgalactosamine or mannose, but not sialic acid or galactose, which provides selectivity for bacterial, viral, fungal, and parasitic cell surfaces. Each pathway leads to the formation of unstable protease complexes, named C3 convertases that cleave and activate the central component C3 protein into C3a and C3b fragments. Notably, activation of both CP and LP results in formation of the C3 convertase C4bC2a, while AP activation generates the central AP C3 convertase C3bBb, in which converge all three pathways. C3b molecules deposited on pathogens or damaged self cells provide a molecular platform for the formation of
the surface AP C3 convertase, C3bBb, which enzymatically generates many more C3b molecules, resulting in a positive amplification feedback loop of complement activation. The binding of C3b to the C3 convertases forms the C5 convertases that cleave the complement component C5 producing C5a and C5b. C5b initiates the “late” events of complement activation, in which the terminal complement components C6 to C9 interact to each other to form the membrane-attack complex C5b-9, also called MAC (Membrane Attack Complex), which creates a pore in the membranes of unprotected cells leading to their lysis. The soluble cytolytically inactive form of this complex, called SC5b-9 and stabilized by S-protein and clusterin, has been shown to bind to endothelial cells and to induce expression of adhesion molecules and cytokines (Tedesco et al. 1997). C3a and C5a, released from the cleavage of C3 and C5 respectively, are important anaphylatoxins and act as chemoattractants to recruit phagocytes to the site of complement activation, generate inflammatory environment and modulate adaptive immunity (Morigi et al. 2011). Different cell types carry specific complement receptors, which play major role in the phagocytosis of pathogens and apoptotic/necrotic cells, in the processing and clearance of immune complexes and in mounting immune responses.

To regulate activation, several inhibitors of complement pathways, are expressed on the surface of host cells and also circulate in plasma, with sometime overlapping functionality (Zipfel and Skerka 2009). FH, C1 inhibitor (C1INH), C4b-binding protein (C4BP), clusterin and vitronectin work as soluble inhibitors of the complement cascade, while complement receptors 1 and 2 (CR1 and CR2), MCP (also known CD46), DAF (also known CD55), and protectin (also known CD59) act as membrane-bound inhibitors protecting self surfaces (Hourcade et al. 1999). In addition, some plasmatic regulators, which control complement in the fluid phase, also attach to lipid bilayers or
to biomembranes and maintain complement-regulatory activities. FH, MCP, CR1, and THBD also act as cofactors for the irreversible proteolytic inactivation of C3b by plasma protease FI (Delvaeye et al. 2009). Of note, despite their common activity, these complement regulators have different structural requirements for their FI-mediated activity (Martinez-Barricarte et al. 2010). Properdin is the only known complement regulator that enhances the stability of the C3bBb convertase and the activity of the AP (Lesher et al. 2013). Interestingly, FH-related proteins were recently described to modulate both C3 and C5 convertases (Hebecker and Jozsi 2012; Jozsi et al. 2015; Valoti et al. 2015; Chen et al. 2016), but their physiological relevance is not yet defined.
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Figure 1-6. Schematic representation of the three pathways of the complement activation: the classical (CP), the lectin (LP) and the alternative (AP) pathways.

The complement cascade can be activated through the classic pathway, the alternative pathway, and the mannose binding lectin pathway. The CP is activated by the binding of Fc region of IgG or IgM antibodies to the complement component C1. The LP is triggered by mannose binding lectin (MBL), Collectin11, Ficolins1-3. The CP and LP converge into the cleavage of complement components C2 and C4 leading to the formation of the CP/LP C3 convertase (C4bC2a). The AP is continuously activated in plasma by low-grade hydrolysis of C3 forming C3(H2O). The latter binds to factor B (FB), which in turn is cleaved by factor D (FD) to form the AP fluid-phase C3 convertase. Activation through each of these pathways generates C3-convertases, enzyme complexes that cleave C3 and generate anaphylotoxin C3a and C3b. Further amplification of complement activation through the alternative pathway generates additional C3b. C3b combines with the C3-convertases to create C5-convertases, enzyme complexes that cleave C5, producing the anaphylatoxin C5a and C5b. C5b initiates the late events of complement activation leading to the formation of the membrane-attack complex (MAC or C5b-9 complex) by interacting with C6-C9 proteins.

Self surfaces are protected from complement damage by protein regulators (squared in green): C1INH, C1 inhibitor; FI, complement factor I; C4BP, C4 binding protein; DAF, decay accelerating factor; MCP, membrane cofactor protein; CR1, complement receptor 1; FH, complement factor H; THBD, thrombomodulin.
1. INTRODUCTION

1.2.6.1 The Alternative Pathway C3 convertase

The alternative pathway is the phylogenetically oldest and most important activation pathway of the complement system since it is always activated at a low rate level for instantaneous complement activation, and since it autoamplifies the entire complement cascade. It assists in the maintenance of the integrity of an organism with spontaneous and continuous slow-rate activation through the “tick-over” hydrolysis of C3 into C3b-like molecule C3(H₂O) (rate 1% of total C3 per h). The plasma glycoprotein C3 is the pivotal complement component and the central element of the activation cascade, which leads to the formation of the AP C3 convertase, the key enzyme of the complement system responsible for the autoamplification of the entire cascade. The generation of the AP C3 convertase begins in vivo with the interaction between C3b-like molecule C3(H₂O) and FB, in a Mg²⁺-dependent manner, to form the C3 proconvertase or C3(H₂O)B. In the presence of FD, FB is cleaved and the N-terminal Ba fragment is released from the C3bB complex, resulting in the active AP initiation C3 convertase, C3(H₂O)Bb. This initial spontaneously formed C3(H₂O)Bb complex is the first fluid-phase convertase which cleaves circulating C3 into C3a and C3b. C3a is an anaphylatoxin while C3b binds covalently to any positively charged surfaces including pathogens and endogenous molecules, interacts with FB and forms firstly the amplification C3bB proconvertase and then C3bBb convertase (Fig. 1-7). These steps in turn provide the amplification of AP complement cascade leading to formation of further convertases and deposition of C3b molecules on the surfaces of particles.
The convertase has a very short half-life (about 90 sec) (Pangburn and Muller-Eberhard 1986) and released Bb cannot rebind to C3b, due to the loss of the anchoring Ba part. The convertase can be stabilized 5 to 10 fold by the binding of the only positive complement regulator, properdin. Properdin can also bind directly to microbial targets and altered self structures, providing a platform for the formation of C3 convertases (Hourcade 2006).

In order to avoid complement hyperactivation, the AP C3bBb convertase is tightly controlled by either soluble or membrane-bound complement regulators (Zipfel and Skerka 2009). The role of these complement regulators is both to prevent C3 convertase formation, to dissociate already formed convertase and/or degrade and eliminate C3b from the cell surface. FH is the main fluid-phase complement regulator (Makou et al. 2013). FH decays the C3 convertase, inhibits the conversion from the C3 proconvertase to the active form C3 convertase, and acts efficiently as a cofactor for FI in the inactivation of C3b. However, whilst FH binds and inactivates C3b promptly in the fluid phase, its affinity for surface-bound C3b increases in the presence of polyanions, such as sialic acids, glycosaminoglycans or sulphated polysaccarides (heparins) (Loeven et al. 2015).
Mutations in C3 have been described in ~10% of aHUS (Fremeaux-Bacchi et al. 2008; Noris et al. 2010). Functional analysis of 5 of the 9 mutations revealed increased resistance to complement regulation and in another two, the mutations resulted in a decreased secretion of C3 (Fremeaux-Bacchi et al. 2008). The mechanism by which impaired C3 secretion results in aHUS is as yet unclear. The frequency of \textit{CFB} mutations in the cohorts of aHUS patients examined to date is low at less than 3% (Goicoechea de Jorge et al. 2007). Functional analysis of the mutants has demonstrated that some \textit{FB} genetic changes resulted in increased generation or enhanced stability of the alternative pathway convertase, while 9 out of 15 mutations, identified in aHUS patients, are unrelated to disease pathogenesis (Marinozzi et al. 2014).

\subsection{1.2.6.1.1 C3}

The gene encoding C3 is located on chromosome 19 and comprises 41 exons, 16 of which encode the \( \beta \)-chain and 25 the \( \alpha \)-chain. With a concentration of 1.0-1.5 mg/ml, C3 is one of the most abundant human plasma proteins, and the hepatocytes are by far the major source of circulating C3. It is now clear that almost all cells, including the renal cells, in the human body can produce complement proteins, and bone marrow-derived cells contribute 40% of total C3 in the serum (Morgan and Gasque 1997). Besides, the local production of complement C3 can be induced and/or increased by pro-inflammatory cytokines, which suggests that extrahepatic production, in particular in the renal tissue, evolved to respond to immune requirement (Shavva et al. 2013).
Locally occurring complement activation is triggered when a cell-activating signal, such as T cell, initiates the generation and secretion of C3, FB and FD, leading to C3 and in turn C5 convertases formation in the extracellular space and/or on cell surface (Kolev et al. 2014).

C3 is synthesized as single-chain precursor (190 kDa) and the native protein (187 kDa) is composed of the two disulfide-bounded polypeptide chains: the 115 kDa α-chain and the 75 kDa β-chain (Fig. 1-8). Central to the function of C3 is the internal thioester bond formed between Cys988 and Gln991. The crystal structure of native C3 has been resolved at atomic resolution using X-ray crystallography, and reveals an intricate arrangement of 13 domains, including an anaphylatoxin domain (ANA; C3a), a small link (LNK) domain, a core of eight homologous macroglobulin domains (MG1-8) forming a ring (MG ring), a CUB domain connecting this ring with ThioEster Domain (TED) that contains the reactive thioester moiety, and the C345C domain (Fig. 1-8).
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(Janssen et al. 2005). As indicated previously, the reactive thioester is shielded in native C3 so that it is not accessible.

Activation of C3 into C3b (or C3(H2O)) induces a huge conformational displacement of the TED domain exposing the reactive group to nucleophilic reagents such as hydroxyl or amine groups (Janssen et al. 2006). However, this thioester is exposed for a brief period of time (60 µs) before the molecule is inactivated. This short half-life restricts AP activation locally and, in time, ensures that activation proceeds within the immediate vicinity. The newly formed C3b expresses multiple binding sites for complement components which are not accessible in the C3 molecule. Release of C3a (ANA domain) from the α-chain of C3 generates a novel C3 domain termed α’NT. The α’NT and C345C domains in C3b include binding sites for FB required for the AP C3 convertase formation. These domains are located in a part of the C3 molecule which undergoes large rearrangement upon activation of C3 into C3b, which explains why C3 does not interact with FB. Similarly, structural analyses have suggested that formation of the AP C3 convertase probably depends on the structure and orientation of the CUB domain of C3b and that the interaction between C3b and FB is independent of the TED domain (Gros et al. 2008). So far four separate sites on C3b have been determined that may either represent a binding site or residues that are indirectly involved in the binding of FB (Janssen and Gros 2007). These putative binding sites are: residues 727-745 of α’NT, residues 933-942 in the CUB domain, residues 200-220 in MG2-MG3 loop and C345C domain. Various studies have shown that residues 727-767, located in α’NT and MG6 domains of C3b, form an important interaction site for both FH and CR1. Notably, the fore-mentioned residues are the same or are close to residues important for FB interaction, which implies that convertase dissociation may be based in part on steric hindrance between FH and FB. In addition, FH has other binding sites on C3b, situated...
on TED domain (Perkins et al. 2012). FI irreversible cleavage takes place in three sites inside the CUB domain of C3b (Arg932-Ser933, Arg1281-Ser1282 and Arg1298-Ser1299), resulting in the formation of C3c, C3dg and C3f fragments (Fig. 1-8, bottom) (Gros et al. 2008).

### 1.2.6.1.2 Factor B

The gene encoding factor B (*CFB*) is located on chromosome 6p21.3 and consists of 18 exons coding for a five domain glycoprotein. FB is a single-chain 93 kDa polypeptide and circulates in plasma as an inactive pro-enzyme at a concentration ranging between 93 and 380 mg/l (Waters and Licht 2011). As for C3, the expression of FB is not restricted to the liver, but involved also: monocytes, macrophages, fibroblasts, endothelial cells, muscle and epithelial cells, adipocytes and astrocytes. Besides, local expression of FB can be either constitutively or under cytokine drive (Morgan and Gasque 1997).

![Figure 1-9. FB domain structure.](image)

Cartoon representing domain organization of human complement component Factor B (FB). CCP1-3, three complement control protein domains; VWA, von Willebrand type A domain; SP, serine protease domain. The Arg234-Lys235 scissile bond is indicated.

The structural analyses of FB crystal reveal that it is composed of five structural domains (Fig. 1-9) (Milder et al. 2007). The Ba fragment (33 kDa) at the N-terminus comprised three short complement control protein domains (CCPs), whereas the larger
Bb fragment (60 kDa) at the C-terminal comprised a von Willebrand type A (VWA) domain followed by a serine protease (SP) domain (Fig. 1-9). The SP domain is responsible for the cleavage of new C3 molecules by C3 convertase complex, so that it is necessary for amplification of the alternative pathway cascade. FB also possesses the Mg$^{2+}$-dependent metal ion-dependent adhesion site (MIDAS) in the $\alpha_1$ helix in the VWA domain of the Bb fragment, which mediates the initial recognition of C3b by FB. This initial interaction also involves contacts between C3b and the SCR1-3 domain of Ba fragment (Milder et al. 2007).

Recently, it has been described that properdin contacts both the C345C domain in C3b and the VWA in Bb, indicating that properdin stabilizes the C3bBb convertase by holding together the two components of this enzymatic complex.
1.3 Differential diagnosis of microangiopathic haemolysis

The clinical distinction between thrombotic thrombocytopenic purpura and haemolytic uraemic syndrome has not been always a clear-cut.

TTP and HUS conditions were been considered part of a disease spectrum for years, since their clinical symptoms are often overlapping. The fundamental lesion which consists of vessel wall thickening with swelling and detachment of the endothelial cells from the basement membrane and intraluminal thrombosis, is identical in the two diseases (Remuzzi 1987; George 2000). However, specific clinical features could be distinguished between TTP and HUS patients. Although both conditions are characterized by thrombotic occlusion of small arteries and arterioles, the pattern of distribution of this abnormality is different, being largely confined to the kidney in HUS but widespread (in heart, pancreas, kidney, adrenal, and brain) in TTP (Hosler et al. 2003). There are also evident clinical differences. HUS is the prevalent clinical form in childhood, characterized by renal involvement, often with renal failure; TTP occurs mostly in adults and manifests with marked thrombocytopenia but minimally impaired renal function, almost invariably involves the central nervous system, and frequently recurs. Anyway, several authors reported exceptions to these definitions, including cases of adults in HUS (Hosler et al. 2003), children in TTP (Schneppenheim et al. 2003; Pecoraro et al. 2015), HUS patients with multiorgan disease (Sheth et al. 1986), and TTP patients with acute renal failure (Shibagaki et al. 2006; Zafirani et al. 2015). Consequently, the pathologic features appear important to make a distinction between the two entities: thrombi in TTP are mainly composed of numerous, variably degranulated and altered platelets, and a small amount of amorphous materials (Asada
et al. 1985); instead, in HUS fibrin/red cell-rich thrombi are present with few platelets (Morel-Maroger et al. 1979).

Altogether these observations suggest that symptoms alone cannot differentiate TTP from HUS given the potential for overlapping clinical manifestations.

1.3.1 Is deficiency of ADAMTS13 specific for TTP?

Approximately 80% of TTP cases is triggered by deficient activity of ADAMTS13, and its importance in the pathogenesis of the disease is well established. However, ADAMTS13 deficiency alone is not sufficient to cause TTP. Indeed, targeted deletion of ADAMTS13 in mice does not result in a spontaneous thrombotic phenotype (Desch and Motto 2007). Only with the introduction of a predisposing genetic background that induces elevated plasma VWF levels and the treatment with microbe-derived Shigatoxins, as a trigger, ADAMTS13-deficient mice exhibit a striking syndrome closely resembling human TTP (Motto et al. 2005).

Compound heterozygous or homozygous mutations in the ADAMTS13 gene result in congenital severe ADAMTS13 deficiency, which is considered the underlying cause of hereditary TTP. However, mutations or polymorphisms within ADAMTS13 gene have been described for some diseases such as HUS (Feng et al. 2013)a, venous thrombosis (Lotta et al. 2013), coronary heart disease (Schettert et al. 2010), ischaemic stroke (Hanson et al. 2009), response to ACE inhibitors (Rurali et al. 2013) and malaria (Kraisin et al. 2014). However many of the studies were performed in a small number of patients and the effect of some of these polymorphisms or mutations in vivo still remains to be determined.
Evaluation of ADAMTS13 activity in patient plasma is performed using tests based on the capability of the protease to cleave standard VWF multimers *in vitro* (Gerritsen et al. 1999; Kokame et al. 2005). In patients presenting with TMA, if ADAMTS13 activity is <10%, a diagnosis of TTP is established, if ADAMTS13 activity is >10% and Shiga toxin assay is negative, a diagnosis of aHUS must be considered. Unfortunately, there is no definitive test to make a diagnosis of aHUS, and genetic screening can predict an underlying complement cause only in 60% cases of aHUS (Noris and Remuzzi 2009). Severe deficiency of ADAMTS13 activity is typically not found in individuals affected by HUS (Galbusera et al. 2006), and this feature, together with platelet count and serum creatinine, is nowadays used to discriminate TTP from HUS (Cataland et al. 2012; Cataland and Wu 2013), especially for treatment choice. However, there are some exceptions. Severe deficiency of ADAMTS13 activity is also found in adult patients with clinical diagnosis of HUS (Remuzzi et al. 2002; Remuzzi 2003; Veyradier et al. 2003), or in individuals carrying both ADAMTS13 and CFH pathogenic variants (Noris et al. 2005). Moreover, VWF-cleaving protease deficiency has also been observed in individuals who have recovered from or never had TTP, such as disseminating intravascular coagulation (DIC) (Ono et al. 2006), thrombocytopenic disorders other than TTP (Moore et al. 2001), various inflammatory conditions and even in healthy controls (Mannucci et al. 2001). On the other hand, not all of the patients with clinically defined TTP diagnosis present with severely reduced ADAMTS13 activity (Sadler 2006). Although a severe deficiency of the ADAMTS13 protease is more consistent with a diagnosis of TTP over aHUS, all the above observations suggest that measurable ADAMT13 activity alone could not exclude the clinical diagnosis of TTP.
In TTP patients with severe ADAMTS13 deficiency, the pathologic presence in the circulation of uncleaved ultralarge VWF multimers is considered as the mechanism responsible for VWF-mediated platelet aggregation and thrombosis (Moake et al. 1982). Consequently, the clinical severity of TTP is heterogeneous both in terms of short and long term prognosis as it directly depends on the ratio between high-molecular-weight (HMW) to low-molecular-weight (LMW) multimers (Remuzzi et al. 2002). However, this could not be a reliable indicator since VWF multimer size distribution is determined by a kinetic balance among secretion, proteolysis, and VWF-platelets binding, and its expression is broadly heterogeneous in endothelial cells (Pusztaszeri et al. 2006). Interestingly, it has been reported a group of TTP patients in which circulating ULVWF multimers were never been found either in the acute phase of the disease or in remission (Galbusera et al. 1999). Moreover, the reduced plasma ADAMTS13 activity and increased plasma ULVWF are risk factors for the development of other arterial and inflammatory diseases, including myocardial infarction (Andersson et al. 2012), ischemic stroke (Bongers et al. 2006), preeclampsia (Stepanian et al. 2011), and cerebral malaria (Lowenberg et al. 2010). In addition, in some patients with TTP, there is enhanced or alternative cleavage of VWF (Mannucci et al. 1989; Galbusera et al. 1999). Thus, it has been hypothesized that in individuals who have severely deficient ADAMTS13 activity without manifestations of TTP, the VWF proteolytic activity of activated leukocytes may be sufficient to prevent platelet thrombosis. Conversely, in patients with active TTP, there may be deficiencies in both leukocyte protease-mediated and ADAMTS13-mediated proteolysis of VWF (Thompson and Howard 1986; Raife et al. 2009). As alternative VWF proteolysis, certain tumor cells have armed themselves against VWF accumulation via the production of ADAM-28 (Mochizuki et al. 2012).
All these observations clearly indicate that ADAMTS13 deficiency does not always differentiate TTP from aHUS, speculating that these two acute disorders are based on common principles with a similar pathophysiology (Zipfel et al. 2011; Noris et al. 2012).

1.3.2 Complement activation in TTP

Activation of the alternative pathway of complement and dysregulation of the complement activity are central to the pathophysiology of aHUS, with low levels of C3 and normal C4 (Noris and Remuzzi 2009). With the understanding of aHUS as a disorder of complement activation, it is reasonable to presume that more specific biomarkers of complement may be able to help in the differentiation of aHUS from TTP and other TMAs. Recently several published data however suggested that complement system may also play a role in the pathophysiology of ADAMTS13 deficiency in TTP, but its biochemical explanation is not already clear.

Previous studies have indeed shown that complement is activated during acute episodes of TTP, with reduced levels of serum C3 or elevated platelet-associated C3 (Weisenburger et al. 1977; Garvey and Freedman 1983; Noris et al. 1999; Wright et al. 1999), with the reservation that older studies did not apply ADAMTS13 evaluation when making the diagnosis of TTP. More recently, many authors investigated complement activation markers in TTP patients with documented severe ADAMTS13 deficiency. Ruiz-Torres et al. showed lower serum levels of C3 during the acute phase of the disease, and C3 and C5b-9 deposition on microvascular endothelial cells, suggesting a selective activation of the alternative pathway of the complement system (Ruiz-Torres et al. 2005). Réti et al. studied 23 patients with a diagnosis of TTP and
ADAMTS13 activity <5% at presentation and found evidence for activation of complement as defined by significantly increased levels of C3a and C5b-9 compared to healthy controls, normalizing at remission, although this study did not find a parallel decrease in serum C3 (Reti et al. 2012). Wu et al. have also demonstrated complement activation, as defined by increased levels of C3a and C5a, in patients with acquired TTP at the presentation with an acute episode (Wu et al. 2013). The acute event from which the patients did not survive were characterized by significantly increased factor Bb, C5a, and C5b-9 compared to those episodes from which the patients survived. Moreover, in a recent report of a patient with acute TTP associated with acquired severe ADAMTS13 deficiency, a skin biopsy revealed endothelial deposition of C3d, C4d and C5b-9 (Chapin et al. 2012). The patient described in the case report was successfully treated with the anti-human C5 antibody eculizumab, which is the gold standard therapy for aHUS, also suggesting a role for complement activation in TTP in this case (Tsai et al. 2013). Anti-C5 treatment success for TTP was confirmed in a 12-year-old boy suffering from a congenital form of the disease, which achieved complete remission both at the onset and during recurrences, supporting the idea that the complement pathway is activated in the presence of ADAMTS13 deficiency (Pecoraro et al. 2015). Furthermore, it has been reported that endothelial microparticles and VWF-platelets strings isolated from plasma samples of TTP patients are coated with complement C3 and C9 (Tati et al. 2013); and plasma from 8/49 patients with ADAMTS13 deficiency induced haemolysis of sheep erythrocytes (Feng et al. 2013)b. The latter is a common in vitro assay to test complement-mediated haemolysis by using serum samples (Sanchez-Corral et al. 2004), otherwise the authors didn’t reported the reasons why they used citrate platelet-poor plasma samples instead of serum, since citrate would block complement activation by chelating divalent cations as Ca^{2+}. However, some studies
from Japan documented that complement can be activated in citrate plasma collected in clinic tubes, since their citrate concentration is not inhibiting (Kobayashi et al. 1999). Besides, Mg\(^{2+}\) ions are not chelated by citrate, thus allowing the activation of the alternative pathway of the complement system, which can promote the haemolysis of sheep erythrocytes.

An additional link between complement activation and severe ADAMTS13 deficiency was reported by Turner et al., who studied the role of ULVWF in complement activation (Turner and Moake 2013). C4 released from human umbilical vein endothelial cells (HUVEC) did not attach to the ULVWF multimer strings, ruling out activation of the classical and lectin complement pathways by ULVWF. In contrast, components of the alternative pathway (C3, FB, C5 and properdin) attached to the ULVWF multimer strings in quantitative patterns consistent with the assembly of the alternative pathway components into active complexes (Turner et al. 2014). This would provide a plausible mechanism for the activation of the alternative pathway in condition with severe deficiency of the ADAMTS13 protease that induce ULVWF accumulation in the bloodstream.

All these observations raise the possibility of additional factors besides ADAMTS13 deficiency involved in the pathophysiology of TTP.

### 1.3.3 Coagulation and complement system interplay

ADAMTS13 is an important regulatory protease of the coagulation cascade, while the alternative pathway is a pivotal activation system of the complement cascade. The coagulation and complement systems are linked through both direct and indirect interactions and serve as innate defense against foreign invasion (Fig. 1-10). The
1. INTRODUCTION

endothelium itself and the vascular bed on which they reside probably play an important role in this interaction.

It is well documented that, during thrombus formation and progression, both clotting cascade and innate immunity are coordinately activated and several complement factors, including C3, C4, C3a, C5a and FH are incorporated into the blood clot (Distelmaier et al. 2009; Howes et al. 2012). Besides, Polley et al showed that the presence of complement proteins C3, C5, C6, C7, C8, and C9 enhanced thrombin-mediated platelet aggregation and the release of serotonin (Polley and Nachman 1978).

1.3.3.1 The complement system stimulates coagulation cascade and immune response

The complement system directly modulates thrombus stability and development by enhancing thrombogenic properties of endothelium: C3a reduces thrombomodulin expression on the endothelial surface, and exocytosis of P-selectin and VWF from intracellular Weibel-Palade bodies (Morigi et al. 2011), while C5a amplifies endothelium activation through loss of heparan sulfate and other glycosaminoglycan from the plasma membranes of endothelial cells, reducing the anticoagulant property of the endothelial surface (Platt et al. 1991). Terminal complement pathway complexes formed as C5 is activated, have also multiple procoagulant properties: C5b-9 deposition on endothelial cells induces exposure of VWF and upregulation of adhesion molecules for platelets on endothelial cell surface, which favor platelet adherence to the vessel wall (Tedesco et al. 1999); incorporation of the C5b-9 complex into the cell membrane activates platelets and results in the exposure of procoagulant lipids (Sims and Wiedmer 1995) and of the procoagulant extracellular matrix (Saadi and Platt 1995). In addition, C5a has also been found to induce the expression of plasminogen activator inhibitor-1
in human mast cells and basophyls, indicating that the complement may also contribute to the mechanisms of thrombosis by inhibiting fibrinolysis (Wojta et al. 2002).

Furthermore, FH, the major negative regulator of the complement system, has been proposed to control also coagulation cascade through the interaction with VWF. FH, bound to VWF, increases ADAMTS13-mediated cleavage of VWF-A2 domain, probably due to a conformational change in VWF that potentially makes the latter more accessible to the metalloprotease (Feng et al. 2013). However another study showed an opposite inhibitory effect of FH on VWF cleavage by ADAMTS13 (Rayes et al. 2014).

Complement also modulates proinflammatory cytokines response and promotes infiltration of immune mediators. Release of anaphylatoxins C3a and C5a following complement activation is considered a critical determinant of neutrophil recruitment and activation in thrombosis (Distelmaier et al. 2009). C5a and the cytolytically inactive form of the MAC, SC5b-9, directly induce the expression of tissue factor (TF), one of the primary initiators of blood coagulation, by leucocytes and human endothelial cells (Ritis et al. 2006). C3a and C5a, through C3aR and C5aR signalling, influence production and secretion of tumour necrosis factor-alpha (TNF-α), interleukin-8 (IL-8) and interleukin-6 (IL-6) by macrophages (Markiewski et al. 2007) and upregulate adhesions (Schraufstatter et al. 2002). In turn, TNF-α is a potent enhancer of TF expression on monocytes surface, which facilitates the interaction with activated platelets and endothelial cells via binding of P-selectin. IL-6 increases the production and thrombogenicity of platelets (Oleksowicz et al. 1994). Microparticles, secreted by activated endothelium, express negatively charged phospholipids and TF, and thus promote coagulation (Combes et al. 1999). Inflammatory cytokines also decrease the level of several anticoagulants (Shebuski and Kilgore 2002).
1.3.3.2 **The complement system activates platelets**

Complement products are also able to directly influence platelets activation. C3 and C5b-9 deposition on the surface of platelets markedly increase the rate of conversion of prothrombin and thrombin, cause the secretion of dense α-granules, which are essential component of primary hemostasis (Sims and Wiedmer 1991), and stimulate the formation of platelets microparticles, that enhance thrombus formation (Tans et al. 1991). The pathophysiological relevance of complement activation on platelets has also been demonstrated by the finding that platelet function is impaired in C3-deficient mice (Gushiken et al. 2009).

1.3.3.3 **The coagulation pathway influences complement activation**

On the other hand, also coagulation system directly modulates complement activation. Both C3 and C5 could be activated in a convertase-independent manner, by several specific proteases of the coagulation cascade such as cathepsin L (Liszewski et al. 2013), cathepsin D (Huber-Lang et al. 2012), thrombin (Huber-Lang et al. 2006), factor Xa and XIa (Amara et al. 2008), trypsin (Ekdahl and Nilsson 1999), kallikrein (Wiggins et al. 1981) and factor VII-activating protease (Kanse et al. 2012). However, C5 is a poor substrate for these proteases, raising questions as to its importance in contributing to C5a generation during thrombosis formation *in vivo*. What is becoming physiologically important is that plasmin, the strongest effector enzyme in the fibrinolytic cascade, is able to cleave C5 at a similar rate as the canonical C5 convertase formed from complement components (Foley et al. 2016). This finding is important since fibrinolysis occurs after and regulates coagulation and therefore the C5 cleavage products will be present during clot resolution as well as clot formation and regulation.
Besides, plasmin generation is increased acutely in blood of patients suffering for thrombotic disorders (Wada et al. 1989), suggesting an important regulatory role of plasmin in proinflammatory complement-mediated events. However, some recent findings described plasmin as a negative regulator of the complement activation, since it is able to inactivate C3b into several fragments similarly to FI-mediated cleavage (Foley et al. 2015). Anyway, further validation studies are required for both mechanisms. Notably, activation of complement by a “single” protease is rapid, requires less energy expenditure and may have evolved before convertase enzyme complex-mediated “activation”, suggesting that it may have important far-reaching functions that are yet to be discovered. However, research into convertase-independent complement activation poses an interesting area to explore and may deliver some novel and unexpected therapeutic targets to control complement (Kolev et al. 2014).

**1.3.3.4 Thrombomodulin: a key protein between the coagulation cascade and the complement system**

Another important modulator of the complement system is thrombomodulin (THBD), a crucial regulatory protein of the coagulation cascade. THBD is a transmembrane glycoprotein, highly expressed in endothelial cells and numerous other cell types. THBD has an anticoagulant role by acting as a cofactor for thrombin, which activates protein C. Activated protein C in turn inactivates factor Va and factor VIIIa of the coagulation cascade and limits clot development (Conway 2012). THBD provides also protection against inflammation: THBD sequesters thrombin on the cell membrane surface thus limiting its pro-inflammatory activities, mediated by protease activated receptors (PARs), in expression and/or release of chemotactic molecules for monocytes and neutrophils. Besides, THBD-thrombin complex promotes TAFI activation and
hence down-regulates fibrinolysis by reducing plasmin generation and function on fibrinogen and complement C3 and C5. Interestingly, the activated form of TAFI (TAFIa) also inhibits the potent anaphylatoxins C3a and C5a by their des-argination. Thus, well-known activities of THBD in protecting against inflammation and thrombosis require mediation of thrombin. However, different studies reported also a direct interaction of THBD with the proteins of the complement system. The lectin-like domain of THBD interferes with in vitro activation of the classical and the lectin pathways (Van de Wouwer et al. 2006). In vitro, THBD binds FH and C3b-FH complexes, and negatively regulates complement by accelerating FI-mediated inactivation of C3b (Delvaeye et al. 2009) and supporting FH in its regulatory activities on the cell surface (Heurich et al. 2016). In contrast to all these results describing THBD as a negative regulator of complement system, a recent publication proposed THBD as a complement activator by enhancing C3 activation in a similar fashion to properdin (Tateishi et al. 2016). Nevertheless, the physiological meaning of the dual modulating functions of THBD in the AP complement system remains still unclear.

Additionally, THBD can be release from endothelial cell surfaces, in a mechanism referred as shedding, mediated by neutrophil-derived proteases and accompanied by cell damage. THBD shedding is thought to contribute to increase risk of thrombosis associated with inflammation, and it is described in TTP/HUS patients (Mori et al. 2001), in coagulation-related syndromes or in several autoimmune disorders (Boehme et al. 2000; Ware et al. 2003). Thus, soluble THBD can be considered a reliable marker for endothelial damage in conditions where coagulation and/or complement are dysregulated.
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1.3.3.5  **VWF: a new player in complement modulation**

More recent studies offer an innovative link between the coagulation cascade and complement activation, provided by VWF. The multimeric VWF, which initiates haemostasis by promoting platelet adhesion, also interacts at various levels with complement components, including C3 and C5, and acts as a complement amplifier on endothelial cell surface (Turner et al. 2009; Turner and Moake 2013). Contrary to these findings, there are also some evidence providing a role for VWF as a negative regulator of the complement system. VWF has been shown to favor C3b inactivation by enhancing FH cofactor activity for FI-mediated cleavage (Rayes et al. 2014). Another study proposed that normal plasma VWF multimers alone may exert cofactor activity, while ULVWF multimers lacked cofactor activity and did not inhibit the generation of C3b from C3 (Feng et al. 2015). Since FH binds to VWF, and VWF-FH complexes were detected in normal plasma (Feng et al. 2013)c, the contribution of contaminating FH in plasma derived VWF preparation to the observed cofactor activity cannot be excluded. Finally, Noone et al recently suggested that VWF has a role in protecting the endothelium from complement deposition, based on findings of increased C3c deposition on cultured blood outgrowth endothelial cells (circulating endothelial progenitor isolated from peripheral blood) from patients with type 3 VWF disease that have severe deficiency of VWF (Noone et al. 2016). However, the precise molecular mechanism through which VWF modulates complement, and its pathophysiological implications for human diseases have not been clarified yet, and further studies are needed.
1.3.3.6 *Intracellular complement system: a new concept*

As mentioned before, the complement system is one of the oldest components of immunity and is central to the detection and destruction of invading pathogens. Finding that complement activation is not confined to the extracellular space but occurs also within cellular compartments (Liszewski et al. 2013), suggest new functions for complement cascade in different cell effectors immune-related and non-immune-related systems. Recently, it has been demonstrated that peripheral blood cells are able to uptake C3(H₂O) from the circulation, and this uptake allows for recycling of the activated C3 as a source of C3a, which in turn can modulate cell activation phenomena and result in enhanced proinflammatory and survival phenotypes. This new concept, called Intracellular Complement System (ICS), is proposed to be an original gateway to activation of complement system in cells, on membranes and in the interstitium, in order to assist in a rapid and local response of the host to danger (Elvington et al. 2017). Nevertheless, much still remains to be discovered about this ancient and pivotal system, and there are many open questions to be fully addressed about its new functions.
Figure 1-10. Coagulation and complement system interplay.
Cartoon summarizing the effects of complement proteins on the coagulation systems, and vice versa. The anaphylatoxins C3a and C5a induce release of TNF-α, IL-6 and IL-8 from macrophages, which stimulate tissue factor (TF) expression, activate platelets, recruit neutrophils, and, together with C5b-9, activate endothelial cells (EC) through loss of heparin sulphate (HS), glycosaminoglycans (GAG) and thrombomodulin (THBD), and secretion of von Willebrand Factor (VWF) and P-selectin from Weibel-Palade bodies. C5b-9 induces TF expression, providing clot development. After activation, platelets release α-granules and microparticles, which stimulate thrombin generation and promote clot formation. Thrombin and plasmin promote C3 and C5 cleavage. Thrombin binds THBD on cell surface and promotes activation of thrombin-activatable fibrinolysis inhibitor (TAFI in TAFIa), which inactivates C3a and C5a, and stimulates fibrinolysis for clot breakdown. THBD inhibits lectin and classical pathway activation, and supports factor H (FH) in its regulatory activity on C3b. VWF modulates complement activation via C3 and FH binding.
1. INTRODUCTION
AIM OF THE STUDY
A disruption of the endothelial cell lining by mechanical or chemical stimuli may activate the complement system and clotting. The focal nature of haemostatic control and thus the thrombotic tendency of each organ vary depending on the distribution of the anticoagulant and procoagulant factors throughout its vascular tree (Rosenberg and Aird 1999). With regard to environmental triggers, otherwise innocuous seasonal infections, pregnancy and certain drugs have all been reported to induce endothelial perturbation which promotes complement activation and ULVWF multimers release. In healthy individuals, these events are self-limiting as a result of multiple, redundant regulatory mechanisms which control complement inflammatory response and ULVWF thrombogenic potential. Individuals carrying genetic abnormalities affecting complement regulation or ADAMTS13 activity on ULVWF cleavage are particularly vulnerable to these events and predisposed to microvascular thrombosis. Although complement system and ADAMTS13/VWF belong to two distinct pathways, their crosstalk is recently proposed and it is gaining importance in the understanding of the underlying causes of thrombosis. Besides, hyperactivation of the complement system, with its prothrombotic and proinflammatory effects, is emerging as a common pathogenetic effector in different TMA manifestations, such as HUS and TTP.

Several publications have highlighted the fact that complement activation via the alternative pathway may occur in TTP patients (Chapin et al. 2012; Reti et al. 2012; Feng et al. 2013; Tati et al. 2013; Tsai et al. 2013; Wu et al. 2013; Pecoraro et al. 2015). The study reported in this thesis is basically focused to elucidate whether complement activation in TTP is a pathogenetic factor or rather an epiphenomenon. The overall objectives of this thesis were to clarify whether direct interactions could exist between ADAMTS13 and/or VWF and complement proteins, and to investigate the
contribution of impaired VWF cleavage due to ADAMTS13 deficiency in complement activation in TTP.

**Specific Aims**

1. To verify whether complement is activated also in congenital forms of TTP (cTTP), since most patients analyzed in published reports are affected by acquired deficiency of ADAMTS13 and it could not be excluded that complement activation found in them is secondary to the presence of autoantibodies.

2. To evaluate whether, in cTTP, complement is activated on microvascular endothelium, considering that this disease, similarly to HUS, is a disorder of endothelial injury.

3. To study whether ADAMTS13 directly modulates complement activation through the interaction with FB, which together with C3b is responsible for the formation of the C3 convertase, the key enzyme of the alternative pathway of complement cascade.

4. To study and clarify whether VWF modulates complement activation through its interaction with FH, the major regulatory protein of the AP of complement.
5. To study and verify whether VWF influences complement activation through its interaction with C3b, the key protein of the complement cascade, and whether this interaction promotes complement activation.

6. To study whether VWF-mediated complement activation modulates thromboresistant endothelial phenotype and promotes thrombus formation.
2. METHODS
2. METHODS

2.1 Patient recruitment

All patients with TTP or aHUS referred to the International Registry of HUS/TTP (http://www.marionegri.it/it_IT/home/medico/ricerca_clinica/registri_patologia) were evaluated for inclusion in this study. The International Registry has been established in 1996 under the coordination of the Clinical Research Center for Rare Diseases Aldo e Cele Daccò and till now has included patients from Italian, European and extra-European Centers. The patients have been enrolled in the study under an approved protocol by the Ethical Committee of the Azienda Sanitaria Locale Bergamo, Italy (U0148858/III). All participants (patients and their relatives) or their legal guardians have received information on the purpose and design of the study, and have provided written informed consent to the study.

The HUS/TTP Registry has been established in order to study the genetic and biochemical abnormalities of HUS/TTP, to collect clinical and genetic data of patients and their families, to find the best therapeutic approach for patients and to give up-to-date information to physicians and families. For this purpose, a Case Report Form, which is completed for each patient by the referring physician, is prepared. The Form included information on signs, symptoms, associated features, plasma and urine routine biochemical analysis, follow-up data and family history. Each patient and their relatives are pseudonymized by assignment of a DNA code and a family number. Only this code is used to label the biomaterial samples. Only the local physicians in charge of the study know the code annotation.
2. METHODS

2.1.1 TTP or aHUS Diagnosis

TTP or aHUS, firstly identified as TMA, was diagnosed in all cases reported to have one or more episodes of microangiopathic haemolytic anemia and thrombocytopenia defined on the basis of hematocrit (Ht)<30%, hemoglobin (Hb)<10 g/dl, platelet count <150,000/µl, serum lactate dehydrogenase (LDH)>460 IU/l, undetectable haptoglobin and the presence of fragmented erythrocytes in the peripheral blood smear, with or without neurological symptoms and with or without acute renal impairment during bouts.

In patients with TMA (low platelet count, increased lactate dehydrogenase and haemolytic anemia) it is important to establish the correct diagnosis (TTP or aHUS). As first, plasma ADAMTS13 activity was measured by the Collagen Binding Assay (CBA); severe deficiency of ADAMTS13 activity (<10% as measured) in remission and absence of inhibitory anti-ADAMTS13 auto-antibodies as tested by CBA leads to diagnosis of TTP. If ADAMTS13 activity is normal (>50%), tests for Shiga toxin-producing bacteria in stools and/or serum antibodies against Shiga-toxin (ELISA) and/or LPS O157, O26, O111, or O145 (ELISA) are performed. Positive tests are consistent with diagnosis of STEC HUS. Diagnosis of aHUS is done by exclusion in patients in whom these tests are negative and with no evidence of secondary conditions (autoimmune diseases, drugs, malignancies). In aHUS patients genetic screening for aHUS-associated genes (Noris and Remuzzi 2009) was performed.

Congenital TTP (cTTP) was diagnosed in TTP patients with homozygous or compound heterozygous *ADAMTS13* mutations. Details of the *ADAMTS13* mutations (most of them have been functionally characterized previously (Donadelli et al. 2006; Lotta et al. 2012; Rurali et al. 2015) and the clinical parameters of the 20 cTTP patients are
reported in Table 4 3-1. For these patients, ADAMTS13 antigen levels were measured in plasma using a commercial ELISA (Imubind, American Diagnostica).

An important inclusion criterion of this study was the availability of plasma and/or serum taken either during the acute phase (before plasma treatment) and/or in remission, at least 30 days after the last plasma treatment. Complete remission was defined as normalization of both hematological parameters (Ht >30%; Hb >10 g/dl; LDH <500 IU/l; platelets >150,000/µl) and renal function (serum creatinine <1.3 mg/dl for adults, <0.5 mg/dl for children below 5 years of age and <0.8 mg/dl for children 5-10 years old). TTP patients with renal involvement were screened for common aHUS associated genes, which allowed us to exclude the presence of genetic abnormalities predisposing to complement dysregulation (Prestidge et al. 2012; Pecoraro et al. 2015; Rurali et al. 2015).

### 2.2 Collection of biological samples from patients and healthy controls

For each patient and healthy subject controls, 6 ml of whole blood were collected in a BD Vacutainer K2EDTA Blood Collection Tube (Becton, Dickinson & Co., Mountain View, CA, USA) and stored at -20°C until DNA extraction. In parallel, 8 ml of whole blood were collected also in ice-cold K2EDTA, Sodium Citrate (105M / 3.2%) and serum silicone-coated Blood Collection tubes, all from BD (Vacutainer), and immediately centrifuged at 4°C to avoid ex vivo complement activation. Plasma and serum from K2EDTA, Sodium Citrate and silicone tubes respectively, were quickly separated and frozen at -80°C until assay.
2.3 Determination of ADAMTS13 activity: Collagen Binding Assay (CBA)

ADAMTS13 activity was measured using the residual Collagen Binding Assay (CBA) previously described by Gerritsen et al. (Gerritsen et al. 1999) with some modifications. This is an indirect assay based on the decreased collagen binding activity of the small VWF multimers compared to that of the fully multimerized VWF (Veyradier and Girma 2004). Briefly, VWF prepared from normal human plasma was used as substrate for the protease and patient’s serum sample was used as source of ADAMTS13 to measure patient’s ADAMTS13 cleaving activity of VWF (Gerritsen et al. 1999; Franchini and Mannucci 2008). Moreover, a pool of normal human sera (NHS) obtained from about 30 healthy donors with known ADAMTS13 activity levels selected to obtain a pool containing 100% of protease activity was used as reference for the assays. The steps are described following.

2.3.1 VWF substrate preparation

Four fresh-frozen plasma bags from four healthy donors (250 ml/each, Azienda Ospedaliera Papa Giovanni XXIII, Bergamo, Italy) were pooled in aliquots of 20 ml and stored at -80°C. After thawing, an aliquot of this pool was added with 230 µl of 2 mM Pefabloc SC buffer (Sigma Aldrich srl) and 1.6 ml of 0.2 M EDTA pH 7.4. After incubation for 3 hours at room temperature, the mixture was exhaustively dialyzed in a Visking cellulose tubing (Prodotti Gianni) against dialysis buffer (4.5% polyethylene glycol 20,000, 5 mM Trizma base, pH 8.0) to remove EDTA and Pefabloc. The dialysis was performed three times: initially for 1 hr, then overnight, and finally for 3 hr, each time against a 25-fold volume of dialysis buffer (500 ml). The resulting cloudy plasma
was free from ADAMTS13 proteolytic activity and was stored in 4.3 ml aliquots at -80°C. Before use, the substrate was thawed for 10 min at 37°C and added with 1 ml of substrate dilution buffer 1 (8 M urea, 5 mM Trizma base, pH 8.0). The substrate was then centrifuged for 10 min at 1100 x g and the supernatant was used in the assay.

2.3.2 VWF substrate digestion by ADAMTS13

Patients’ serum samples were diluted 1:10, 1:20, and 1:40 with sample dilution buffer 2 (1.5 M urea, 5 mM Trizma base, pH 8.0). Aliquots of 50 µl were then pre-incubated with 5 µl of 93 mM BaCl$_2$ for 30 min at 37°C to achieve partial degradation of patient’s endogenous VWF. Subsequently, 100 µl of VWF substrate was added to the sample in the presence of 3 mM BaCl$_2$ and incubated for 1 hr 45 min at 37°C. The VWF digestion was stopped by adding 10 µl of 0.825 M Na$_2$SO$_4$ and the incubation mixtures were centrifuged for 3 min at 4200 x g. The obtained supernatants were used to quantify the residual VWF substrate by its collagen binding activity.

Fifty µl aliquots of 1:5, 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320 NHS dilutions in sample dilution buffer 1 characterized by 400%, 200%, 100%, 50%, 25%, 12.5% and 6.25% ADAMTS13 activity level, respectively, were used for the calibration curve.

2.3.3 Collagen binding activity of the digested VWF substrate

Nunc CovaLink multiwell plate (Thermo Fisher Scientific) was coated with 100 µl of Collagen Solution (0.5 mg/ml Human Placenta Villi Collagen type III, Southern Biotechnology) for 2 hr and blocked with 2.5% BSA in PBS. Forty µl aliquots of digested samples were diluted with 160 µl of sample dilution buffer 2 using a Nunc-Immuno Maxisorp microtiter plate (Thermo Fisher Scientific) that had been previously
blocked with 0.5% BSA, 0.05% Tween20 in PBS 1X for 30 min. Subsequently, 100 µl of each diluted digested sample were transferred into wells of the Nunc CovaLink multiwell plate previously coated with Human Collagen type III. After a 1 hr 45 min incubation, the bound VWF multimers were incubated with 100 µl of a peroxidase-conjugated anti-human VWF antibody diluted 1:4000 in sample dilution buffer 2. Between each step, the wells of the microtiter plates were washed three times with Wash buffer (0.05% Tween20 in PBS 1X). The chromogenic reaction was performed by addition of 3 tablets of o-phenylenediamine (OPD, Dako) dissolved in 13.5 ml ddH₂O containing 7.5 µl of 30% (v/v) H₂O₂. The reaction was stopped by adding 100 µl of 2 M H₂SO₄ and the absorbance was read at 492 nm with the MDL680 Microplate Reader (BioRad).

The values of ADAMTS13 activity were read from a dose-response curve obtained for each assay run by testing serial dilutions from 1:5 to 1:320 of the reference NHS. The within-assay (n=18) coefficient of variation was 8%, the between-assay (n=100) coefficient of variation was 14%, and the lower limit of sensitivity of the method was 6% of the normal protease levels (Mannucci et al. 2001). Interference of endogenous VWF in test samples has been excluded by finding similar protease levels in normal plasma and in plasma after cryoprecipitation to remove a large amount of VWF, particularly of the larger multimers more reactive with type III collagen (Mannucci et al. 2001). Patient samples with activity levels below the detection limit of the assay (6%) were defined as having complete deficiency of ADAMTS13 (Remuzzi et al. 2002).
2.4 Genetic screening

Genetic screening of all exons and intronic flanking regions of aHUS associated genes (CFH, CD46, CFI, CFB, C3 and THBD) and ADAMTS13 were performed through Next Generation Sequencing (NGS) by my colleagues of the Laboratory of Rare Diseases, coordinated by Dr. Marina Noris. Genomic DNA was extracted from blood leukocytes (NucleoSpin Blood L, Macherey-Nagel) and sequenced using the Ion Torrent Personal Genome Machine (PGM) platform (Thermo Fisher Scientific) which works with the Ion semiconductor sequencing method. Ion Torrent technology is capable of directly translating chemical signals into digital information. The Ion semiconductor sequencing is a method of DNA sequencing based on the detection of hydrogen ions that are released during the polymerization of DNA. Naturally, a proton is released when a nucleotide is incorporated by the polymerase in the DNA molecule, resulting in a detectable local change of pH. Each micro-well of the Ion Torrent semiconductor sequencing chip contains approximately million copies of a DNA molecule. The PGM sequencer sequentially floods the chip with one nucleotide after another. If a nucleotide complements the sequence of the DNA molecule in a particular micro-well, it will be incorporated and hydrogen ions are released. The pH of the solution changes in that well and is detected by the ion sensor, essentially going directly from chemical information to digital information. If there are two identical bases on the DNA strand, the voltage is double, and the chip records two identical bases. If the next nucleotide that floods the chip is not a match, no voltage change is recorded and no base is called. Since this detection is direct, each nucleotide incorporation is measured in sec enabling very short run times.
2. METHODS

The Ion next generation sequencing requires a very simple work-flow:

1) **DNA preparation.** DNA must be pure and not degraded. The purity was evaluated as 260/280 and 260/230 absorbance ratios, which must be in the range of 1.8 – 2.2 and 1.5 – 2.2 respectively, using the NanoDrop ND-1000 spectrophotometer. The degradation and RNA contamination were evaluated by agarose gel electrophoresis. DNA concentration was then confirmed by Qubit 3.0 Fluorometer.

2) **Genomic DNA Library preparation.** Libraries were prepared by ultra-high multiplex PCRs using the Ion Ampliseq Library Kit v2 and the Ion Plus Fragment Library Kit (both from Thermo Fisher Scientific) containing 311 primer pairs (sub-divided into 7 pools). Then, PCR products were pooled per individual DNA sample, and subjected to end-repair (to create blunt ends), to barcode adapter ligation (important for the simultaneous sequencing of several samples) and to nick repair reaction (important to complete the linkage between the adapters and the amplicons).

3) **Template preparation (Emulsion PCR).** The emulsion PCR was performed using the Ion PGM Template OT2 200 Kit (Thermo Fisher Scientific) to amplify a single DNA fragment on a single Ion Sphere Particles (ISP) in an aqueous droplet (micelle). As result of this step each ISP is coated with millions of copies of a single DNA fragment.

4) **Sequencing on Ion Chip.** In this step the templated ISP were loaded into a 318 Ion chip (Thermo Fisher Scientific) and sequenced using the Ion Sequencing Kit (Thermo Fisher Scientific). In Ion sequencing, all four nucleotides were provided in a stepwise fashion, and their incorporation into the nascent strand results in a shift in the pH of the surrounding solution, converted to a voltage and digitized by off-chip electronics.

5) **Data analysis.** Once data were generated on the Ion PGM sequencer, they were automatically transferred to the required Torrent Server, that produce the DNA sequences associated with individual reads. Read alignment and variant calling were
performed using the Torrent Suite software, mapping reads to the reference human genome (UCSC build hg19). Genetic variants were functionally annotated using the ANNOVAR software: dbSNPbuild137, 1000 genomes ESP6500 and ExAC databases were used to distinguish new variants from those already reported; new coding variants, coding variants MAF <1% in cases of autosomal recessive inheritance and coding variants with MAF <0.1% in cases of autosomal dominant inheritance were analyzed by SIFT and Polyphen-2 softwares to identify potentially damaging variants. Intronic variants localized 10bp from junctions were analyzed by the Human Splicing Finder and Genscan Softwares. Putative mutations and coding regions not adequately covered by NGS were validated and analyzed by Sanger sequencing, respectively.

2.5 Serum and plasma complement profile

Complement C3 and C4 levels in serum were measured by nephelometry, using a standard procedure and commercially available diagnostic kits (Siemens Healthcare Diagnostics or Beckman Coulter). SC5b-9 levels were evaluated in EDTA plasma samples by MicroVue SC5b-9 Plus EIA-(Quidel). Levels below or above the limits of normal ranges (defined as mean ± 2 SD of the laboratories of the “Azienda Ospedaliera Papa Giovanni XXIII”, Bergamo for C3 and C4: C3, 83-180 mg/dl; C4, 15-40 mg/dl, and as mean ± 2 SD of 50 healthy controls tested at least twice for SC5b-9, 127-400 ng/ml) were considered abnormal. Healthy subjects, considered as controls, were recruited by “Azienda Ospedaliera Papa Giovanni XXIII”, Bergamo, and by the Clinical Research Center for Rare Diseases Aldo e Cele Daccò, Ranica BG. The protocol was approved by the Ethical Committee of the Azienda Sanitaria Locale
Bergamo, Italy (U0148858/III). Participants or their legal guardians provided written informed consent.

2. METHODS

2.6 **Ex vivo studies with HMEC-1 and cTTP serum**

The *ex vivo* studies used here were set up for aHUS and described in detail previously (Noris et al. 2014). The human microvascular endothelial cell line of dermal origin (HMEC-1, a kind gift from Dr. Edwin Ades and Francisco J. Candal of CDC and Dr. Thomas Lawley of Emory University, Atlanta, GA) was cultured as described (Ruiz-Torres et al. 2005; Noris et al. 2014). In a previous work of the same Laboratory where this study was performed, it has been demonstrated that in primary human microvascular endothelial cells of dermal origin (HDMECs) treated with Verotoxin-1, expression of endothelial markers such as vitronectin receptor, P-selectin, and PECAM-1, and thrombus formation were similar to those observed in the HMEC-1 line (Morigi et al. 2001). These results indicated that HMEC-1 line manifests the physiological sensitivity of the primary cells and could be considered a reliable and representative *ex vivo* model for microvascular endothelium. The growth medium consisted of MCDB 131 (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies), 1 mg/ml hydrocortisone, 100 U/ml penicillin (Life Technologies), 100 mg/ml streptomycin (Life Technologies), 2 mM glutamine (Life Technologies), and 50 mg/ml endothelial cell growth factor (TEBU-BIO srl) (Maciag et al. 1979). For the test, HMEC-1 (about 75,000 cells) were plated on glass coverlips and used when confluent. Cells were washed three times with test medium (HBSS: 137 mmol/l NaCl, 5.4 mmol/l KCl, 0.7 mmol/l Na<sub>2</sub>HPO<sub>4</sub>, 0.73 mmol/l KH<sub>2</sub>PO<sub>4</sub>, 1.9 mmol/l CaCl<sub>2</sub>, 0.8 mmol/l MgSO<sub>4</sub>, 28 mmol/l Trizma base pH 7.3, 0.1% dextrose; with 0.5% BSA) and then
activated with 10 µM ADP (Sigma Aldrich srl) in test medium for 10 min (ADP-activated cells) or incubated with test medium alone (resting cells). Then, cells were washed three times with test medium and then incubated for 4 hr with serum diluted 1:2 with test medium (final volume 300 µl). At the end of the incubation, HMEC-1 were washed two times with PBS, fixed in 3% paraformaldehyde, washed again twice with PBS and then blocked with PBS with 2% BSA for 1 hr. The cells were stained with the following specific antibodies: FITC-conjugated rabbit anti-human C3c-complement (Dako, which recognizes C3c, part of C3 and C3b), or rabbit anti–human complement C5b-9 complex (Calbiochem, which recognizes new epitopes of human C5b-9 complement complex) followed by FITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories), or goat anti-human C4α (Santa Cruz Biotechnology, which recognizes an internal region of C4 of human origin) followed by donkey anti-goat Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories), or rabbit polyclonal anti-human VWF (Dako) followed by Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories), or mouse anti-human thrombomodulin (R&D System) followed by Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). Isotype-matched irrelevant antibodies were used as negative controls. In selected experiments, soluble complement receptor 1 (sCR1, TP-10, 150 µg/ml, a gift from CellDex), an inhibitor of the classical, alternative, and lectin pathways of complement activation, or CR2-CFH (TT30 150 µg/ml; a gift from Taligen Therapeutics), or a FH concentrate from human plasma (230 µg/ml; LFB Biotechnologies), or rADAMTS13 (4 µg/ml; R&D Systems), or eculizumab (100 µg/ml, Alexion), or an anti-VWF antibody (100 µg/ml, Dako), or an irrelevant antibody (normal rabbit IgG, 100 µg/ml, Santa Cruz Biotechnology), or the serine protease
inhibitor aprotinin (200 KIU, Sigma Aldrich srl) were added to serum (before dilution with test medium) that was incubated with HMEC-1 for 4 hr.

In each experiment, serum from a healthy control was tested in parallel with cTTP serum; the background (non-specific staining, usually less than 100 pixel²/field) was defined in a sample run in parallel of HMEC-1 cells incubated with test medium alone without serum and then processed with the same antibodies. The effect of 10 min exposure of HMEC-1 to 10 µM ADP on C3 and VWF deposition / expression, and VWF-C3 costaining was evaluated by rabbit polyclonal anti-human VWF antibody (Dako) followed by a Cy3-conjugated secondary antibody and by mouse anti-human C3 monoclonal antibody (Abbiotec) followed by a FITC-conjugated secondary antibody.

C5aR expression on HMEC-1, either resting or activated with 10 µM ADP for 10 min, was analyzed using a mouse anti-human CD88 (anti-C5aR, AbD Serotec) followed by a Cy3-conjugated secondary antibody. The effect of C5a on VWF and thrombomodulin staining was evaluated by challenging HMEC-1 with 200 ng/ml of C5a (Complement Technology Inc.) for 10 min. The cells were then treated with rabbit polyclonal anti-human VWF antibody (Dako) or mouse anti human thrombomodulin (R&D System) followed by the specific Cy3-conjugated secondary antibodies.

Negative controls run in parallel with isotype-matched antibodies showed no staining.

A confocal inverted laser microscope (LSM 510 Meta, Zeiss) was used for the acquisition of the fluorescent staining on the endothelial cell surface. Fifteen fields, systematically digitized along the surface, were acquired using a computer-based image analysis system. The area occupied by the fluorescent staining was evaluated by automatic edge detection using built-in specific functions of the software Image J (NIH, Bethesda, MD), and expressed as pixel² per field analyzed. For each sample the mean of the 15 fields was calculated. In order to obtain unbiased quantification of
immunofluorescence stainings, the technician who routinely acquired the images from the microscope and quantified the fluorescent signal of each of them, did not know details about serum samples used, such as phase of disease, mutations or treatment.

2.7 Computational studies on FB, VWF and ADAMTS13 structures

2.7.1 In silico comparison between von Willebrand type A domain of Factor B and A2 domain of VWF

Published 3D crystal structures of VWA domain of FB and A2 domain of VWF were collected from the free open-source Protein Data Bank (PDB) website. 1Q0P is the PDB reference number for the crystal of A domain of Factor B (VWA, FB), while 3ZQK is the PDB reference number for the crystal of Von Willebrand Factor A2 domain (VWF-A2), the latter obtained in the presence of calcium. Aminoacidic sequence alignment of the two domains was performed by UNIPROT (http://www.uniprot.org/align). The results of the alignment are expressed as a degree of identity, calculated as the ratio between the number of identical aminoacids and the length of the considered sequence x 100, and display the following symbols denoting the scale of conservation observed in each column (refering to Fig. 3-7 in Results):

- An * (asterisk) indicates positions which have a single, fully conserved residue.
- A : (colon) indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix.
- A . (period) indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix.
2.7.2 Three dimensional structural model of ADAMTS13

Structural models of ADAMTS13 were designed using I-TASSER software (http://zhanglab.ccmb.med.umich.edu/I-TASSER) (Zhang 2008; Roy et al. 2010) (Fig. 2-1).

Aminoacids 75-1427 (protein sequence without signal peptide and propeptide) of human ADAMTS13 were submitted to I-TASSER server and the structural models of the sequence were built based on the alignment with the structural coordinates of proteins chosen as threading templates, reported in Table 2-1. The best structural prediction of ADAMTS13 obtained a C-score of -0.73 and an expected TM-score of 0.62±0.14, indicating a model of correct topology and folding. C-score is typically in
the range of \([-5\) to \(2]\), where a C-score of higher value signifies a model with a high confidence and vice-versa. In general, models with C-score \(>-1.5\) have a correct fold (Roy et al. 2010).

Table 2-1. Templates used for the reconstruction of the tridimensional model of human ADAMTS13.

<table>
<thead>
<tr>
<th>Rank</th>
<th>PDB-ID</th>
<th>Ident1</th>
<th>Ident2</th>
<th>Z-score</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3gawA</td>
<td>0.12</td>
<td>0.18</td>
<td>2.49</td>
<td>Solution structure of Human Complement Factor H in 250 mM NaCl buffer</td>
</tr>
<tr>
<td>2</td>
<td>2eroA</td>
<td>0.16</td>
<td>0.06</td>
<td>2.13</td>
<td>Crystal structure of vascular apoptosis-inducing protein-1 (orthorhombic crystal form)</td>
</tr>
<tr>
<td>3</td>
<td>3ghnA</td>
<td>0.99</td>
<td>0.27</td>
<td>2.63</td>
<td>Crystal structure of the exosite-containing fragment of human ADAMTS13 (form-2)</td>
</tr>
<tr>
<td>4</td>
<td>3ghmA</td>
<td>1.00</td>
<td>0.27</td>
<td>3.71</td>
<td>Crystal structure of the exosite-containing fragment of human ADAMTS13 (form-1)</td>
</tr>
<tr>
<td>5</td>
<td>2rjqA</td>
<td>0.30</td>
<td>0.06</td>
<td>2.34</td>
<td>Crystal structure of ADAMTS5 with inhibitor bound</td>
</tr>
<tr>
<td>6</td>
<td>3gauA</td>
<td>0.10</td>
<td>0.18</td>
<td>6.14</td>
<td>Solution structure of Human Complement Factor H in 50 mM NaCl buffer</td>
</tr>
<tr>
<td>7</td>
<td>2rjqA</td>
<td>0.30</td>
<td>0.06</td>
<td>3.53</td>
<td>Crystal structure of ADAMTS5 with inhibitor bound</td>
</tr>
<tr>
<td>8</td>
<td>2v4bA</td>
<td>0.30</td>
<td>0.07</td>
<td>3.85</td>
<td>Crystal structure of human ADAMTS1 catalytic domain and Cysteine-rich domain (apo-form)</td>
</tr>
<tr>
<td>9</td>
<td>3gavA</td>
<td>0.12</td>
<td>0.18</td>
<td>2.24</td>
<td>Solution structure of Human Complement Factor H in 137 mM NaCl buffer</td>
</tr>
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<td>10</td>
<td>3q2gA</td>
<td>0.30</td>
<td>0.07</td>
<td>1.81</td>
<td>ADAMTS1 in complex with a novel N-hydroxyformamide inhibitors</td>
</tr>
</tbody>
</table>

Template ranks represent the top ten threading templates used by I-TASSER; \(^{2}\)PDB-ID is the ProteinDataBank (PDB) reference number of the template proteins; \(^{3}\)Ident1 is the percentage sequence identity of the templates in the threading-aligned region with the query sequence; \(^{4}\)Ident2 is the percentage sequence identity of the whole template chains with the query sequence; \(^{5}\)Cov. represents the coverage of the threading alignment and is equal to the number of aligned residues divided by the length of query sequence; \(^{6}\)Norm. Zscore is the normalized Z-score of the threading alignments (>1 reflects a good alignment and vice versa); \(^{7}\)The name of the crystal structures used as template.

The model was then manipulated using the program PyMOL (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrodinger, LLC).

### 2.7.3 In silico prediction of ADAMTS13-FB interaction

The computational docking of target protein structures was perfomed by the fully automated web-based program ClusPro 2.0 (https://cluspro.bu.edu/login.php) (Comeau et al. 2004; Comeau et al. 2004) (Fig. 2-2).
ClusPro is one of the most efficient software for the automatic prediction of protein-protein docking. ClusPro is a web server developed by the laboratory of Structural Bioinformatics of the Boston University. It provides many different options for molecular docking because good results go hand-in-hand with experimental knowledge of the complex.

Models are ranked by cluster size, which is how the models coming out of Cluspro, and which is the most efficiently used method in Critical Assessment of Predicted Interactions (CAPRI) and on various protein docking benchmarks. The score results as a translation of the ligand rotations on a grid relative to the receptor, and refers to binding site RMSDs (root-mean-square deviation of atomic positions, the measure of the average distance between the atoms of the ligand relative to the receptor). The ligand position with the most "neighbors" in 9 angstroms becomes a cluster center (Center in Figure 2-2. Screenshot of ClusPro web interface.)
the Table 2-2). The “stability” of a cluster was then evaluated by testing the lowest local energy minimum in Monte Carlo simulation (Lowest Energy in the Table 2-1).

Coordinate files of ADAMTS13 structural model, obtained with I-TASSER software, and PDB code of FB structure (2OK5) were uploaded through ClusPro’s web interface as Receptor and Ligand, respectively. The docking algorithms evaluated billions of putative complexes, retaining a preset number with favourable surface complementarities, and good electrostatic and desolvation free energies. Since it is unknown of which forces govern ADAMTS13-FB complex, docking results have been chosen by balanced coefficients, which took into consideration electrostatic, hydrophobic and van der Waals forces concurrently (Table 2-2).

### Table 2-2. List of putative ADAMTS13-FB complexes.

<table>
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<tr>
<th>Cluster</th>
<th>Members</th>
<th>Representative</th>
<th>Weighted Score</th>
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</thead>
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<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>34</td>
<td>Center</td>
<td>-1052.1</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>-1221.1</td>
</tr>
<tr>
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<td>31</td>
<td>Center</td>
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<td>-1041.5</td>
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<td></td>
<td></td>
<td>Lowest Energy</td>
<td>-1114.3</td>
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<tr>
<td>6</td>
<td>17</td>
<td>Center</td>
<td>-1125.7</td>
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<td>7</td>
<td>15</td>
<td>Center</td>
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<tr>
<td>8</td>
<td>15</td>
<td>Center</td>
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<tr>
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<td>12</td>
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<tr>
<td>13</td>
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<td>Center</td>
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<tr>
<td>14</td>
<td>11</td>
<td>Center</td>
<td>-1026.0</td>
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2. METHODS

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<td>26</td>
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</table>

The models were manipulated using the Educational-use-only program PyMOL (https://www.pymol.org/, The PyMOL Molecular Graphics System, Version 1.2r3pre). PyMOL is a molecular visualization system on an open source foundation, maintained and distributed by Schrödinger.

### 2.8 rADAMTS13-FB interaction

#### 2.8.1 SPR binding assays

In order to study whether ADAMTS13 interacts with FB, Surface Plasmon Resonance (SPR) experiments were performed using a BIAcore 2000 system in collaboration with Dr. Marco Gobbi and coworkers of the laboratory of Pharmacodynamics and Pharmacokinetics in “Mario Negri” Institute in Milano. Commercial human rADAMTS13 (R&D Systems) was diluted in sodium acetate pH 5.0 and immobilized
onto a flow channel surface of a Proteon GLC sensor chip (Biorad) by amine coupling chemistry. Three different concentrations (1, 2 and 4 µM) of purified human FB (Complement Technology Inc.) were flowed over immobilized rADAMTS13 at a flow rate of 30 ml/min. To evaluate the binding specificity, a single concentration (1µM) of purified human C3b or C3 (Complement Technology Inc.) was injected over immobilized rADAMTS13. All the assays were performed at 25°C. The sensorgrams were normalized to a base-line value of 0. The signal observed in the surfaces immobilizing the protein was corrected by subtracting the nonspecific response observed in the reference surface.

2.8.2 ELISA binding assays

Different amounts of FB 50-0 nM (dilution 1:2) were incubated over rADAMTS13 coated wells (2.5 ug/mL), and the ADAMTS13-FB complexes were detected by goat anti-human FB antibody (1:10000, Quidel) followed by HRP-conjugated anti-goat antibody (1:40000, Sigma Aldrich srl). Colour was developed using TMB substrate (3,3′,5,5′-Tetramethylbenzidine, Bethyl) and stopped with H₂SO₄ 2M, and absorbance was measured at 450 nm. Each reaction was performed in duplicate and the OD values averaged. Bovine and human albumin serum (BSA and HSA, Sigma Aldrich srl) were considered as irrelevant negative controls.
2. METHODS

2.9 rADAMTS13 cleavage assays

2.9.1 Cleavage of FB, FD and C3b

A mixture of FB (27 nM or 5.6 nM, Complement Technology Inc.) or C3b (14 nM, Complement Technology Inc.) or FD (110 nM, Complement Technology Inc.) was incubated in the presence of different concentrations of rADAMTS13 (R&D Systems; 0.56 nM, 100 ng/ml; 0.168 nM, 30 ng/ml; 1.4 nM, 250 ng/ml; 2.8 nM, 500 ng/ml; 5.6 nM, 1000 ng/ml), for 2hr at 37°C in 5 mM Tris-HCl buffer containing 1.5 M urea and 3 mM BaCl₂, pH 8. For FB cleavage assay, ADAMTS13 concentrations range from the physiological molar ratio 330:1=FB:ADAMTS13 to non-physiological 10:1. For C3b and FD cleavage assays, ADAMTS13 amount correspond to the concentration that provides the cleavage of 100 ng of rVWF-A1A2A3. The reactions were stopped by adding Na₂SO₄ and the cleavage products were subjected to 10% SDS-PAGE and visualized by Silver Staining (Sigma Aldrich srl) or western blot (WB) with rabbit anti-FB antibody (1:500, ATLAS) followed by HRP conjugated anti-rabbit antibody (1:30000; Vector Lab.) and ECL system (Amersham).

2.9.2 Cleavage of rVWF fragment

Recombinant ADAMTS13 activity was evaluated by cleavage of a rVWF fragment. In particular, a mixture of purified recombinant VWF fragment comprising A1-A2-A3 domains (residues 1271-1874) of VWF (rVWF-A1A2A3) (Donadelli et al. 2006) was incubated and processed as previously described (Remuzzi et al. 2002). Briefly, rVWF-A1A2A3 (10 ng/ml) was mixed with 0.2 nM of rADAMTS13 (R&D system) and incubated for 2 hr at 37°C in the presence of 5 mM tris buffer containing 1.5 M urea and
2. METHODS

3 mM BaCl₂, pH 8.0. The cleavage was stopped by adding 5.7 µl of 0.825 M Na₂SO₄. Fifteen µl of the reaction were subjected to 10% SDS-PAGE and transferred to a PVDF membrane (Biorad). The membrane was then stained with the primary mouse monoclonal antibody directed against the carboxy-terminal of the rVWF-A1A2A3 (dilution 1:500, kindly provided by Prof. Z. M. Ruggeri, The Scripps Research Institute, La Jolla, CA), followed by the secondary HRP-conjugated rabbit anti-mouse IgG antibody (1:2000, Zymed). Signals were developed using ECL chemiluminescence detection system (Amersham). The amount of proteolytic fragment formed (about 30 kDa) from the cleavage of the rVWF A1-A2-A3 (80 kDa) by rADAMTS13. The results were expressed as the mean of n=3 experiments.

2.10 C3 proconvertase and C3 convertase formation assays

2.10.1 C3bBb(Mn²⁺) C3 proconvertase and C3bBb(Mg²⁺) C3 convertase formation in microplate/WB assays

Novel user-friendly methods based on combined microplate and WB techniques were set up to selectively generate and detect AP C3bB proconvertase and C3bBb convertase (Marinozzi et al. 2014). The setup is explained in details as follows.

For the generation of C3bB and C3bBb complexes in vitro, an ELISA assay was first tested as described previously by Hourcade et al. (Hourcade et al. 1995). ELISA plates were coated with 3 µg/ml C3b (Complement Technology Inc.) in PBS by overnight incubation at 4°C, blocked with 1% BSA, 0.1% Tween20 in PBS for 1h at 37°C, and washed with wash buffer (8.1 mM Na₂HPO₄, 1.8 mM NaH₂PO₄, 0.1% Tween20 and 25 mM NaCl) supplemented with 2 mM NiCl₂. C3bB and C3bBb complexes were formed by incubating C3b-coated wells at 37° C for 2hr with different concentrations of FB.
(Complement Technology Inc., 0–500 ng/ml, 1:2) in the absence or in the presence of FD (Complement Technology Inc., 25 ng/ml), respectively, both diluted in assay buffer (8.1 mM \( \text{Na}_2\text{HPO}_4 \), 1.8 mM \( \text{NaH}_2\text{PO}_4 \), 4% BSA, 0.1% Tween20 and 75 mM NaCl) containing 2 mM NiCl₂. After washes, the C3bB and C3bBb complexes were detected by ELISA using polyclonal goat anti-human FB antibody (1:10000, Quidel) followed by HRP-conjugated anti-goat antibody (1:40000, Sigma Aldrich srl), both diluted in antibody buffer (8.1 mM \( \text{Na}_2\text{HPO}_4 \), 1.8 mM \( \text{NaH}_2\text{PO}_4 \), 4% BSA, 0.1% Tween20 and 25 mM NaCl) supplemented with 2 mM NiCl₂. Colour was developed using TMB substrate and stopped with \( \text{H}_2\text{SO}_4 \) 2 M, and absorbance was measured at 450 nm. Each reaction was performed in duplicate and the OD values averaged. As shown in Fig. 2-3A, the ELISA curves from the reaction of coated C3b with either FB alone or FB plus FD showed dose-dependent superimposable profiles. In parallel experiments, the complexes originated on wells were detached from microplate by incubation with 10 mM EDTA and 1% SDS for 1hr at room temperature, subjected to 10% SDS-PAGE, transferred by electroblotting to a PVDF membrane (Biorad), and analyzed on WB with polyclonal rabbit anti-human FB antibody (1:500, Atlas) followed by HRP-conjugated anti-rabbit antibody (1:30000, Vector Laboratories) and the ECL chemiluminescence detection system (Amersham). C3bB and C3bBb formation was evaluated by the visualization by WB of the B band (93 kDa) and the Bb band (60 kDa), respectively. When the complexes that formed in the presence or in the absence of FD were detached from the wells both B and Bb bands were found in both conditions (Fig. 2-3A, right side) indicating that this experimental protocol could not selectively discriminate between C3bB and C3bBb complexes formation. Indeed, in the presence of FD only a portion of C3bB was converted to C3bBb. Conversely, in the absence of FD, some C3bBb also formed besides C3bB, possibly due to FD contamination in commercial FB
plasma-purified protein (Harris et al. 2005). As a result, this protocol, based on Ni\textsuperscript{2+} ions, was abandoned due to the simultaneous stabilization of both enzymes.

In an effort to specifically generate either C3bB or C3bBb complexes in an in vitro assay, selective stabilization abilities of different divalent cations have been taken into account. Hourcade and Mitchell (Hourcade and Mitchell 2011) documented that Mn\textsuperscript{2+} stabilizes C3bB in a form susceptible to FD cleavage, but the C3bBb(Mn\textsuperscript{2+}), once formed, is highly unstable and dissociates immediately, so that only the C3bB(Mn\textsuperscript{2+}) complex could be detected. At variance, Mg\textsuperscript{2+} stabilizes C3bBb but not the proenzyme C3bB (Fishelson and Muller-Eberhard 1982). On the basis of this evidence, the ELISA described above was repeated replacing Ni\textsuperscript{2+} with Mn\textsuperscript{2+} or Mg\textsuperscript{2+} ions to stabilize either the C3bB or the C3bBb, respectively. ELISA plates were coated with C3b, blocked, and then washed with wash buffer supplemented with 2 mM MnCl\textsubscript{2} or 10 mM MgCl\textsubscript{2} for C3bB or C3bBb, respectively. C3bB(Mn\textsuperscript{2+}) complexes were assembled by incubating C3b-coated wells at 37 °C for 2 hr with FB (500 ng/ml), diluted in assay buffer containing 2 mM MnCl\textsubscript{2}. C3bBb(Mg\textsuperscript{2+}) complexes were formed by incubating C3b-coated wells at 25 °C for 30 min with FB (500 ng/ml) and FD (25 ng/ml), in assay buffer with 10 mM MgCl\textsubscript{2}. In parallel with ELISA, the complexes were detached from microplate and analyzed on WB as above. As shown in Fig. 2-3, B and C, in either condition the amount of the complexes formed was too small to be detected by ELISA (left panels), likely due to degradation of the C3bB(Mn\textsuperscript{2+}) and the C3bBb(Mg\textsuperscript{2+}) during post-reaction incubation times with primary and secondary antibodies. At variance, when we detached the complexes immediately at the end of the reaction, and analyzed them on WB, the B and Bb bands of C3bB(Mn\textsuperscript{2+}) and C3bBb(Mg\textsuperscript{2+}), respectively, could be visualized well (Fig. 2-3, B and C, right panels). It is relevant that only the B band of C3bB was visualized in the product of the reaction with Mn\textsuperscript{2+}, even when FD
was added to the incubation mixture. Similarly, only the Bb band of C3bBb was detected in the reaction with Mg$^{2+}$ and FD.

![Figure 2-3. Detection of C3bB C3 proconvertase and C3bBb C3 convertase complex formation by ELISA and microplate/WB assays.](image)

A. Detection of Ni$^{2+}$-dependent C3bB and C3bBb. C3b-coated microtiter wells were incubated with increasing amounts of FB (0–500 ng/ml) and 2 mM NiCl$_2$ in the absence or in the presence of 25 ng/ml FD and analyzed by ELISA by using an anti-FB antibody (left side). The product of the reaction with 500 ng/ml FB, once detached from the wells, was also analyzed by WB with an anti-FB antibody (right side). B and C. Selective formation of C3bB and C3bBb with Mn$^{2+}$ and Mg$^{2+}$, respectively. C3b-coated wells were incubated with 500 ng/ml FB in the absence or in the presence of 25 ng/ml FD, 2 mM MnCl$_2$, or 10 mM MgCl$_2$ to obtain C3bB(Mn$^{2+}$) and C3bBb(Mg$^{2+}$), respectively. Complexes were detected both by ELISA (left side) and WB (right side). C3bB and C3bBb formation was evaluated by the visualization of the B band (93 kDa) and the Bb band (60 kDa), respectively. One representative experiment of three is shown.
Then, time course of C3bB(Mn$^{2+}$) and C3bBb(Mg$^{2+}$) complexes formation was analyzed, and the two novel microplate/WB procedures were optimized. Microplates were coated with C3b, blocked and washed as above. C3bB(Mn$^{2+}$) complexes were assembled by incubating C3b-coated wells at 37 °C for 1, 2, 4 and 8hr with FB (1000 ng/ml, 10.8 nM), diluted in assay buffer containing 2 mM MnCl$_2$ and 4 % BSA. C3bBb(Mg$^{2+}$) complexes were formed by incubating C3b-coated wells at 25 °C for 5, 10, 30 and 45 min with FB (1000 ng/ml, 10.8 nM) and FD (5 ng/ml, 0.22 nM; physiological FB/FD molar ratio of 50:1), in assay buffer with 10 mM MgCl$_2$ and 0.5 % BSA. After washes, the complexes were detached from microtiter wells and analyzed on WB as described in the previous section. C3bB and C3bBb formation was evaluated by the visualization on WB of the B (93 KDa) and Bb (60 KDa) bands, respectively, and estimated by densitometry using NIH Software ImageJ (NIH, USA). As shown in Fig. 2-4A, the B band of C3bB(Mn$^{2+}$) was clearly detected after 2hr of incubation and was still evident at 8hr independently of the presence of FD, further confirming the specificity of the test for C3bB assembly. The kinetics of C3bBb(Mg$^{2+}$) (Fig. 2-4B) formation was faster than that of C3bB(Mn$^{2+}$), and indeed the Bb band was already well detected after 10 min of incubation and the intensity further increased at 45 min of incubation.
2. METHODS

In the attempt to evaluate ADAMTS13 effect on C3bB C3 proconvertase and C3bBb C3 convertase formation, these two novel microplate/WB assays were used. Microtiter wells were coated with 3 µg/ml C3b in PBS by overnight incubation at 4°C, blocked with 1 % BSA, 0.1 % Tween20 in PBS for 1h at 37 °C, and washed with wash buffer supplemented with 2 mM MnCl₂ or 10 mM MgCl₂. C3bB(Mn²⁺) complexes were formed by incubating C3b-coated wells at 37 °C for 2hr with FB (1000 ng/ml, 10.8 nM), in the absence or in the presence of rADAMTS13 (R&D Systems; 0.04 nM, 6.6 ng/ml; 0.11 nM, 19.8 ng/ml; 0.55 nM, 99 ng/ml), diluted in the assay buffer containing 2 mM MnCl₂ and 4 % BSA. C3bBb(Mg²⁺) complexes were formed by incubating C3b-coated wells at 25 °C for 10 min with FB (1000 ng/ml, 10.8 mM) and FD (5 ng/ml, 0.22 mM), in the absence or in the presence of rADAMTS13 (0.04 nM, 6.6 ng/ml; 0.11 nM, 19.8 ng/ml; 0.55 nM, 99 ng/ml), both diluted in the assay buffer with 10 mM MgCl₂ and 0.5 % BSA. After washes, the protein complexes were detached from microtiter wells and analysed by WB as above.

Figure 2-4. Time course of selective C3bB(Mn²⁺) C3 proconvertase assembly (A) and C3bBb(Mg²⁺) C3 convertase formation (B) detected by microplate/WB assays. C3bB(Mn²⁺) and C3bBb(Mg²⁺) complexes were obtained by incubating C3b-coated wells at time points indicated with 1000 ng/ml FB in the presence or in the absence of 5 ng/ml FD and 2 mM MnCl₂ at 37 °C or 10 mM MgCl₂ at 25 °C, respectively. The amount of C3b assembled and of C3bBb formed was calculated as the intensity of B (93 kDa) and Bb (60 kDa) bands, respectively, and results are reported in the bottom graphs as pixel²*10⁶. Results of a representative microplate/WB experiment of n = 3 are shown.
2. METHODS

2.10.2 Spontaneous and FH-mediated decay of C3bBb(Mn²⁺) C3 proconvertase and C3bBb(Mg²⁺) C3 convertase in microplate/WB assays

In order to study spontaneous or FH-mediated decay of C3 proconvertase and C3 convertase over time, C3bB(Mn²⁺) and C3bBb(Mg²⁺) were allowed to form for 2 hr at 37°C and 10 min at 25°C, respectively as described above, then were washed and incubated with selective assay buffers in the presence or in the absence of FH (2640 ng/ml; molar ratio FB:FH=1:1.6) for the following time periods: C3bB(Mn²⁺), 30, 60, 120 and 240 min; C3bBb(Mg²⁺), 2, 4, 8 and 16 min.

The intensity of the B bands of C3bB(Mn²⁺) did not change over 240 min decay vs. baseline, either in the absence or in the presence of FH, indicating that in these conditions FH did not displace FB from C3b (Fig. 2-5A). Conversely, Bb bands of C3bBb(Mg²⁺) disappeared in a time-dependent manner, and faster in the presence of FH (Fig. 2-5B) confirming that FH is very efficient in Bb displacement from C3b.

To investigate the effect of VWF on FH decay accelerating activity on C3 convertase, rVWF-A1A2A3 was tested in this new microplate/WB assay. C3bBb(Mg²⁺) complexes were obtained as described above by incubating C3b-coated wells at 25 °C for 10 min with FB (1000 ng/ml, 10.8 mM) and FD (5 ng/ml, 0.22 mM), in the absence or in the presence of rVWF-A1A2A3 (17.6 nM, 1408 ng/ml; 0.176 nM, 14.08 ng/ml), diluted in the assay buffer with 10 mM MgCl₂ and 0.5 % BSA. After washes, the protein complexes were incubated at 25 °C for 4 min with assay buffer alone in the presence or in the absence of FH (17.6 nM, 2640 ng/ml) and rVWF-A1A2A3 (17.6 nM, 1408 ng/ml). At the end of incubation, the complexes were detached from microtiter wells and analysed by WB as above.
2. METHODS

2.10.3 C3 proconvertase and C3 convertase formation in SPR assays

SPR experiments were carried out in collaboration with Dr. Marco Gobbi, as mentioned before, by using BIAcore 2000 (Biorad). Purified human C3b (Complement Technology Inc.) was diluted to 30 µg/ml in sodium acetate pH 5.0 and immobilized using amine coupling chemistry over a surface of a GLC sensor chip (Biorad), as previously described (Jokiranta et al. 2001; Harris et al. 2005), to obtain a final immobilization level of about 7000 RU (1RU = 1 pg/mm²). A reference surface was always prepared in parallel by performing the same immobilization procedure but without the addition of any protein. The signal observed in the surfaces immobilizing the protein was corrected by subtracting the nonspecific response observed in the

Figure 2-5. Spontaneous and FH-mediated decay of selective C3bB(Mn²⁺) C3 proconvertase (A) and C3bBb(Mg²⁺) C3 convertase (B) detected by microplate/WB assays.

C3bB(Mn²⁺) and C3bBb(Mg²⁺) complexes were obtained by incubating C3b-coated wells with 1000 ng/ml FB in the absence or in the presence of 5 ng/ml FD and 2 mM MnCl₂ or 10 mM MgCl₂ for 2 h at 37 °C or 10 min at 25 °C, respectively. Time-course of spontaneous and FH-mediated decay of C3bB(Mn²⁺) or C3bBb(Mg²⁺) was evaluated by further incubation with buffer alone or with 2640 ng/ml FH for 30, 60, 120 and 240 min, or 2, 4, 8 and 16 min, respectively. The amount of C3bB and C3bBb formed was calculated as the densitometry of the B (93 kDa) and Bb (60 kDa) bands and results are reported in the bottom graphs as pixel² * 10⁶. The percentage of residual B and Bb bands was calculated as the ratio of the densities (in Pixel²) of each band after decay and the corresponding baseline band density before decay x 100. Results of a representative microplate/WB experiment of n = 3 are shown.

2.10.3 C3 proconvertase and C3 convertase formation in SPR assays

SPR experiments were carried out in collaboration with Dr. Marco Gobbi, as mentioned before, by using BIAcore 2000 (Biorad). Purified human C3b (Complement Technology Inc.) was diluted to 30 µg/ml in sodium acetate pH 5.0 and immobilized using amine coupling chemistry over a surface of a GLC sensor chip (Biorad), as previously described (Jokiranta et al. 2001; Harris et al. 2005), to obtain a final immobilization level of about 7000 RU (1RU = 1 pg/mm²). A reference surface was always prepared in parallel by performing the same immobilization procedure but without the addition of any protein. The signal observed in the surfaces immobilizing the protein was corrected by subtracting the nonspecific response observed in the
reference surface. After rotation of the microfluidic system, FB (200 nM, Complement Technology Inc.) was injected in the presence or in the absence of FD (4 nM, Complement Technology Inc.) and rADAMTS13 (1 nM, R&D Systems) over the immobilized C3b for 180 s at a flow rate of 30 ml/min. All of these assays were done at 25°C. The sensorgrams were normalized to a base-line value of 0.

2. METHODS

2.11 FH cofactor activity for FI-mediated proteolysis of fluid phase C3b

The fluid-phase cofactor activities of FH and VWF were determined in a C3b proteolytic assay using purified proteins. C3b and FI were from Complement Technology Inc.; FH was purchased from Merck; rVWF was a gift from Baxter; LFB-VWF was from LFB Biotechnologies (Mazurier 1998); Emoclot-VWF was from Kedrion S.p.A. (Peyvandi et al. 2013); rA2-VWF was from R&Dsystems.

In brief, C3b, FH, VWF and FI were mixed in 10 mM HEPES (pH 7.5), 150 mM NaCl, and 0.02 % Tween20. Final concentrations were: C3b 0.42 µM, FH 31.3 nM, FI 43 nM, and VWF 0.313 nM (1X, physiological molar ratio with FH) or 3.13 nM (10X) or 156.5 nM (500X). Molarities were calculated using the following masses: C3b, 185 KDa; FH, 155 KDa; FI, 88 KDa; VWF, 250 KDa; A2-VWF, 20 KDa. Mixtures were incubated at 37°C in a water bath for 10 min or 1 min, in the absence or in the presence of FH respectively. The reactions were stopped by the addition of 4x Laemmlli protein sample buffer for SDS-PAGE (Biorad) supplemented with 10% 2-mercaptoethanol (Sigma Aldrich srl). Samples were analyzed in 10% SDS-PAGE under reducing conditions. Gels were stained with ProteoSilver Stain Kit (Sigma Aldrich srl), and proteolysis of
C3b was determined by analyzing the disappearing of the α' chain (110 KDa) band and the appearance of the α65 KDa and α43 KDa bands.

### 2.12 C3b-VWF interaction

#### 2.12.1 SPR binding assays

SPR experiments were carried out in collaboration with Dr. Marco Gobbi, as mentioned before, by using BIAcore 2000. Recombinant multimeric human VWF (rVWF, kindly provided by Baxter) was reduced (20 min at 56 °C with 65 mM dithiothreitol, DTT) and then covalently immobilized onto a flow channel surface of a Proteon GLC sensor chip (Biorad), using amine coupling chemistry. Three different concentrations (1µM, 500 nM or 100 nM) of purified human C3b (Complement Technology Inc.) were injected over immobilized rVWF at the rate of 30 µl/min for 10 min (associating phase). The dissociating phase was evaluated in the following 10 min. The running buffer, also used to dilute analytes, was phosphate-buffered saline pH 7.4, 0.005% Tween20. A reference channel was always prepared in parallel by the same immobilization procedure without the addition of the ligand. The signal observed in the surfaces immobilizing the protein was corrected by subtracting the nonspecific response observed in the reference surface. All the assays were performed at 37 °C.

#### 2.12.2 ELISA binding assays

Microtiter wells were coated with 5 µg/ml rVWF either multimeric or monomeric (reduced with DTT 30 mM) or purified rVWF-A1A2A3 domains (a kind gift of Prof. Z.M. Ruggeri, The Scripps Research Institute, La Jolla, Ca) rVWF-A1 (U-Protein
Express BV), rVWF-A2 (R&D Systems), rA3-VWF (U-Protein Express BV) in PBS by overnight incubation at 4°C. Then, coated wells were blocked with 1% BSA for 1hr at R.T., and washed with PBS supplemented with Tween20 0.1% and MgCl₂ 10 mM. Different amounts of C3b ranging from 0.16 µg/ml to 50 µg/ml were incubated with coated wells for 2hr at 37°C. Protein complexes were detected by mouse anti-human C3 antibody (1:16000, Abbiotec) followed by HRP-conjugated anti-mouse antibody (1:10000, Zymed). Colour was developed using TMB substrate (Bethyl), stopped with H₂SO₄ 2 M, and absorbance was measured at 450 nm. Each reaction was performed in duplicate and the OD values averaged. BSA was considered as an irrelevant-negative control.

In additional experiments microtiter wells were coated with 5 µg/ml C3b (Calbiochem) in PBS by overnight incubation at 4°C, blocked with 1% BSA for 1hr at R.T., and washed with PBS supplemented with Tween20 0.1% and MgCl₂ 10 mM. Different amounts of purified rVWF-A1A2A3 domains of VWF ranging from 25 µg/ml to 0 µg/ml (dilution 1:2 in PBS) were incubated with coated wells for 2hr at 37°C. Protein complexes were detected by HRP-conjugated anti-human VWF antibody (1:4000, Dako). Colour was developed using TMB substrate (Bethyl), stopped with H₂SO₄ 2 M, and absorbance was measured at 450 nm. Each reaction was performed in duplicate and the OD values averaged. BSA was considered as an irrelevant-negative control.

### 2.12.3 In silico prediction of C3b – VWF-A2 interaction

The computational docking between protein structures of C3b and A2 domain of VWF was performed by ClusPro 2.0 as described above in Paragraph 4.7.3.
PDB codes of C3b (2I07) and VWF-A2 (3ZQK:A) structures were uploaded through ClusPro's web interface, and results of protein-protein docking prediction are reported in Table 2-3. Since it is unknown of what forces govern C3b-VWF complex, docking results have been chosen by the balanced coefficients, which took into consideration electrostatic, hydrophobic and van der Waals forces concurrently.

Table 2-3. List of putative C3b – VWF-A2 complexes.

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The models were manipulated using the Educational-use-only program PyMOL (https://www.pymol.org/, The PyMOL Molecular Graphics System, Version 1.2r3pre). PyMOL is a molecular visualization system on an open source foundation, maintained and distributed by Schrödinger.
2. METHODS

2.13 AP C3 Convertase and C5b formation

2.13.1 C3bBb(Mg\(^{2+}\)) C3 convertase and C5b-9 terminal complex formation in the presence of normal human serum or serum from cTTP patients

To obtain C3 convertase formation by using a physiological mixture of complement components, microtiter wells were coated with 5 µg/ml of purified rA1A2A3 wt or rA1A2A3 mutant (a kind gift of Prof. Z.M. Ruggeri, The Scripps Research Institute, La Jolla, Ca), properdin (Complement Technology Inc.) or rA2-VWF (R&D Systems) in PBS by overnight incubation at 4 °C, blocked with 0.5% BSA in PBS for 1hr at R.T., and washed with wash buffer supplemented with 5 mM MgCl\(_2\). C3bBb(Mg\(^{2+}\)) complexes were formed by incubating coated wells at 37 °C for 30 min with 20% NHS (normal human serum, a pool obtained from 15 healthy volunteers) or cTTP serum diluted in PBS containing 5 mM MgCl\(_2\) in the presence or in the absence of 1 mM EGTA. After washes, formed C3bBb(Mg\(^{2+}\)) complexes and bound FH and complement components were detached from wells by incubation with 10 mM EDTA and 1% SDS for 1hr at R.T., subjected to 10% SDS-PAGE, and transferred by electroblotting to a PVDF membrane (Biorad). Proteins were detected with polyclonal rabbit anti-human FB antibody (1:500, ATLAS) or rabbit anti-C3c antibody (1:12000, Dako) both followed by HRP-conjugated anti-rabbit antibody (1:30000, Vector Laboratories Inc.), or with goat anti-FH (1:1000, Calbiochem) followed by HRP-conjugated anti-goat antibody (1:15000, Sigma Aldrich srl) and the ECL chemiluminescence detection system (Amersham). C3bBb formation was evaluated by the visualization by WB of the Bb band (60 kDa) and C3b β chain (75 KDa). In addition, C5b formation was evaluated by using polyclonal rabbit anti-human C5/C5b (1:300, Abcam) antibody, followed by
HRP-conjugated anti-rabbit antibody (1:15000, Vector Laboratories Inc.). C5b was visualized as α’ chain C5b (104 kDa) band. C6 and C9 were detected with rabbit polyclonal anti-C6 (1:1000, ABGENT) or goat-polyclonal anti-C9 (1:250, Origene) antibodies respectively, followed by HRP-conjugated anti-rabbit (1:30000, Vector Laboratories Inc.) and anti-goat (1:10000, Sigma Aldrich srl) antibodies, respectively.

In additional experiments, properdin or C3b or rVWF-A2-coated wells were incubated with NHS in the absence or in the presence of 1 mM EGTA or 150 µg/ml sCR1 or with PBS only. C5b-9 complexes were detected by HRP conjugated anti-C5b-9 antibody (1:100, Quidel) followed by TMB substrate (Bethyl). Color was stopped with H$_2$SO$_4$ 2 M, and absorbance was measured at 450 nm. Each reaction was performed in triplicate and the OD values averaged.

### 2.13.2 Detection of rVWF-A2 coated on plastic wells

The amount of rVWF-A2 that coated plastic wells after incubation with PBS in the absence or in the presence of 1 mM EGTA was detected by ELISA using a rabbit anti-VWF antibody (1:4000, Dako), followed by HRP-conjugated anti-rabbit antibody (1:30000, Vector Laboratories Inc.) and the TMB substrate (Bethyl). Color was stopped with H$_2$SO$_4$ 2 M, and absorbance was measured at 450 nm. Each reaction was performed in triplicate and the OD values averaged.

### 2.14 Thrombus formation under flow condition

The assay was performed as previously described (Morigi et al. 2011) and depicted in Fig. 2-6. HMEC-1 were plated on glass slides and used when confluent. Cells were activated with 10 µM ADP for 10 min and, after washes, exposed for 4 hr to serum from
patients or healthy controls diluted 1:2 with test medium in static conditions. Perfusion of heparinized whole blood (10 UI/ml) obtained from healthy subjects (prelabelled with the fluorescent dye mepacrine 10 µM, that marks platelets) was then performed in a thermostatic flow chamber (37°C) in which one surface of the perfusion channel was a glass slide seeded with a monolayer of endothelial cells at a constant flow rate of 1500 sec$^{-1}$ (60 dynes/cm$^2$). After 3 min, perfusion was stopped, and the slide with the endothelial cell monolayer was dehydrated and fixed in acetone for 20 min. In selected experiments the adhesion test was performed with patients’ sera added with sCR1 (150 µg/ml, as positive control of complement inhibition), or a FH concentrate (230 µg/ml), eculizumab (100 µg/ml), or the C5aR antagonist (W-54011, 1 µM; Calbiochem), or rADAMTS13 (4 µg/ml), or a polyclonal rabbit anti-human VWF antibody (100 µg/ml), or an irrelevant antibody (100 µg/ml) before incubation with HMEC-1. Slides were examined under confocal inverted laser microscopy (LSM 510 Meta, Zeiss) as described above. Fifteen fields for each slide were systematically digitized along the surface and the area covered by thrombi was quantified by Image J (NIH, Bethesda, MD), and expressed as pixel$^2$ per field analyzed. In each experiment, serum from one healthy control was tested in parallel to cTTP serum. For each sample the mean of fifteen fields (excluding the lowest and the highest values) was calculated.
2. METHODS

2.15 Statistics

Results are expressed as mean ± SE. Data were analyzed by ANOVA (two-tailed) or Kruskall-Wallis test. Statistical significance was set at P<0.05.
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3.1 Circulating complement profile in cTTP

20 TTP patients with congenital ADAMTS13 deficiency were recruited among those referred to the International Registry of HUS and TTP coordinated by the Clinical Research Centre for Rare Disease in Ranica (Bergamo) (Table 3-1). Inclusion criteria were: 1) diagnosis of cTTP; 2) homozygous or compound heterozygous ADAMTS13 mutations; 3) availability of plasma and serum samples; 4) absence of genetic abnormalities in aHUS associated complement genes (see Methods 2.1). For each of these patients sex, diagnosis, mutations, phase disease and clinical parameters (platelets count, LDH, Hemoglobin and s-Creatinine levels) referred at the time of sampling were collected and reported in Table 3-1. Undetectable ADAMTS13 activity levels were observed by the collagen binding assay for all recruited patients except for F1518#728, who had 9% activity. Undetectable plasmatic ADAMTS13 antigen levels were observed for all recruited patients except for F48#002, who had 79 ng/ml.
### Table 3-1. Clinical parameters of cTTP patients.

<table>
<thead>
<tr>
<th>Patient's code</th>
<th>Sex</th>
<th>age of onset (years)</th>
<th>ADAMTS13 mutation (mutated domain)</th>
<th>ADAMTS13 activity (%) (CBA)</th>
<th>ADAMTS13 (ng/ml) (ELISA)</th>
<th>Disease phase</th>
<th>Clinical parameters</th>
<th>Renal impairment</th>
</tr>
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<tbody>
<tr>
<td>F760#239</td>
<td>F</td>
<td>21</td>
<td>p.I143F (MP)</td>
<td>p.I143F (MP)</td>
<td>&lt;6%</td>
<td>&lt;62.5</td>
<td>acute rem</td>
<td>platelets (150-400*10^3/µl)</td>
</tr>
<tr>
<td>F1116#239</td>
<td>M</td>
<td>18</td>
<td>p.I143F (MP)</td>
<td>p.I143F (MP)</td>
<td>&lt;6%</td>
<td>&lt;62.5</td>
<td>rem</td>
<td>LDH (186-500 IU/l)</td>
</tr>
<tr>
<td>S1312</td>
<td>F</td>
<td>0.3</td>
<td>p.R257Fasl*13 (MP)</td>
<td>c.1786+1G&gt;A (Spacer)</td>
<td>&lt;6%</td>
<td>&lt;62.5</td>
<td>rem</td>
<td>Hemoglobin (14-18 g/dl)</td>
</tr>
<tr>
<td>F908#226</td>
<td>F</td>
<td>28</td>
<td>p.W28Lfs*111 (SP)</td>
<td>p.R1060W (TSP1-7)</td>
<td>&lt;6%</td>
<td>&lt;62.5</td>
<td>rem</td>
<td>s-Creatinine (0.55-1.25 mg/dl)</td>
</tr>
<tr>
<td>F711#226</td>
<td>F</td>
<td>20</td>
<td>p.W28Lfs*111 (SP)</td>
<td>p.R1060W (TSP1-7)</td>
<td>&lt;6%</td>
<td>&lt;62.5</td>
<td>rem</td>
<td>s-Creatinine (0.55-1.25 mg/dl)</td>
</tr>
<tr>
<td>F1519#728</td>
<td>F</td>
<td>3.5</td>
<td>p.E1382Rfs*6 (CUB2)</td>
<td>p.E1382Rfs*6 (CUB2)</td>
<td>&lt;6%</td>
<td>&lt;62.5</td>
<td>rem</td>
<td>s-Creatinine (0.55-1.25 mg/dl)</td>
</tr>
<tr>
<td>F1518#728</td>
<td>M</td>
<td>35</td>
<td>p.G769#239</td>
<td>p.E1382Rfs*6 (CUB2)</td>
<td>9%</td>
<td>&lt;62.5</td>
<td>n.a.</td>
<td>s-Creatinine (0.55-1.25 mg/dl)</td>
</tr>
<tr>
<td>F1057#403</td>
<td>M</td>
<td>7</td>
<td>p.E1382Rfs*6 (CUB2)</td>
<td>p.E1382Rfs*6 (CUB2)</td>
<td>&lt;6%</td>
<td>&lt;62.5</td>
<td>rem</td>
<td>s-Creatinine (0.55-1.25 mg/dl)</td>
</tr>
<tr>
<td>F1209#403</td>
<td>F</td>
<td>8</td>
<td>p.E1382Rfs*6 (CUB2)</td>
<td>p.E1382Rfs*6 (CUB2)</td>
<td>&lt;6%</td>
<td>&lt;62.5</td>
<td>rem</td>
<td>s-Creatinine (0.55-1.25 mg/dl)</td>
</tr>
<tr>
<td>R002</td>
<td>F</td>
<td>0.25</td>
<td>p.Q429* (TSP1-1)</td>
<td>p.R1095Q (TSP1-8)</td>
<td>&lt;6%</td>
<td>&lt;62.5</td>
<td>rem</td>
<td>s-Creatinine (0.55-1.25 mg/dl)</td>
</tr>
<tr>
<td>R564</td>
<td>F</td>
<td>24</td>
<td>p.D235Y (MP)</td>
<td>p.D235Y (MP)</td>
<td>&lt;6%</td>
<td>&lt;62.5</td>
<td>rem</td>
<td>s-Creatinine (0.55-1.25 mg/dl)</td>
</tr>
<tr>
<td>F1689#922</td>
<td>M</td>
<td>12</td>
<td>p.G769#239</td>
<td>p.E1382Rfs*9 (CUB2)</td>
<td>&lt;6%</td>
<td>n.a.</td>
<td>acute</td>
<td>platelets (150-400*10^3/µl)</td>
</tr>
<tr>
<td>F1628#873</td>
<td>M</td>
<td>birth</td>
<td>p.N1345Tfs*14 (CUB2)</td>
<td>p.N1345Tfs*14 (CUB2)</td>
<td>&lt;6%</td>
<td>n.a.</td>
<td>rem</td>
<td>platelets (150-400*10^3/µl)</td>
</tr>
<tr>
<td>F1627#873</td>
<td>F</td>
<td>1.83</td>
<td>p.N1345Tfs*14 (CUB2)</td>
<td>p.N1345Tfs*14 (CUB2)</td>
<td>&lt;6%</td>
<td>n.a.</td>
<td>rem</td>
<td>platelets (150-400*10^3/µl)</td>
</tr>
<tr>
<td>F1833#985</td>
<td>F</td>
<td>28</td>
<td>p.L120V#1(MP)</td>
<td>p.L120V#1(MP)</td>
<td>&lt;6%</td>
<td>n.a.</td>
<td>acute</td>
<td>platelets (150-400*10^3/µl)</td>
</tr>
<tr>
<td>F48#002</td>
<td>F</td>
<td>23</td>
<td>p.V88M (MP)</td>
<td>p.G1239V (CUB1)</td>
<td>&lt;6%</td>
<td>79</td>
<td>rem</td>
<td>platelets (150-400*10^3/µl)</td>
</tr>
<tr>
<td>R012</td>
<td>M</td>
<td>3</td>
<td>p.R1123C (TSP1-8)</td>
<td>p.R1123C (TSP1-8)</td>
<td>&lt;6%</td>
<td>&lt;62.5</td>
<td>acute rem</td>
<td>platelets (150-400*10^3/µl)</td>
</tr>
<tr>
<td>F101#019</td>
<td>M</td>
<td>35</td>
<td>p.R1219W (CUB1)</td>
<td>p.R1219W (CUB1)</td>
<td>&lt;6%</td>
<td>&lt;62.5</td>
<td>acute</td>
<td>platelets (150-400*10^3/µl)</td>
</tr>
<tr>
<td>F102#019</td>
<td>M</td>
<td>28</td>
<td>p.R1219W (CUB1)</td>
<td>p.R1219W (CUB1)</td>
<td>&lt;6%</td>
<td>&lt;62.5</td>
<td>acute</td>
<td>platelets (150-400*10^3/µl)</td>
</tr>
<tr>
<td>R019</td>
<td>M</td>
<td>5.5</td>
<td>p.G236C (MP)</td>
<td>p.W1016* (TSP1-7)</td>
<td>&lt;6%</td>
<td>&lt;62.5</td>
<td>acute rem</td>
<td>platelets (150-400*10^3/µl)</td>
</tr>
</tbody>
</table>

Rem, remission. For domain abbreviations see Introduction 1.1.6.1. ¹This patient was not studied in remission since he was treated with eculizumab (Pecoraro et al. 2015); ²Limits of normal ranges; ³New mutation; ⁴At the time of sampling. Normal value of s-creatinine for children < 1-5 yrs, 0.3-0.5 mg/dl, children >10 yrs/adults, 0.55-1.25 mg/dl. Increased abnormal range are in bold. ⁵Renal impairment: acute kidney injury, need for dialysis and/or urinary abnormalities (hematuria and/or proteinuria) in patients without previous renal involvement.
Firstly, I attempted to characterize circulating complement profile of all available plasma/serum samples by measuring complement activation markers such as C3, C4 and soluble terminal complement complex (SC5b-9) (Fig. 3-1 and Table 3-2).

During the acute phase we found lower than normal serum C3 level in only 1 out of 9 patients (11%). Lower than normal serum C3 levels were found in 2 out of 17 patients in remission (12%). C4 levels were normal in all patients both during the acute phase and at remission. During the acute phase, plasma levels of SC5b-9 were higher than normal in 57% of patients. At remission, increased SC5b-9 plasma levels were found in 43% of patients.

Mean values of plasma SC5b-9 in cTTP patients both in acute and in remission phases were significantly higher than in healthy controls and comparable to values recorded in aHUS cohort patients referred to the International Registry of HUS and TTP (Fig. 3-2).
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3.2 cTTP serum induced C3 and C5b-9 deposition on microvascular endothelial cells

To evaluate whether complement is activated at endothelial cell level in cTTP, resting or ADP-activated HMEC-1 were incubated with serum from cTTP patients or healthy subjects by using an ex vivo test that my Supervisors and I have recently set up for patients with aHUS (Noris et al. 2014). This new assay is based on the incubation of serum from aHUS patients on ADP-activated HMEC cells for 4hr at 37°C. After washes, C3 and C5b-9 complement deposits were evaluated and quantified by immunostaining with anti-human C3c and anti-human C5b-9 antibodies. In previous
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studies we documented that all sera from aHUS patients induced abnormal C3 and C5b-9 deposits on ADP-activated endothelial cells *ex vivo*. ADP activation was used, before incubation with serum, to mimic an activated/perturbed endothelium (Moake et al. 1988), resulting in exocytosis of P-selectin (Morigi et al. 2011), an adhesive molecule that can bind and activate C3, and VWF (Fig. 3-3).

![Figure 3-3. ADP stimulation on endothelial cells (HMEC-1).](image)
VWF (red) and C3 (green) staining on unstimulated (resting) or ADP-activated HMEC-1 (nuclei: DAPI, blue), not exposed to serum. Original magnification 400x. White scale bar: 20 µm

I found that sera from all cTTP patients studied during the acute phase (n=4; cTTP patients: F1518#728, F1057#403, R002, F1689#922) induced more C3 and C5b-9 deposits (Table 3-2 and Fig. 3-4) than control sera both on resting and on ADP-activated HMEC-1. On resting HMEC-1, C3 and C5b-9 deposits were comparable following exposure to serum from cTTP patients in remission (n=4; selected cTTP patients: F1518#728, F1057#403, R002, R564) or from controls (Fig. 3-4A and C), whereas on activated HMEC-1 cTTP sera taken both during the acute phase (n=4; cTTP
patients as above) and in remission (n=11; cTTP patients: F769#239, F1116#239, F908#226, F711#226, F1518#728, F1057#403, F1209#403, R002, R564, F1833#985, F48#002) induced more C3 and C5b-9 deposits than control sera run in parallel, further documenting that activation of complement proceeded till the terminal pathway (Table 3-2 and Fig. 3-4B and D).

Figure 3-4. C3 and C5b-9 deposition on microvascular endothelial cells (HMEC-1).
Endothelial surface area covered by C3 (A-B) and C5b-9 (C-D) staining after incubation for 4 hr of resting (A and C) or ADP-activated HMEC-1 (B and D) with 50% serum from healthy subjects (ctr, n=15) or cTTP patients (cTTP), studied both during the acute phase (n=4; acute) and at remission (A and C, n=4; B and D, n=11; rem), or from aHUS patients (acute n=3; remission, rem n=4), with or without sCR1 (150 µg/ml). Thereafter, cells were stained with anti-human C3c or anti-human C5b-9 antibodies and complement deposits were analyzed by confocal microscopy. Data are expressed as mean ± SE of area covered by C3 or C5b-9 deposits. °P<0.01, °°P<0.05 vs ctr; *P<0.01, **P<0.05 vs cTTP untreated, ANOVA.

Notably, the intensities of complement deposits on HMEC-1 exposed to cTTP sera were comparable to those on cells incubated with aHUS sera (Fig. 3-4B-D) (Noris et al.)
3. RESULTS

HMEC-1 exposed to ADP alone, without serum, showed no C3 staining (Fig. 3-3). C3 and C5b-9 deposits were blocked by adding the pan complement inhibitor, soluble complement receptor 1 (sCR1) to cTTP serum (Fig. 3-4).

In addition, the AP inhibitors CR2FH (Risitano et al. 2012), and FH (230 µg/ml, which approximately doubled the FH serum concentration (Noris et al. 2014)) significantly reduced cTTP serum-induced C3 deposits (Fig. 3-5A, n=4; selected cTTP patients: F1116#239; F1209#403; R564; F48#002). These findings, together with the observation that sera from cTTP patients, studied during remission phase, and healthy subjects induced comparable C4 staining on ADP-activated HMEC-1 (Fig. 3-5B, n=3; selected cTTP patients: F1116#239; F1057#403; R564), confirm selective activation of the alternative pathway of the complement system by cTTP serum on endothelial cells.

Figure 3-5. C3 and C4 deposition on endothelial cells (HMEC-1), and the effect of some complement inhibitors.

A Effect of inhibitors of the complement alternative pathway on cTTP serum-induced C3 deposits on ADP-activated HMEC-1. Cells were exposed to 50% serum from healthy subjects (ctr, n=4), or from cTTP patients in remission (cTTP, n=4) with or without CR2FH (150 µg/ml) or exogenous FH (230 µg/ml). B. Endothelial surface area covered by C4 staining after incubation of ADP-activated HMEC-1 with 50% serum from healthy subjects (ctr, n=3) or cTTP patients (cTTP, n=3), studied at remission, with or without sCR1 (150 µg/ml). Data are mean ± standard error (SE). ° p<0.0001 vs ctr; * p<0.0001 vs TTP.

In addition, the AP inhibitors CR2FH (Risitano et al. 2012), and FH (230 µg/ml, which approximately doubled the FH serum concentration (Noris et al. 2014)) significantly reduced cTTP serum-induced C3 deposits (Fig. 3-5A, n=4; selected cTTP patients: F1116#239; F1209#403; R564; F48#002). These findings, together with the observation that sera from cTTP patients, studied during remission phase, and healthy subjects induced comparable C4 staining on ADP-activated HMEC-1 (Fig. 3-5B, n=3; selected cTTP patients: F1116#239; F1057#403; R564), confirm selective activation of the alternative pathway of the complement system by cTTP serum on endothelial cells.
3. RESULTS

3.3 Restoration of ADAMTS13 activity prevented cTTP serum-induced C3 and C5b-9 deposition on microvascular endothelial cells

To investigate whether cTTP serum-induced complement activation was related to ADAMTS13 deficiency, recombinant ADAMTS13 (rADAMTS13, at the concentration of 4 µg/ml, which restored ADAMTS13 activity to 100% normal levels, as measured by collagen binding assay, CBA, in cTTP serum) was added to cTTP serum taken in remission. Recombinant ADAMTS13 fully normalized cTTP serum-induced C3 and C5b-9 endothelial deposits (Fig. 3-6, n=3; selected cTTP patients for C3 deposits: F1209#403; R564; F48#002; selected cTTP patients for C5b-9 deposits: F711#226; F1029#403; R564). At variance the same amount of rADAMTS13 did not affect complement deposits in sera from an healthy subject (control serum). Interestingly, rADAMTS13 blocked both C3 and C5b-9 deposition on endothelial cells at the same level of what can be observed with a complement inhibitor.
Together, these findings strongly suggest an important role for ADAMTS13 in the regulation of the alternative pathway of the complement system.

Figure 3-6. Effects of rADAMTS13 on C3 and C5b-9 deposition on endothelial cells.
rADAMTS13 added to cTTP serum normalized C3 and C5b-9 deposits on HMEC-1. ADP-activated HMEC-1 were exposed to 50% serum from healthy subjects (ctr, n=3) or cTTP patients in remission (cTTP, n=3) in the absence or in the presence of rADAMTS13 (4 µg/ml).
For each patient’s sample a serum from an healthy subject was run in parallel to correct for assay variability. Data are expressed as mean ± SE of area covered by C3 or C5b-9 deposits. °P<0.01, vs ctr; *P<0.01 vs cTTP untreated, ANOVA.
Table 3-2. Complement activation markers of cTTP patients.

<table>
<thead>
<tr>
<th>Patient’s code</th>
<th>Sex</th>
<th>ADAMTS13 mutation (mutated domain)</th>
<th>Disease phase</th>
<th>Complement parameters¹</th>
<th>Endothelial complement deposits⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>on 1st allele</td>
<td></td>
<td>serum C3 (83-180 mg/dl)²</td>
<td>C3 deposits (% of control)⁴</td>
</tr>
<tr>
<td></td>
<td></td>
<td>on 2nd allele</td>
<td></td>
<td>serum C4 (15-40 mg/dl)²</td>
<td>C5b-9 deposits (% of control)⁴</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>serum SC5b-9 (127-400 ng/ml)²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F769#239</td>
<td>F</td>
<td>p.I143F (MP)</td>
<td>acute</td>
<td>205</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p.I143F (MP)</td>
<td>rem</td>
<td>139</td>
<td>184% ⁶</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>48</td>
<td>287% ⁶</td>
</tr>
<tr>
<td>F1116#239</td>
<td>M</td>
<td>p.I143F (MP)</td>
<td>rem</td>
<td>99</td>
<td>486% ⁶</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p.I143F (MP)</td>
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<td>15</td>
<td>206% ⁶</td>
</tr>
<tr>
<td>S1312</td>
<td>F</td>
<td>p.R257As*13 (MP)</td>
<td>rem</td>
<td>89</td>
<td>n.d.</td>
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<tr>
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<td>c.1766+1G&gt;A (Spacer)</td>
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<td>15</td>
<td>n.d.</td>
</tr>
<tr>
<td>F9088#226</td>
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<td>p.W28Lfs*111 (SP)</td>
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<td>118</td>
<td>176% ⁶</td>
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<tr>
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<td>p.R1060W (TSP1-7)</td>
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<td>228% ⁶</td>
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<td>F711#226</td>
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<td>p.W28Lfs*111 (SP)</td>
<td>rem</td>
<td>182</td>
<td>199% ⁶</td>
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<td>262% ⁶</td>
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<td>n.d.</td>
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<td>124</td>
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</tr>
<tr>
<td>F1518#728</td>
<td>M</td>
<td>p.G761S (TSP1-3)</td>
<td>acute</td>
<td>133</td>
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<tr>
<td></td>
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<td>p.E1382Rfs*6 (CUB2)</td>
<td>rem</td>
<td>27</td>
<td>668% ⁶</td>
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<td>101</td>
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<td>F1057#403</td>
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<td>224</td>
<td>224% ⁶</td>
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<tr>
<td></td>
<td></td>
<td>p.E1382Rfs*6 (CUB2)</td>
<td>rem</td>
<td>83</td>
<td>1016% ⁶</td>
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<td></td>
<td></td>
<td>1688</td>
<td>245% ⁶</td>
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<tr>
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<td>F</td>
<td>p.E1382Rfs*6 (CUB2)</td>
<td>acute</td>
<td>141</td>
<td>355% ⁶</td>
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<td>p.E1382Rfs*6 (CUB2)</td>
<td>rem</td>
<td>30</td>
<td>216% ⁶</td>
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<td>R002</td>
<td>F</td>
<td>p.Q429+ (TSP1-1)</td>
<td>acute</td>
<td>97</td>
<td>287% ⁶</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p.R1095Q (TSP1-8)</td>
<td>rem</td>
<td>23</td>
<td>403% ⁶</td>
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<td>316% ⁶</td>
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<td>R564</td>
<td>F</td>
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<td>acute</td>
<td>107</td>
<td>242% ⁶</td>
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<td></td>
<td></td>
<td>p.D235Y (MP)</td>
<td>rem</td>
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<tr>
<td>F1689#922</td>
<td>M</td>
<td>p.C1084Y (TSP1-8)</td>
<td>acute</td>
<td>156</td>
<td>244% ⁶</td>
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<td></td>
<td>p.E1351Rfs*9 (CUB2)</td>
<td>rem</td>
<td>29</td>
<td>159% ⁶</td>
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<tr>
<td>F1628#873</td>
<td>M</td>
<td>p.N1345Tfs*14 (CUB2)</td>
<td>acute</td>
<td>230</td>
<td>362% ⁶</td>
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<td>p.N1345Tfs*14 (CUB2)</td>
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<td>42</td>
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<td>F1627#873</td>
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<td>acute</td>
<td>238</td>
<td>424% ⁶</td>
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<td>869</td>
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<td>F1833#985</td>
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<td>acute</td>
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<td>109</td>
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<td>p.R1123C (TSP1-8)</td>
<td>rem</td>
<td>44</td>
<td>224% ⁶</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n.a.</td>
<td></td>
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<tr>
<td>F101#019</td>
<td>M</td>
<td>p.R1219W (CUB1)</td>
<td>acute</td>
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<td>n.d.</td>
</tr>
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<td>p.R1219W (CUB1)</td>
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<td>16</td>
<td>n.d.</td>
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<td>F102#019</td>
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<td>acute</td>
<td>97</td>
<td>n.d.</td>
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<tr>
<td></td>
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<td>p.R1219W (CUB1)</td>
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<td>20</td>
<td>n.d.</td>
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</table>

N.a., not available; n.d., not done; rem, remission. For domain abbreviations see Introduction 1.1.6.1. ¹This patient was not studied in remission since he was treated with eculizumab (Pecoraro et al. 2015); ²Limits of normal ranges (see Methods 2.5); ³New mutation; ⁴At the time of sampling. ⁵On ADP-activated HMEC-1. ⁶P< 0.05 vs control (statistical comparisons were made for each patient by comparing deposits in pixel² recorded in the 15 fields analyzed for the patient and for the corresponding control run in parallel).
3.4 ADAMTS13-FB interaction

3.4.1 Structural homology between FB and VWF

The Bb proteolytic fragment of FB contains a von Willebrand type A (VWA) domain structurally homologous with A2 domain in VWF (VWF-A2), which contains ADAMTS13 binding and cleavage sites. *In silico* comparison between VWA-FB and VWF-A2 reveals a similar overall folding of their crystal structures (Fig. 3-7A).

![Figure 3-7. In silico comparison between von willebrand type A domain of Factor B (VWA-FB) and A2 domain of VWF (VWF-A2).](image)

A. Top, Primary sequences of FB (Milder et al. 2007) and VWF (Haberichter 2015). Bottom, crystal structures of VWA-FB (1Q0P) and VWF-A2 (3ZQK) domains. B. Results of aminoacidic sequence alignment obtained by UNIPROT. CS: Cleavage Site for ADAMTS13. Numbers indicate exosites.
Furthermore, aminoacid sequence alignment of the two domains shows 18.5% identity (UNIPROT, Fig. 3-7B), with conservation of the peculiar exosites regions (in Fig. 3-7B as numbers) that are important for the recognition and interaction with ADAMTS13:

- exosite 1, Asp1614-Glu1615 (DE), is complementary to a functional exosite on the ADAMTS13 disintegrin-like domain, and helps orientating the scissible bond toward the active center of the protease (de Groot et al. 2009);

- exosite 2, Gln1624-Arg1668 (depicted as an α-helix, α6), is referred as the VWF A2 domain exosites for the binding with spacer domain of ADAMTS13 (Gao et al. 2008);

- exosite 3, residues 1596-1668, together with exosite-1 and exosites-2, is reported to make cooperative and modular contacts with the catalytic cleft of ADAMTS13 (Gao et al. 2008; Akiyama et al. 2009).

Of note, the cleavage site (CS) Tyr1605-Met1606 of VWF-A2 is replaced by Leu-Met in VWA of FB (Fig. 3-7B).

### 3.4.2 Protein structure prediction of ADAMTS13

Since the crystal structure of whole ADAMTS13 molecule has not been obtained yet, it was modeled by I-TASSER. The software provided an high-quality structural model, since C-score (-0.73) is higher than -1.5, meaning a correct fold based on availability of good threading templates in protein library, and TM-score (0.62) is higher than 0.5, meaning correct topology for all sizes of protein. This model was then analyzed in details by PyMOL (Fig. 3-8).

The proposed three dimensional model shows that ADAMTS13 undertakes an elongated structure like a “big scissor”, without buried regions. (Fig. 3-8A). Thrombospondin repeat modules (T) fold back on themselves, providing the approach
between CUB (CUB) and Spacer (S) domains, both important for the binding to its physiological substrate, VWF. According to the multiple interaction model proposed by Crawley and co-workers for ADAMTS13-VWF binding (Fig. 1-4) (Crawley et al. 2011), it could be speculated that the domains of the protease could have great mobility. This is also supported by the difficulty in obtaining a crystal structure of the entire ADAMTS13 molecule, suggesting that a spectrum of ADAMTS13 conformations exists, with different spatial alignments of the exosites (Akiyama et al. 2009). This mobility could in turn increase the possibility of ADAMTS13 to interact with partially unfolded VWF molecules, which also present a wide spectrum of conformations under shear-stress conditions in the circulation, and with other proteins carrying VWA domain such as FB.

Figure 3-8. ADAMTS13 structural model generated by I-TASSER and manipulated with PyMOL.
A. The image corresponds to the predicted model of ADAMTS13 obtained by submitting 75-1427 aminoacidic sequence of ADAMTS13 to I-TASSER software. B. Cartoon representing domain organization of ADAMTS13 with color correspondence in A. M, metalloprotease domain; D, Disintegrin-like domain; T1-8, Thrombospondin repeat modules; C, Cysteine-rich domain; S, Spacer domain; CUB1-2, CUB domains.
3.4.3 In silico interaction between ADAMTS13 and FB

The predicted three dimensional model of ADAMTS13 structure (Fig. 3-8) was then used to assess the predicted in silico interaction between the protease and FB, by using the free open-source ClusPro software available on the net. In this study, the model referred to the most populated cluster (Model 0, the first cluster in Table 2-2) was chosen and analyzed in details by PyMOL and the results are reported in Fig. 3-9. The docking interface of the predicted complex reveals that, in contrast to what was expected, FB binding sites are mostly restricted to Ba fragment (26-259 aa), while VWA domain seems not to be involved in the binding, remaining on the back of the docking interface (Fig. 3-9B). Interestingly, in this model ADAMTS13 interacts with FB through its MP domain (Fig. 3-9C). Nevertheless, the analysis of the other 1-4 models, corresponding to the subsequent most populated clusters 1-4 generated by ClusPro (Table 2-2), indicates that the binding surface of ADAMTS13 involves Spacer and CUB domains (Fig. 3-10). This heterogeneity among the proposed models reflects the previous observation of the great mobility of ADAMTS13 structure, and further highlights the possibility of a multiple interaction mode between the protease and its binding partners. However, also in these models FB binds ADAMTS13 through its Ba fragment chain, while VWA domain remains exposed to the surface (Fig. 3-10).
3. RESULTS

Figure 3-9. ADAMTS13 - FB docking model generated by ClusPro and manipulated with PyMOL. 
A. The image corresponds to the complex, predicted by ClusPro web server, formed by ADAMTS13 (light grey, I-TASSER model Fig. 3-8) with FB (dark grey PDB code: 2OK5). The binding interface is highlighted in red. B. Tridimensional structure of FB shown as surface distribution (upper panels) and in ribbon representation (bottom panels) with location of von Willebrand type A (VWA) domain in paleyellow in all structures prepared by PyMOL. The aminoacidic residues of FB contained in the binding interface are highlighted in red and indicated as numbers. C. Tridimensional structure of ADAMTS13 shown as surface distribution (upper panels) and in ribbon representation (bottom panel) with location of MetalloProtease (MP) domain (boxed) in the structure model from PyMOL. The aminoacidic residues of ADAMTS13 contained in the binding interface are highlighted in red and indicated as numbers.
Figure 3-10. Other ADAMTS13 - FB docking models generated by ClusPro and manipulated with PyMOL.
The images correspond to the Models 1-4 predicted by ClusPro web server for the complex formed by ADAMTS13 (light grey, I-TASSER model Fig. 3-8) and FB (dark grey PDB code: 2OK5). Tridimensional structure of both proteins are shown as surface distribution. The binding interface is highlighted in red. The clusters corresponding to each model are reported in Table 2.
3. RESULTS

3.4.4 ADAMTS13 interacts with FB in SPR and ELISA

I then investigated ADAMTS13-FB binding by *in vitro* assays. SPR experiments were performed by flowing FB on immobilized recombinant human ADAMTS13. The protein complex assembly results in an increase of light refraction expressed as resonance units (RU), in the first step of FB perfusion, followed by a second dissociation step evidenced by the decrease of the signal, due to buffer perfusion over the chip. The sensorgrams reported in Fig. 3-11ATop show that FB bind immobilized rADAMTS13. The calculated dissociation constant (KD) is $1.89 \times 10^{-6}$ M at room temperature, and $1.88 \times 10^{-7}$ M at 30 °C, suggesting that ADAMTS13-FB binding is dependent on temperature. To evaluate the binding specificity, a single concentration of purified human C3b or C3 was flowed over immobilized rADAMTS13, showing no binding in both conditions (Fig. 3-11ABottom). In this assay, the dissociation phase was then monitored until 900 sec, and the RU values of these curves became negative. Since RU value indicates the total mass on the chip, it can be speculated that the continuous perfusion of the buffer alone on the chip might induce the detachment of the immobilized rADAMTS13 protein, resulting in the decrease of RU under the zero, previously set as background. The meaning of this event could be correlated to the instability of the protein on the surface of the chip.
3. RESULTS

Figure 3-11. Specific interaction between FB and ADAMTS13.
A. SPR experiments. Top, three different concentrations (1, 2 and 4 µM) of FB were flowed over immobilized rADAMTS13. Bottom, a single concentration (1µM) of FB or C3b or C3 was injected over immobilized rADAMTS13. The binding was estimated as the increment of Resonance Unit (RU). B. ELISA experiments. Different amounts of FB 50-0 nM (1:2) were incubated over rADAMTS13 coated wells (2.5 ug/mL), and the ADAMTS13-FB complexes were detected by goat anti-human FB antibody followed by HRP-conjugated secondary antibody and TMB substrate. Each reaction was performed in duplicate and the OD values averaged. BSA and HSA were considered as irrelevant negative controls.

These results were confirmed by ELISA experiments, where it was documented a dose-response interaction of FB with coated rADAMTS13 (Fig. 3-11B).

To better characterize the binding site of FB involved in the interaction with ADAMTS13, the same SPR and ELISA experiments could be repeated by using Ba or Bb fragments chains.
3.5 **ADAMTS13 does not cleave neither FB, nor C3b nor FD**

ADAMTS13 physiologically works as a protease by cleaving VWF, regulating its multimeric length. In order to investigate the mechanism(s) through which ADAMTS13 deficiency in cTTP may result in complement AP activation, rADAMTS13 cleavage activity was tested on complement proteins such as FB, C3b and FD. It was speculated that ADAMTS13 could modulate complement cascade reducing protein availability for complement activation.

![Diagram](image)

**Figure 3-12. rADAMTS13 proteolytic activity on rVWF-A1A2A3, FB, C3b and FD.**  
A. rVWF-A1A2A3 was incubated with rADAMTS13 in the presence of urea and BaCl2. The cleavage product was visualized by WB with a monoclonal antibody anti-rVWF-A1A2A3 carboxy-terminal. At the end of the incubation the band corresponding to rVWF-A1A2A3 (80 kDa) disappeared, while the 30 kDa rVWF-A1A2A3 cleavage product appears.  
B and C. FB was incubated with different concentration of rADAMTS13, starting from the one that provides the cleavage of 100 ng of the VWF, in the presence of urea and BaCl2. rADAMTS13 and FB were visualized by silver stained SDS-PAGE (B) and WB (C) with an anti-ADAMTS13 antibody (upper image) or an anti-FB antibody (lower image). The two last lines on the right show the bands of purified FB and Bb for comparison.  
D and E. C3b or FD was incubated with different concentration of ADAMTS13, starting from the one that provides the cleavage of 100 ng of the VWF, in the presence of urea and BaCl2. The reactions were evaluated by silver stained SDS-PAGE. The physiological molar ratios between FB or FD and rADAMTS13 are squared. The molar ratio between C3b and rADAMTS13 is almost 30 fold lower than the physiological condition. Results of representative experiments of n=3 are shown.
In the presence of urea and BaCl$_2$, rADAMTS13 efficiently cleaved rVWF-A1A2A3 fragment (Fig. 3-12A) but had no effect on either FB, both in silver stained SDS-PAGE and WB (Fig. 3-12B and C), or C3b (Fig. 3-12D) or FD (Fig. 3-12E) since no cleavage fragments could be visualized on the gels.

In normal circulation VWF is unfolded due to shear stress allowing A2 cleavage site exposure to ADAMTS13 proteolysis. In in vitro fluid phase experiments in static condition this phenomenon is reproduced by urea, which was used also in the reactions containing FB or C3b and FD. However, other denaturing agents could also have been considered.

### 3.6 ADAMTS13 does not inhibit the formation of C3 proconvertase and C3 convertase complexes

Relying on the previous evidence that ADAMTS13 binds FB, I hypothesized that ADAMTS13 could modulate complement activation by interacting with FB during the formation of the proenzyme AP C3 proconvertase or the central enzyme C3 convertase complex (Fig. 3-13).
In the attempt to evaluate ADAMTS13 effect on the assembly of C3 proconvertase and the formation of C3 convertase, the recombinant protease was tested in microplate/WB methods, which selectively generate and detect C3bB(Mn$^{2+}$) and C3bBb(Mg$^{2+}$) complexes (see Methods 2.10) (Fig. 3-14A).

**Figure 3-13. Working hypotheses to investigate ADAMTS13 effect on C3bB C3 proconvertase and C3bBb C3 convertase.**

**A.** ADAMTS13 inhibits C3bB C3 proconvertase assembly by binding to FB, thus preventing its association with C3b. **B.** ADAMTS13 inhibits C3bBb C3 convertase formation by interacting with FB bound to C3b (in proconvertase complex), hindering FD cleavage activity on FB.
3. RESULTS

Figure 3-14. ADAMTS13 does not affect C3B proconvertase and C3Bb C3 convertase formation.
A. Experimental design of the assay for testing the effect of rADAMTS13 on C3B(Mn$^{2+}$) proconvertase and C3Bb(Mg$^{2+}$) convertase formation by microplate/WB.
B. Effect of rADAMTS13 on C3B(Mn$^{2+}$) proconvertase assembly. C3B(Mn$^{2+}$) complexes were formed by incubating C3B-coated wells with FB, in the absence or in the presence of increasing concentrations of rADAMTS13. C3 proconvertase was detected as a FB band (93 kDa) visualized by WB with an anti-FB antibody. C. Effect of rADAMTS13 on C3Bb(Mg$^{2+}$) convertase formation. C3Bb(Mg$^{2+}$) complexes were formed by incubating C3B-coated wells with FB and FD, with or without rADAMTS13. C3-convertase formation was evaluated by the visualization of the Bb band (60 kDa) by WB with an anti-FB antibody. ADAMTS13 buffer (right side) was the negative control. The physiological molar ratios between FB or FD and rADAMTS13 are squared. Results of representative experiments of n=3 are shown.
No difference either in C3bB(Mn$^{2+}$) assembly (Fig. 3-14B) or in C3bBb(Mg$^{2+}$) formation (Fig. 3-14C) was observed in the presence of rADAMTS13, either at physiological or at molar excess ratios with FB and FD.

These results indicate that ADAMTS13 does not affect the formation of both C3bB and C3bBb complexes in static conditions. With the aim to reproduce the physiological fluid shear stress, C3 proconvertase and C3 convertase were allowed to form in SPR experiments, thanks to the collaboration with the laboratory of Dr. Marco Gobbi in Mario Negri Institute in Milano. C3bB and C3bBb complexes were obtained by flowing FB over immobilized C3b in the absence or in the presence of FD, respectively (Fig. 3-15). ADAMTS13 effect was evaluated by flowing the recombinant protein together with FB and/or FD over C3b-coated chip.

![Figure 3-15. ADAMTS13 does not affect C3bB C3 proconvertase assembly and C3bBb C3 convertase formation in SPR.](image)

C3b was immobilized by amine coupling on sensor chip. FB (200 nM) was injected in the presence or in the absence of FD (4 nM) and rADAMTS13 (1 nM) or its buffer (buffer) over the immobilized C3b. The binding was estimated as the increment of Resonance Unit (RU). Buffer was considered as irrelevant negative control.
As reported in Fig. 3-15, rADAMTS13 modestly affected C3 proconvertase assembly by reducing the total amount of C3bB complexes generated on chip (violet vs orange curves), suggesting a possible competition among C3b and ADAMTS13 for the binding to FB. However, rADAMTS13 did not influence C3 proconvertase decay, since the dissociation curves of C3bB complexes in the presence or in the absence of the protease have the same slope (Fig. 3-15; violet vs orange curves). On the other hand, rADAMTS13 had no effect on the formation of C3 convertase, while it mildly accelerated C3bBb dissociation (Fig. 3-15; blue vs green curves). The latter indicates that ADAMTS13 may promote C3 convertase decay, which in turn could result in reducing the stability of C3bBb complexes and promoting complement regulation. Notably, these observations alone could not explain the previous results obtained on cells, where the presence of ADAMTS13 completely abolished the complement deposition mediated by serum from cTTP patient. Thus, at this point, the regulatory role for ADAMTS13 in complement cascade remains still unexplained. However, the very modest effect of ADAMTS13 on both complexes obtained in SPR should be confirmed with additional experiments.

In summary, altogether these results indicate that the binding between ADAMTS13 and FB is very weak and this interaction does not substantially impact FB activity in C3bB proconvertase assembly and C3bBb convertase formation. Nevertheless, a limited modulation of complexes formation and decay could be observed only in fluid phase, which could properly emulate the physiological condition in blood circulation. Effects of ADAMTS13 on C3 convertase activity could not be excluded and further experiments are needed.
3. RESULTS

3.7 VWF does not modulate complement regulatory activities of FH

3.7.1 Neither ULVWF multimers nor normal plasma VWF multimers inactivate C3b

Since VWF is the physiological substrate of ADAMTS13, and in TTP the deficiency of the protease results in the VWF accumulation into blood of the patients, it was then hypothesized that VWF could also interact with complement proteins and in turn affect the complement system. Different groups have recently reported that VWF binds FH, the major negative regulator of the alternative pathway of the complement cascade, and together with or without FH it modulates the complement activation (Rayes et al. 2014; Feng et al. 2015). With the aim to verify whether VWF had any effect on complement system, I tested different VWF preparations in the FH-cofactor assay for FI-mediated C3b degradation, as suggested by previous studies.

As reported in Fig. 3-16, neither normal plasma-derived VWF from two different commercial sources (LFB-VWF or Emoclot-VWF), nor rVWF (which includes ULVWF multimers) nor the rVWF-A2 domain, either alone or in the presence of FH, had any effect on C3b cleavage, when tested both at physiological molar ratios with FH and FI and in large excess. Faint bands of iC3b products were only observed with 500 molar excess of plasma VWF (Emoclot-VWF), which however also contained small amounts of contaminating FH, as shown by a faint 150 kDa band in Fig. 3-16C. FH contamination was determined by analysing Emoclot-VWF, LFB-VWF and rVWF on WB with a goat anti-FH antibody. The presence of FH in Emoclot-VWF was demonstrated by the visualization of the FH 150 kDa band, while no 150 kDa band could be visualized for both LFB-VWF and rVWF (Fig 3-16D).
Notably, LFB-VWF concentrate reflects the “physiological state” of VWF in normal human plasma (the presence of intermediate and high molecular forms of VWF), indeed it has been shown to have a multimeric pattern similar to plasma VWF giving evidence that this preparation strongly preserves physiological functions of plasma VWF (Mazurier 1998). The other concentrate, Emoclot-VWF has been shown to exhibit a lower content of high molecular weight VWF multimers than normal plasma (Peyvandi et al. 2013), while the rVWF contains also UL-multimers (Turecek et al. 2009) and has been used to mimic the multimers pattern in TTP plasma that lacks ADAMTS13 activity.
3. RESULTS

Figure 3-16. VWF does not exert cofactor activity in FI-mediated C3b cleavage.
C3b was incubated with FI, in the absence (A and C) or in the presence (B) of FH for 10 min (A and C) or 1 min (B), respectively. Different preparations of VWF were tested in the assays at 1X (0.313 nM, physiological molar ratio with FH), 10X (3.13 nM) or 500X (156.5 nM) molar concentrations: rVWF and LFB-VWF (A-B); Emoclot-VWF and rVWF-A2 (C). The reactions were analyzed on SDS-PAGE with Silver staining detection. C3b was detected as α’-chain (110 kDa) and β-chain (76 kDa) bands. FH was visualized as 150 kDa band. C3b cleavage products were detected as α65 kDa and α43 kDa bands. VWF full length (rVWF, LFB-VWF and Emoclot-VWF) was detected as 250 kDa band. rVWF-A2 was not visualized on SDS-PAGE since it run out the lower limit of the gel. The second lane in panel B (0 min) shows the reaction baseline. Results of a representative assay of n=3 are shown. D. Western Blot for FH contamination in Emoclot-VWF, LFB-VWF or rVWF preparations. The colors and numbers (200, 400 or 800) indicate VWF source and ng of the protein loaded on gel; 200 ng correspond to VWF amount presents in the 500X reaction.
3. RESULTS

3.7.2 VWF does not affect FH decay accelerating activity on C3 convertase

It is well known that FH regulates complement activation by promoting C3 convertase decay (Rodriguez de Cordoba et al. 2004; Makou et al. 2013). In order to investigate whether VWF-FH interaction could affect FH activity in dissociating C3 convertase, VWF fragments were tested in the new microplate/WB assay, which selectively generate and detect C3bBb(Mg$^{2+}$) convertase complexes (Marinozzi et al. 2014) (see Methods 2.10).

As shown in Fig. 3-17A, the presence of rVWF-A1A2A3 at two different concentrations during the formation of C3bBb(Mg$^{2+}$) complexes did not modulate FH-mediated C3 convertase decay, indicating that VWF does not affect FH activity on C3 convertase.

Also when rVWF-A1A2A3 fragments were added both during formation and decay of C3 convertase complexes, no effect was observed on FH accelerating activity on C3bBb dissociation (Fig. 3-17B), suggesting that also the direct VWF-FH interaction, if it occurs, does not influence FH regulatory activity in this static condition.
3. RESULTS

Figure 3-17. rVWF-A1A2A3 does not prevent both spontaneous and FH-mediated decay of C3 C3bBb(Mg^{2+}) convertase.

C3bBb(Mg^{2+}) complexes were formed by incubating C3b-coated wells with FB and FD, in the absence or in the presence of rVWF-A1A2A3. Spontaneous and FH-mediated decay of C3bBb(Mg^{2+}) was evaluated by further incubation with buffer alone or with 2640 ng/ml FH in the absence (A) or in the presence (B) of rVWF-A1A2A3 for 4 minutes at 25 °C. The amount of C3bBb formed and remained on well after decay incubation was evaluated by the visualization of the Bb band (60 kDa) by WB with an anti-FB antibody. Molar ratios between FH and rVWF-A1A2A3 are indicated. Results of representative experiments of n=3 are shown.
3.8 **VWF-C3 interaction**

3.8.1 **VWF accumulation on ADP-activated microvascular endothelial cells exposed to cTTP serum favors C3 deposition**

ULVWF multimeric strings secreted by, and anchored to, activated endothelial cells were found to bind C3b, other components of the AP (FB, FD, properdin), and C5 (Turner and Moake 2013). Moreover, the evidence that VWF possesses A2 domain that shares a high homology with typeA domain of FB (see Results 3.4.1), the natural C3b binding protein, highlights the possibility of a direct interaction between C3b and VWF.

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*A. Representative confocal microscopy images of co-localization of C3 (green) and VWF (red) on ADP-activated HMEC-1 (nuclei: DAPI, blue) exposed to 50% serum from a healthy subject (ctr) or a cTTP patient (cTTP). Cells were immunostained with an anti-C3 antibody, and with an anti-VWF antibody. Original magnification 630x, white scale bar: 20 µm.*

*B. ADP-activated HMEC-1 were exposed to serum from healthy subjects (ctr, n=3) or from cTTP patients in remission (cTTP, n=3), the latter with or without a polyclonal anti-human VWF antibody (anti-VWF) or an irrelevant antibody (irrel), or sCR1 (negative control). Data are expressed as mean ± SE of area covered by C3 deposits. *P<0.01 vs ctr, *P<0.01 vs cTTP, ^P<0.01 vs cTTP+irrel, ANOVA.*
Based on the above considerations, I then investigated whether the severe ADAMTS13 deficiency in cTTP patients leads to VWF accumulation on endothelial cell surface and whether VWF deposits may act as nuclei for C3 deposition. Incubation with cTTP serum taken during remission resulted in intense VWF staining on ADP-activated HMEC-1 (Fig. 3-18), as expected since VWF multimers could not be cleaved by ADAMTS13 which is deficient. Interestingly, VWF deposition partially colocalized with C3 (Fig. 3-18A), suggesting active interaction between VWF and C3 activation products on endothelial cell surface.

The addition of an anti-VWF antibody to cTTP serum during incubation with HMEC-1 completely prevented the excessive C3 deposition on ADP-activated HMEC-1 as the complement inhibitors sCR1 did, while an irrelevant antibody had no effect (n=3, Fig. 3-18B).

Altogether these results indicate that in cTTP, VWF strings accumulating on endothelial cells due to severe ADAMTS13 deficiency participate in complement activation through C3b binding.

### 3.8.2 VWF interacts with C3 in SPR and ELISA

Through SPR Feng et al (Feng et al. 2015) documented that C3b binds VWF with high affinity under flow; the C3b-VWF interaction was reversible, with a dissociation constant of 218 nM. In order to verify and characterize VWF-C3b interaction, different fragments of VWF were tested for C3b binding in SPR and ELISA experiments (Fig. 3-19). Thanks to the collaboration with the laboratory of Dr. Marco Gobbi in Mario Negri Institute in Milano, we firstly tried to immobilized rVWF on SPR chip in order to evaluate the binding between the multimeric form of VWF and C3b in a condition that
reproduce physiological shear flow. However, we observed that multimeric rVWF was not able to covalently bind the chip. Thus, rVWF was reduced with DTT, immobilized on the chip and subsequently used to binding assays in SPR. In Fig. 3-19A is reported the sensorgrams (time-course of SPR signal) obtained when flowing different concentrations of soluble C3b over monomeric rVWF immobilized on the sensor chip. A dose-dependent binding signal was detected, with a very slow dissociation rate (Kd = 2x10^{-4} \text{s}^{-1}, on average) suggesting a stable binding between VWF and C3b. The association rate constant (Ka) and the equilibrium dissociation constant (KD) could not be determined in a reliable manner since, in these conditions, binding saturation was not reached. However, these sensorgrams are consistent with KD values ranging 50-2000 nM, suggesting a specific interaction between monomeric rVWF and C3b.

Then, VWF-C3b interaction was studied in static conditions using ELISA. Recombinant multimeric VWF did not interact with C3b (Fig. 3-19B), possibly due to its non-permissive close conformation, while dose-response interaction of C3b with coated VWF monomer was observed (Fig. 3-19B). To examine whether VWF binds C3b through its typeA region, ELISA experiments were performed using either rA1A2A3-VWF or rA2-VWF coated onto the solid phase. Recombinant VWF-A1A2A3 and C3b dose-dependently interacted with each other (Fig. 3-19C and D) and the same was observed between rVWF-A2 and C3b (Fig. 3-19E and F). In competition studies, addition of rVWF-A2 (at equimolar concentration with C3b) to the C3b solution, inhibited C3b binding to coated rVWF-A2, confirming the specificity of the observed interaction (Fig. 3-19E, black circle). In contrast, rVWF-A1 and rVWF-A3 domains did not bind C3b (Fig. 3-19G). No binding was found between the rVWF-A2 domain and C4b (Fig. 3-19H).
3. RESULTS

Figure 3-19. VWF interacts with C3b in SPR and ELISA experiments.
A. High-affinity binding of C3b to rVWF by SPR. The graph shows the sensorgram (time course of the SPR signal in resonance unit, RU) obtained by simultaneously injecting three concentrations of C3b (1 µM, 500 nM or 100 nM) over sensor surface immobilizing rVWF-B-H. ELISA assays of the interaction between VWF and C3b or C4b. B. C3b was added at increasing concentrations to either multimeric rVWF- (rVWF) or monomeric rVWF- (rVWF monomer) coated wells. C. C3b was added at increasing concentrations to rVWF-A1A2A3-coated wells. D. rVWF-A1A2A3 was added at increasing concentrations to C3b-coated wells. E. C3b was added at increasing concentrations to rVWF-A2-coated wells. In separate wells C3b (5 µg/ml) was mixed with an equimolar amount (black circle) of rVWF-A2. F. rVWF-A2 was added at increasing concentrations to C3b-coated wells. G. C3b was added at increasing concentrations to either rVWF-A1- or rVWF-A3- coated wells. C3b binding was detected with a mouse anti-human C3 antibody followed by HRP-conjugated anti-mouse antibody; rVWF-A1A2A3 or rVWF-A2 binding was detected with an HRP-conjugated anti-human VWF antibody. H. C4b was added at increasing concentrations to either rVWF-A1- or rVWF-A2- or rVWF-A3- coated wells. Binding was detected with an anti-C4α antibody.
B-H. The open circles show the background binding with the corresponding amounts of BSA.
The graphs show the mean of triplicate measurements from a representative experiment of n=3.
In summary, SPR and ELISA experiments documented that VWF binds C3b in a conformation dependent manner, where fluid phase act as an important VWF-activating factor by exposing important binding exosites contained in the A2 domain.

### 3.8.3 In silico interaction between C3b and VWF-A2 domain

The three dimensional structures of C3b (2I07) and VWF-A2 domain (3ZQK:A) were docked by ClusPro software to investigate the molecular binding sites between VWF and C3b. ClusPro output provided 29 models for C3b - VWF-A2 docking prediction (see Methods 2.12.3). In this study, the model referred to the most populated cluster (the first cluster in Table 2-3) was chosen and analyzed in details by PyMOL (Fig. 3-20).
3. RESULTS

The docking interface of the predicted model reveals that C3b interacts with C-terminal of A2 domain of VWF through its TED domain (Fig. 3-20A-B). Interestingly, thioester moiety, contained in TED domain of C3b, seems to be involved in the binding, while the cleavage site Tyr1605-Met1606 of VWF remains buried inside the closed conformation of A2 domain (Fig. 3-20C).
3.9 VWF, interacting with C3b through its A2 domain, favors the formation of the alternative pathway C3 convertase

In order to investigate the effect of VWF-C3b interaction on complement activation, I then studied whether the binding between VWF and C3b results in the formation of the C3bBb AP C3 convertase. Recombinant VWF-A1A2A3-coated wells were incubated with 20% normal human serum (NHS) as a source of complement. The products were detached from wells and analyzed by WB (Fig. 3-21A). C3bBb formation occurred on rVWF-A1A2A3-coated wells, as documented by specific C3b β-chain and Bb bands, respectively (Fig. 3-21B). Notably, the C3b and Bb bands of C3bBb were more intense when the reaction was performed on coated mutant rVWF-A1A2A3 (180-200% and 150-171% of the C3b and Bb bands on wild-type rVWF-A1A2A3, respectively, Fig. 3-21B), which could not be cleaved by ADAMTS13 present in NHS since the Tyr1605-Met1606 at the cleavage site have been replaced by two Alanines. C3bBb formation was observed in positive control wells coated with properdin (Fig. 3-21B) (Lesher et al. 2013). No C3 convertase was formed on rVWF-A1A2A3- or properdin-coated wells incubated with NHS pre-treated with EDTA or sCR1 (Fig. 3-21B), which both block complement activation.

The C3 convertase assay was repeated on rVWF-A2-coated wells. Results showed specific bands of C3b and Bb that were fully abolished by EDTA (Fig. 3-21C). FH added in excess (higher than the physiological concentration) to NHS dose-dependently reduced the amount of Bb recovered from rVWF-A2-coated wells (Fig. 3-21D), confirming the specific formation of the AP C3 convertase on rVWF-A2 coated surface.
The addition of EGTA to NHS greatly reduced the C3b and Bb bands from rVWF-A2-coated wells (Fig. 3-21C-D), without altering the amount of rVWF-A2 coated on wells (Fig. 3-21E). While EDTA chelates all divalent cations, EGTA binds Ca\(^{2+}\) with a greater affinity than Mg\(^{2+}\) and the other divalent cations, and at the concentration we used should not affect the formation of the Mg\(^{2+}\)-dependent AP C3 convertase (Fishelson and Muller-Eberhard 1982). Indeed EGTA did not affect the C3b and Bb bands recovered from properdin-coated wells (Fig. 3-21C). Conversely, limited amount of C3b was recovered from rVWF-A2-coated wells in the presence of EGTA. Thus, it is likely that EGTA treatment inhibited C3b binding to coated rVWF-A2, suggesting a Ca\(^{2+}\)-dependent interaction between the two molecules.

Altogether these results indicate that rVWF-A2 binds C3b and acts as a platform for the formation of the AP C3 convertase.
3. RESULTS

Figure 3-21. Formation of C3 alternative pathway convertase on VWF.
A. Experimental design of the assay for testing the formation of the C3 convertase by microplate/WB. B. Microtiter wells were coated with wild-type rVWF-A1A2A3 or rVWF-A1A2A3, mutated at the ADAMTS13 cleavage site (YM>AA), or PBS or properdin, and incubated with 20% normal human serum (NHS) with or without EDTA 5 mM or sCR1 150 µg/ml. C. Microtiter wells were coated with rVWF-A2 or properdin, and incubated with 20% NHS with or without EGTA 1 mM or EDTA 5 mM. D. Microtiter wells were coated with rVWF-A2, and incubated with 20% NHS with or without EGTA 1 mM or increasing amounts of exogenous FH (100 and 200 µg/ml). FH was visualized as a 150 kDa band by WB. In B, C and D C3bBb formation was detected by C3b β-chain (76 kDa) and Bb (60 kDa) bands by WB. Results of a representative experiment of n=3 are shown. E. Absorbance values of the staining of the carboxymethylation of rVWF-A2 coated on microplate wells in the absence or in the presence of EGTA 1 mM, by using a rabbit anti-human VWF antibody followed by an HRP-conjugated anti-rabbit antibody.

3.10 VWF-C3b interaction forms the alternative pathway C5 convertase and activates the terminal pathway

I previously documented that immobilized C3b in the microplate/WB assay formed an active C5 convertase in the presence of NHS. To evaluate the impact of VWF-C3b interaction on the terminal complement pathway, the products recovered from rVWF-A2-coated wells after incubation with NHS were analyzed on WB with anti-C5, anti-C6, or anti-C9 antibodies (Fig. 3-22A). Bands corresponding to the C5b α’-chain, C6
and C9 were observed in samples recovered from wells coated with rVWF-A2 or with properdin (Fig. 3-22B). No C5b, C6 or C9 bands could be recovered from rVWF-A2-coated wells exposed to EGTA-treated NHS (Fig. 3-22B). No activation products of C3 and C5 convertases could be recovered following the incubation of NHS in wells coated with fibrinogen, a protein that does not bind C3b (Andersson et al. 2005), supporting the specificity of these findings. Finally, labeling the products on rVWF-A2-coated wells, after incubation with NHS, with an anti-C5b-9 antibody in an ELISA assay, showed the formation of C5b-9 complexes (Fig. 3-22C). Notably, the absorbance values of C5b-9 formed in rVWF-A2-coated wells were comparable to absorbance values in C3b- or properdin-coated wells. The addition of sCR1 reduced the absorbance values in all three conditions, while EGTA only reduced the C5b-9 signal in rVWF-A2-coated wells (Fig. 3-22C).

These results indicate that rVWF-A2 domain favors the assembly of an active C5 convertase, the formation of C5b and the activation of the terminal complex (Fig. 3-22D).
3. RESULTS

**Figure 3-22. Formation of C3 and C5 alternative pathway convertases on VWF.**

**A.** Experimental design of the assay for testing the formation of the C3 and C5 convertases by microplate/WB. **B.** Microtiter wells were coated with properdin or C3b or rVWF-A2 or fibrinogen, and incubated with 20% NHS with or without EGTA 1 mM. C3bBb and C5b formation, and C6 and C9 binding were detected by the bands of C3b β-chain (76 kDa), Bb (60 kDa) and C5b α’-chain (104 kDa), C6 (105 kDa) and C9 (66 kDa), respectively, with specific antibodies. FH was visualized as a 150 kDa band. Results of a representative experiment of n=3 are shown.

**C.** ELISA-based detection of C5b-9 deposits formed on properdin or C3b or rVWF-A2 in the presence of NHS, or NHS plus EGTA 1 mM, or NHS plus sCR1 150 µg/ml, by using an HRP-conjugated anti-C5b-9 antibody. PBS was used as negative control. The graphs show the mean of triplicate measurements from a representative experiment of n=3.

**D.** Drawing describing C3, C5 convertases and C5b-9 complex formation on rVWF-A2 coated wells in the presence of NHS.
3. RESULTS

3.11 cTTP serum exerts a pro-thrombogenic effect on endothelial cells

3.11.1 cTTP serum induces VWF deposition and thrombomodulin shedding mediated by complement terminal pathway products

To investigate whether in the presence of ADAMTS13 deficiency, VWF-induced complement activation perturbed the thromboresistant endothelial phenotype, I then tested cTTP serum for VWF staining and thrombomodulin expression on ADP-activated HMEC-1, in the absence and in the presence of complement inhibitors.

HMEC-1 incubated with cTTP serum (taken in remission, n=5) showed more intense VWF staining than cells incubated with control serum (Fig. 3-23A). The VWF staining intensity was normalized through supplementation of cTTP serum with ADAMTS13 (4 µg/ml corresponding to 100% protease activity) that cleaved VWF strings on cell surface (Fig. 3-23A). Blocking either the entire complement cascade with sCR1 or the terminal pathway alone with eculizumab significantly limited the excessive VWF staining induced by cTTP serum (Fig. 3-23A). Following exposure of ADP-activated HMEC-1 to cTTP serum the intensity of thrombomodulin staining was significantly reduced relative to HMEC-1 exposed to control serum (Fig. 3-23B, n=5). Because the cleavage of THBD from the plasma membrane is thought to be dependent on serine protease activity, we examined t-PA, a serine protease present in endothelial Weibel-Palade bodies possibly released upon stimulation by anaphilatoxins. Blockade of t-PA activity by treatment of HMEC-1 with the serine protease inhibitor aprotinin prevented THBD shedding in response to exogenous C3a. (Morigi et al. 2011). Addition of aprotinin to cTTP serum significantly limited the loss of thrombomodulin staining,
suggesting that cTTP serum induced thrombomodulin shedding from HMEC-1 (Fig. 3-23B). Also eculizumab antagonized the effect of cTTP serum on thrombomodulin loss (Fig. 3-23B).

Together the above results indicated that C5 activation products have a role in cTTP serum-induced VWF accumulation on HMEC-1 and in thrombomodulin loss from HMEC-1 cell surface.

Human umbilical vein and human microvascular endothelial cells have been reported to express the receptors for the C5 cleavage product C5a (C5aR) (Schraufstatter et al. 2002). Here we also found that HMEC-1 also express C5aR, and C5aR expression levels increased after stimulation with ADP (Fig. 3-23C).

To investigate whether C5a mimicked the effects of cTTP serum on HMEC-1, HMEC-1 were exposed to C5a in test medium, in the absence of serum (Fig. 3-23D-E). HMEC-1 incubation with C5a resulted in intense VWF staining compared with cells incubated with test medium alone. C5a also caused loss of thrombomodulin staining on the HMEC-1 cell surface (Fig. 3-23D-E).
3. RESULTS

Figure 3-23. cTTP serum-induced activation of the terminal complement pathway alters the thromboreistant phenotype of microvascular endothelial cells.

A. ADP-activated HMEC-1 were incubated with 50% serum from healthy subjects (ctr, n=5) or from cTTP patients in remission (cTTP, n=5), the latter in the absence or in the presence of sCR1 or rADAMST13 or eculizumab (ecu). VWF deposits were evaluated by staining with an anti-VWF antibody, and the surface area covered by VWF staining was quantified on images from 15 high power fields acquired by confocal fluorescence microscopy. Data are expressed as mean ± SE of area covered by VWF deposits. °P<0.01 vs ctr, *P<0.01 vs cTTP untreated, ANOVA.

B. Left panel. Thrombomodulin expression on ADP-activated HMEC-1 incubated with serum from healthy subjects (ctr, n=5) or from cTTP patients in remission (cTTP, n=5), with or without eculizumab (ecu) or aprotinin. Thrombomodulin (THBD) expression was evaluated by staining with an anti-thrombomodulin antibody, and the surface area with THBD staining was quantified on images from 15 high power fields acquired by confocal fluorescence microscopy. Results were expressed as mean ± SE. °P<0.01 vs ctr, *P<0.01 vs cTTP untreated, ANOVA. Right panel. Representative confocal microscopy images of thrombomodulin (red) staining on ADP-activated HMEC-1 (nuclei: DAPI, blue) exposed to serum from healthy subjects (ctr) or cTTP patients (cTTP). Original magnification 400x, white scale bar: 20 µm.

C. C5aR expression (red) on resting and ADP-activated HMEC-1 (nuclei: DAPI, blue). HMEC-1 were incubated for 10 min with medium alone or added with 10 µM ADP, then C5aR were labeled with an anti-human CD88 antibody (or isotype control antibody, negative). Magnification 400x, white scale bar: 20 µm.

D. Effect of C5a on VWF and thrombomodulin expression on endothelial cells. HMEC-1 were incubated (10 min) with 200 ng/ml C5a added to test medium. Staining of VWF and thrombomodulin was evaluated by confocal microscopy. Results were expressed as mean ± SE of 15 high power fields. #P<0.01 vs resting HMEC-1, ANOVA.

E. Representative images of VWF (red) and thrombomodulin (red) staining on HMEC-1 (nuclei: DAPI, blue), either resting or exposed to 200 ng/ml C5a. Magnification 400x. White scale bar: 20 µm.
3. RESULTS

3.11.2 cTTP serum induces microvascular thrombosis

Finally, to evaluate whether the above abnormalities contributed to microvascular thrombosis of cTTP, ADP-activated HMEC-1 were pre-incubated with cTTP serum (n=11, in remission) and then perfused with normal heparinized whole blood (added with mepacrine, Fig. 3-24A, see Methods 2.14) at a shear stress encountered into the microcirculation (60 dynes/cm²). In these experimental conditions blood did not clot and platelet thrombi could be evaluated as green fluorescence signal on HMEC-1. A much wider surface area covered by thrombi was observed upon blood perfusion on HMEC-1 pre-treated with cTTP serum, compared to cells exposed to control serum (Fig. 3-24B-C). The thrombus area was completely normalized by the addition of sCR1, or FH, or eculizumab (Fig. 3-24B-C) during preincubation with cTTP serum. A C5aR antagonist significantly reduced but did not fully normalize the thrombus area (Fig. 3-24B-C), indicating that both terminal complement products, C5a and C5b-9, have a role in the prothrombogenic effect of cTTP serum. cTTP serum-induced thrombus formation was also inhibited by rADAMTS13 (Fig. 3-24B-C) that prevented VWF accumulation on HMEC-1 (Fig. 3-23A), and by an anti-VWF antibody (Fig. 3-24B-C).

Together these data indicate that VWF accumulation and complement activation on endothelium might play a cooperative role in the insorgence of microvascular thrombosis in conditions of ADAMTS13 deficiency.
3. RESULTS

Figure 3-24. cTTP serum exerts a prothrombogenic effect on microvascular endothelial cells mediated by VWF and terminal complement pathway.

A. Experimental design.
B. ADP-activated HMEC-1 were exposed to serum from healthy subjects (ctr, n=11) or cTTP patients in remission (cTTP, n=11) with or without sCR1 (n=11) or FH (n=8) or eculizumab (ecu, n=8), or a C5aR antagonist (C5aRA, n=3), or rADAMTS13 (rA13, n=8), or a polyclonal anti-human VWF antibody (anti-VWF, n=8) or an irrelevant antibody (irrel, n=8); then cells were perfused with whole blood. The area occupied by thrombi was measured on images from 15 high power fields acquired by confocal fluorescence microscopy and data were expressed as mean ± SE. °P<0.01 vs ctr, *P<0.01 vs cTTP untreated, ^P<0.01 vs cTTP+irrel, ANOVA.
C. Representative confocal microscopy images of thrombi (platelet deposition, green) formed on ADP-activated HMEC-1 after exposure to serum from an healthy subject (ctr) or from a cTTP patient (cTTP) with or without sCR1 or FH or eculizumab (ecu) or a C5aRA, or rADAMTS13 (rA13) or a rabbit anti-VWF antibody (anti-VWF) or an irrelevant rabbit antibody (irrel). Original magnification 200x. White scale bar: 50 µm.
4. DISCUSSION
In this study I demonstrated that severe ADAMTS13 deficiency, which results in the accumulation of ULVWF multimers, is associated with activation of the complement AP and hence the terminal pathway on endothelial cell surface.

This is supported by the following findings: 1) sera from all patients with cTTP and ADAMTS13 mutations induced abnormal C3 and C5b-9 deposits on ADP-activated endothelial cells, which were prevented by selective AP inhibitors; 2) abnormal C3 deposits induced by cTTP serum colocalized with VWF; 3) the effect of cTTP serum on C3 and C5b-9 deposition was completely prevented by rADAMTS13 supplementation or by treatment with an anti-VWF antibody; 4) VWF interacted with C3b and provided a platform for the formation of active C3 and C5 convertases of the AP. Notably, circulating C3 and SC5b-9 levels were altered in only a fraction of patients, indicating that complement activation in cTTP occurs at cell surface rather than in the fluid phase.

Altogether these results match the condition of endothelial-restricted complement activation of patients with aHUS (Goicoechea de Jorge et al. 2007; Noris et al. 2014), and support the existence of a cross-talk between ADAMTS13/VWF and the complement system.

Previous studies suggested that complement is activated during acute episodes of acquired TTP. High plasma levels of C3a and SC5b-9 were reported in patients with anti-ADAMTS13 antibodies (Reti et al. 2012), however chronic high levels of C4d indicated secondary activation of the classical complement pathway by autoantibodies (Reti et al. 2012). There are also data suggesting that complement activation may occur in cTTP, as documented by complement-induced hemolysis of sheep erythrocytes in the presence of cTTP plasma (Feng et al. 2013)b, and by C3 and C5b-9 deposition on endothelial cells following exposure to cTTP sera (Ruiz-Torres et al. 2005). In the above reports the diagnosis of cTTP could not be confirmed since no genetic data were
4. DISCUSSION

provided; in addition, samples were taken during an acute episode (Ruiz-Torres et al. 2005; Feng et al. 2013)\(^b\), so the possibility that complement activation was a secondary phenomenon triggered by extensive platelet activation and ischemic tissue injury could not be ruled out. Tati et al (Tati et al. 2013) found that plasma from patients with cTTP and ADAMTS13 mutations contained higher levels of complement-coated endothelial microparticles than control plasma. Exposure of histamine-stimulated glomerular endothelial cells to cTTP plasma caused deposition of C3 on VWF-platelet strings (Tati et al. 2013). Notably, all patients in the above study had renal involvement, a clinical feature that is observed only in a subgroup of cTTP patients, while it is prototypical of aHUS (Noris and Remuzzi 2009). Moreover, the Authors did not investigate the concomitant presence of any aHUS-associated complement abnormalities that could account for the renal phenotype and complement activation (Noris et al. 2005; Chapin et al. 2013; Tsai et al. 2013).

Unlike in the above studies, Gavrilaki et al (Gavrilaki et al. 2015) failed to find increased C5b-9 deposits and complement-mediated killing in cells exposed to TTP serum, however a large majority of patients had acquired TTP and only three of them were studied during the acute phase of the disease. In addition, the tests were done on cell lines (human endothelial hybrid and human erythroblast cell lines) made artificially deficient for glycosylphosphatidylinositol-anchored complement regulatory proteins (CD55 and CD59), an experimental setting that was far from reproducing the conditions of human vascular endothelium. In this regard, and unlike in other studies (Noris et al. 2014), the above tests could not distinguish aHUS patients with active disease from aHUS patients in remission under terminal complement blockade through eculizumab treatment, because of both groups causing the same complement-mediated cell killing.
The aim of the present study was to clarify whether a direct link exists between impaired VWF cleavage due to ADAMTS13 deficiency and complement activation. For this purpose only cTTP patients with identified ADAMTS13 mutations and without mutations in complement genes were studied. It is relevant that all but two ADAMTS13 mutations have been functionally characterized previously and cause a severe reduction in protein secretion and/or activity or result in protein interruption (Donadelli et al. 2006; Lotta et al. 2012; Pecoraro et al. 2015; Rurai et al. 2015). In addition, to separate the relative contribution of ongoing thrombotic microangiopathy from that of ADAMTS13 deficiency per se to complement activation, cTTP patients were studied both during acute episodes and at remission.

Although plasma SC5b-9 is considered a surrogate marker of complement activation (Cataland et al. 2014), it is documented to be unreliable in TMA patients, since most patients in remission continued to have elevated SC5b-9 levels, also after eculizumab therapy that blocks C5 cleavage (Noris et al. 2014). Also in cTTP cohort here considered high SC5b-9 levels detected during acute phase remained elevated in remission. Recent studies reported that SC5b-9 complex could originate also after C5 cleavage mediated by thrombin and plasmin, which act as “alternative” potent C5 convertases (Huber-Lang et al. 2006; Foley et al. 2016). Evidence that thrombin and plasmin generation increases in blood of patients suffering from thrombotic disorders (Wada et al. 1989; Grabowski 2002), where coagulation cascade is activated, suggests that the total amount of SC5b-9 complexes could be the sum of the “alternative” and the canonical C5 cleavage. To address above considerations, other experiments are needed to discriminate the contribution of thrombin- and plasmin- mediated C5 proteolysis in the complement cascade.
To evaluate whether complement is activated at endothelial cell level, we used the new *ex vivo* assay set up for monitoring eculizumab therapy in aHUS patients (Noris et al. 2014) for cTTP cohort here described. Serum from acute cTTP caused C3 and C5b-9 deposits on resting endothelial cells, which could reflect in vivo complement activation triggered by widespread platelet thrombi and coagulation (Huber-Lang et al. 2006; Amara et al. 2008; Speth et al. 2015). In contrast, cTTP serum taken in remission induced excessive complement deposition only on activated endothelial cells. These data fit with the observation that cTTP onset or relapses may occur in concomitance with a triggering event (infections, drugs, pregnancy) that perturbs microvascular endothelium (Galbusera et al. 2006), and suggest that ADAMTS13 deficiency predisposes to AP dysregulation on cell surface. The observation that supplementing cTTP serum with rADAMTS13 fully prevented C3 and C5b-9 deposits supported the above hypothesis. However, mechanistic studies did not reveal any direct regulatory activity of ADAMTS13 on the complement AP. Indeed, rADAMTS13 failed to proteolyse FB and did not alter the assembly of the AP C3 convertase. Some structural homology exists between the A2 domain of VWF that contains the ADAMTS13 cleavage site (Akiyama et al. 2009; Crawley et al. 2011), and the VWA domain of FB that is involved in its binding with C3b (Milder et al. 2007; Torreira et al. 2009). However, the Tyrosine1605 in the ADAMTS13 cleavage site is replaced by Leucine in the VWA FB domain, which could explain failure of FB proteolysis by ADAMTS13. Nevertheless, this work provided for the first time experimental evidence that ADAMTS13 binds FB, and the results of ClusPro docking prediction suggested that Ba fragment chain of FB is responsible for this interaction. Since FB binding to C3b depends on elements contained in Ba chain (Milder et al. 2007), ADAMTS13 could hamper this interaction by competing with C3b for the same binding epitopes on FB,
and it could account for the little inhibition observed on C3 proconvertase assembly in SPR. However, ADAMTS13 did not substantially interfere in the formation of C3bBb convertase complexes, which originate after C3 proconvertase assembly. This consideration together with the evidence that physiological blood concentration of FB is largely higher than that of ADAMTS13 (FB = 2.15 uM, ADAMTS13 = 5.55 nM, FB:ADAMTS13 = 387:1) (Gerritsen et al. 2001; Waters and Licht 2011), could suggests that ADAMTS13 interacts with FB too weakly to efficiently antagonize the binding of FB with C3b in the C3bB and C3bBb complexes. Thus, the physiological meaning of this binding remains still unclear. Further studies will be necessary to better characterize the binding sites of ADAMTS13 and FB by using deletions mutants of both proteins in ELISA and SPR experiments similar to the ones here reported. Besides, any ADAMTS13 effect on C3 convertase activity could be not excluded and must be also investigated.

Once established that ADAMTS13 does not directly modulate complement, I then investigated whether VWF interacted with C3b. Here I documented that a specific interaction between VWF and C3b exists, and that the binding site for C3b is localized in the A2 domain of VWF. ClusPro prediction revealed that C-terminal of VWF-A2 interacts with TED domain of C3b, containing the thioester moiety which is also involved in this binding.

Activation of C3 into C3b in the complement pathway is a crucial step for complement response, since nascent C3b is able to bind covalently to cell and target surfaces via the exposed thioester (Janssen and Gros 2007). I would hypothesize that C3b molecules deposit on endothelial VWF in the same covalent manner in which they attach to stimulated surfaces. Indeed we observed in SPR experiments high affinity binding between VWF and C3b, and this interaction seems to be irreversible, in contrast with
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published data by Feng and coworkers (Turner and Moake 2013; Feng et al. 2015). These results together with the observation that monomeric VWF was immobilized on the SPR chip after reduction with DTT, could properly suggest that thiol free groups of VWF could efficiently link to the exposed thioester group of C3b. However, these speculations should be confirmed by additional experiments.

Nevertheless, of great relevance I confirmed VWF-C3b interaction at the endothelial cell level by colocalization of VWF and C3 deposits on HMEC-1 exposed to cTTP serum. Altogether these results are in line with published data showing the colocalization of C3/C3b with ULVWF on histamine-stimulated HUVEC (Turner and Moake 2013; Feng et al. 2015). I then found no C3 staining on ADP-activated HMEC-1 before exposure to serum, despite the evidence of secreted/attached VWF on cell surface, indicating that the C3 deposited on HMEC-1 following incubation with cTTP serum mostly originated from serum.

How the interaction between VWF and C3b impacts on the activation of the complement AP is a matter of debate. VWF has been shown to favor C3b inactivation by enhancing FH cofactor activity (Rayes et al. 2014). Another study proposed that normal plasma VWF multimers alone may exert cofactor activity, while ULVWF multimers lacked cofactor activity and did not inhibit the generation of C3b from C3 (Feng et al. 2015). Here I documented no cofactor activity either for plasma-derived VWF or rVWF that includes ULVWF multimers, either in the absence or in the presence of FH. Discrepancies between the present and published results could be due to the largely supraphysiological molar ratios of VWF used in published studies, compared to FI and FH (Rayes et al. 2014; Feng et al. 2015). In addition, since FH binds to VWF, and VWF-FH complexes were detected in normal plasma, the contribution of contaminating FH in plasma-derived VWF preparation to the observed
cofactor activity cannot be excluded (Rayes et al. 2014). Furthermore, the interaction between VWF and the FH does not seem to modulate the activity of the FH even in C3 convertase accelerating decay. Altogether these results documented that VWF does not directly affect FH activities on C3b and C3 convertase.

Rather, finding here that the binding of rVWF-A1A2A3 or rVWF-A2 domains to C3b results in the formation of the C3 convertase, indicates that VWF may act as an initiator of the complement AP. This possibility is confirmed by data that either sCR1, or FH, which dissociates the AP C3 convertase (Jozsi and Zipfel 2008), inhibited C3 convertase formation on rVWF, and normalized C3 deposits induced on HMEC-1 by cTTP serum.

The current dogma states that while Ca\(^{2+}\) is required for the initiation of the complement classical pathway (Fishelson and Muller-Eberhard 1982; Major et al. 2010), the AP is Ca\(^{2+}\) independent. A major finding of the present study is the identification of a Ca\(^{2+}\)-dependent mechanism of AP activation that is mediated by VWF, as supported by data that EGTA prevented the formation of C3bBb on the rVWF-A2 domain. Calcium ions bind the VWF-A2 domain, and the calcium binding site and a vicinal disulphide bond act together to maintain the structural integrity of the VWF-A2 domain and to protect against ADAMTS13 proteolysis (Lynch et al. 2014). A plausible explanation of findings here reported could be that the Ca\(^{2+}\)-stabilized conformation of the VWF-A2 domain may favor the interaction between VWF and C3b, either by exposing a functional binding site or preventing cleavage of the subunit. Recent studies reported that single-particle electron microscopy (EM) could provide reliable and high resolution views of how domains of C3b could move and orientate during assembly with its binding partners (Torreira et al. 2009; Alcorlo et al. 2013). Considering all the observations here reported regarding the binding between C3b and VWF, EM
methodology could be an useful tool to describe and better characterize the C3b-VWF complex. On the other hand, it is important to note that rVWF-A2 domain used in this study is produced in bacteria (E.coli, provided by R&D Systems), while rVWF-A1 and rVWF-A3 domains are obtained from mammalian cells (HEK293E, Human Embryonic Kidney Cells; provided by U-Protein Express BV). So, I can not exclude that the results obtained here could be influenced by the non-human source of the recombinant proteins, which do not carry all the post-translational modifications present in mammalian cells. In this context, the expression of rVWF-A2 in human cells, such as HEK293 cells, is needed to confirm the results of this study and to understand the impact of mammalian modifications, such as glycosylation, in the interaction and function of this crucial domain of VWF.

In contrast with our results, Noone et al (Noone et al. 2016) recently suggested VWF has a role in protecting endothelial cells from complement, based on finding of increased C3c deposition on cultured blood outgrowth endothelial cells from patients with type 3 VWF disease that have severe deficiency of VWF. However in this study cells were treated with antibodies that artificially blocked all membrane-anchored complement regulators and induced complement fixation via the classical pathway. It would have been interesting that the Authors had analyzed whether serum of aHUS patients, in which alternative pathway cascade is selectively activated due to mutations in complement components, could induce the same response on the surface of these cells. Furthermore, the in vivo relevance of the above data is debatable since there is no published evidence indicating complement hyperactivation in patients with VWF disease.

Another relevant finding of this study is the formation of C5b and C5b-9 on rVWF-A2 domain, indicating that the AP C3 convertase assembled on VWF formed the C5
convertase that cleaved C5 to C5a and C5b and initiated the terminal pathway. Altogether these results provide a mechanistic explanation of the formation of C5b-9 deposits on cTTP serum-exposed HMEC-1.

C5a and C5b-9 cause profound perturbations of the physiologically thromboresistant endothelial phenotype, including upregulation of tissue factor, loss of thrombomodulin, and exocytosis of P-selectin and ULVWF (Hattori et al. 1989; Platt et al. 1991; Ikeda et al. 1997; Tedesco et al. 1997; Mori et al. 2001). Finding here that treatment with eculizumab prevented the loss of thrombomodulin and the VWF increase on endothelial cells exposed to cTTP serum, supports a role of terminal complement components in the loss of endothelial thromboresistant phenotype. The evidence that treating HMEC-1 with C5a caused VWF exocytosis and thrombomodulin shedding points to C5a as a plausible major player in the cTTP serum-induced prothrombogenic endothelial abnormalities. The pathophysiological relevance of these data to microvascular thrombosis is highlighted by evidence that blocking complement with either sCR1, FH, eculizumab, or a C5aR antagonist consistently reduced the pro-thrombogenic effect of cTTP serum on microvascular endothelium. Besides, results here proposed further support the importance of C5a-C5aR axis in promoting thrombosis in a particular pro-thrombogenic condition (Romay-Penabad et al. 2007).

Endothelial cells express functional C5aRs and delineates the intracellular signal transduction pathway by activating G-proteins and EGFR cascade (Schraufstatter et al. 2002). It has been shown that various proinflammatory mediators can up-regulate C5aR expression (Gasque et al. 1997; Laudes et al. 2002), allowing C5a to directly potentiate proinflammatory events on the microvascular endothelium. Thus, it could be interesting to evaluate whether also cTTP serum stimulate endothelial C5aR expression by evaluating cellular C5aR mRNA levels after serum incubation. What still remains to
understand is the mechanisms by which C5a induces VWF secretion and THBD shedding during cTTP serum stimulation. Since C5a treatment of HMEC induced cytoskeletal changes, cell retraction and gap formation (Schraufstatter et al. 2002), it can be assumed that C5a could induce re-organization of intracellular vesicle trafficking of endothelial cells that results in exocytosis of VWF on cell surface. To evaluate this hypothesis, intracellular cytoskeletal markers such as actin and tubulin fibers could be analyzed in endothelial cells after incubation with cTTP serum. Finally, a novel anaphylatoxin receptor, named C5L2, has also been identified (Cain and Monk 2002) and its transduction signal could be further investigated.

To summarize, the results presented here indicate that VWF multimer accumulation on endothelium, as occurs in cTTP, promotes the activation of the complement AP that proceeds until C5 cleavage with the formation of C5a and the terminal C5b-9 complex. In turn, the terminal complement components, cause the loss of the antithrombotic thrombomodulin and the increase in VWF multimers secreted/anchored on endothelial cells, thus creating a positive amplification loop that results in microvascular thrombosis (Fig. 4-1). Of note, VWF plasma levels and endothelial cells vary from one vascular bed to another and in particular cells may also have different responses to the same signals (Rosenberg and Aird 1999). In patients with TTP, accumulation of ULVWF multimers could have different effects on endothelial cells from different organs. These observations closely correlate with heterogeneous distribution of microthrombi in patients with TTP and suggest that the phenotype is governed by individualistic responses of endothelial cells to a stimulus.

Altogether these findings link hemostasis-thrombosis with the complement AP and open potential therapeutic perspectives of complement-inhibitory drugs in cTTP and in general in thrombotic and inflammatory disorders, such as diabetes, autoimmune
disorders and cardiovascular diseases, associated with endothelium perturbation, VWF release and complement activation (Zhang et al. 2002; Bjerre et al. 2010; Verschoor and Langer 2013; Montoro-Garcia et al. 2014). Therapeutic strategies that include C5a and its receptor are considered an especially promising approach to complement inhibition (Reis et al. 2015). The observation that eculizumab given as sole treatment to a boy with cTTP resulted in prompt disease remission is in keeping with such a possibility (Pecoraro et al. 2015).
4. DISCUSSION

In NORMAL SUBJECTS:

In cTTP PATIENTS with ADAMTS13 deficiency:

Figure 4-1. The interplay between VWF and the complement alternative pathway.

C3 spontaneously undergoes cleavage to C3b at a slow rate, amplified by bacterial and viral products. Upon a trigger event that activates or injures endothelial cells, ULVWF multimers are exocytosed from Weibel-Palade bodies on endothelial cell surface, and by binding C3b may amplify the alternative pathway (AP) cascade by forming the C3 convertase complex. In normal subjects (upper panel) ADAMTS13 cleaves ULVWF multimers from cell surface and prevents AP activation maintaining homeostasis. The low amount of C3b formed is inactivated to iC3b by factor H (FH) and factor I (FI). Thrombomodulin (THBD), a surface protein expressed on all endothelial cells, provides additional protection: by binding to thrombin blocks its ability to activate coagulation and platelets and redirects the activity of thrombin toward the generation of carboxypeptidase B (TAFIa), which inactivates C3a and C5a.

In patients with cTTP and severe ADAMTS13 deficiency (lower panel), ULVWF multimers can not be cleaved and accumulate on endothelial cell surface, providing a nucleus for the C3 and C5 convertases of the AP, leading to C5 cleavage to C5a and C5b. C5a binds to its receptors on endothelial cells, and C5b forms the terminal C5b-9 complex. Both C5a and C5b-9 cause further exocytosis of VWF from Weibel-Palade bodies that favor platelet adhesion and aggregation and amplifies the complement activation cascade. In addition C5a causes the shedding of thrombomodulin. The latter event results in impaired TAFI-mediated inactivation of C5a and thrombin-mediated platelet activation. Together the above events concur to platelet adhesion-aggregation and microvascular thrombosis.
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COLLABORATIONS

Thesis project was carried out in collaboration with other researchers of the IRCCS Istituto di Ricerche Farmacologiche Mario Negri as follow:

- Clinical data and biological samples of the 20 cTTP patients included in this thesis work were collected by MD Elena Bresin, of the medical group of the Clinical Research Center for Rare Diseases "Aldo e Cele D'Accò" in Ranica (BG);

- DNA sequencing and ADAMTS13 mutations identification were performed by the colleagues of the same laboratory (Ph.D. Caterina Mele, Dr. Rossella Piras, Dr. Elisabetta Valoti, Marta Alberti, Ph.D. Matteo Breno, Ph.D. Paola Cucarolo, and Dr. Alessandra Cremaschi,);

- serum C3, C4 and creatinine levels were performed by Pharmacokinetics and Clinical Chemistry laboratory, coordinated by Dr. Flavio Gaspari, of the Clinical Research Center for Rare Diseases "Aldo e Cele D'Accò" in Ranica (BG);

- the technician Sara Gastoldi of the same Department (Molecular Medicine), maintained HMEC-1 cell culture and cooperated with Ph.D. student, Dr. Serena Bettoni, to perform in vitro deposition-expression studies and thrombus formation on endothelial cells;

- Dr. Miriam Galbusera, of the same Department (Molecular Medicine) measure ADAMTS13 activity and collaborated in the interpretation of data.

All other techniques, experiments and analyses described in this thesis were performed by the Ph.D. student, Dr. Serena Bettoni.
LIST OF PAPERS

The thesis is based on the following papers, referred to in the text by their first author’s name and year:

