Studies of ADAMTS13 expression and activity in the kidney

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2013

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Akademisk avhandling
som med vederbörligt tillstånd från Medicinska Fakulteten vid Lunds Universitet för avläggande av doktorsexamen i Medicinsk Vetenskap kommer att offentligen försvaras i Belfragesalen, Biomedicinskt Centrum (BMC), D15
torsdagen den 7 mars 2013, kl 09.00

Fakultetsopponent
Marina Noris
Head of The Laboratory of Immunology and Genetics of Transplantation and Rare Diseases
Mario Negri Institute for Pharmacological Research
Ranica, Bergamo, Italy

Organization	Document name
LUND UNIVERSITY Medical Faculty Department of Pediatrics Clinical Sciences Lund BMC, Tornavägen 10, 221 84 Lund, Sweden	DOCTORAL DISSERTATION
	Date of issue March 7, 2013
	Sponsoring organization

Author(s)
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Title and subtitle

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Abstract

Von Willebrand factor (VWF) is an abundant plasma glycoprotein involved in platelet adhesion and aggregation at sites of vascular injury. ADAMTS13 is the sole physiological VWF-cleaving protease thus regulating the size of thrombus growth. Dysfunctional ADAMTS13 leads to thrombotic thrombocytopenic purpura (TTP), which is either due to mutations (congenital TTP) or auto-antibodies (acquired TTP). The histopathological lesion is termed thrombotic microangiopathy and characterized by disseminated hyaline thrombi in the microvasculature of various organs including the kidney. The present study aimed to investigate ADAMTS13 expression and activity in the kidney.

Cultured renal tubular epithelial cells were shown to express biologically active ADAMTS13. Renal tissues from patients with tubulopathy exhibited an altered ADAMTS13 expression pattern in comparison to controls. ADAMTS13 was detected in urine from patients with tubulopathy suggesting local synthesis. Cultured glomerular endothelial cells were also shown to express biologically active ADAMTS13.

ADAMTS13-deficiency in mice led to glomerular capillary vessel wall thickening and platelet deposition as demonstrated by electron microscopy. ADAMTS13 was also detected in the glomerular basement membrane (GBM), however its role in VWF-cleavage in the GBM appeared to be minimal whereas serine protease activity accounted for most of the cleavage. ADAMTS13 deficiency led to complement deposition in kidney from TTP patients and from ADAMTS13-deficient mice. Plasma from TTP patients contained high levels of complement (C3 and C9)-coated endothelial microparticles. Experiments using plasma/serum samples from the patients with added normal platelets perfused over glomerular endothelial cells demonstrated C3 deposition on VWF-platelet strings and on the endothelial cells. Perfusion of patient plasma induced more release of C3- and C9-coated endothelial microparticles than control plasma.

The experimental work in this thesis provides evidence for ADAMTS13 in renal tissue including tubular cells, GBM and glomerular endothelial cells. Local production of the protease may contribute to protection of glomerular vessels from injury, in addition to circulatory ADAMTS13. ADAMTS13 deficiency promoted complement deposition which may further aggravate the vascular damage.

Key words: ADAMTS13, von Willebrand factor, Thrombotic thrombocytopenic purpura, Kidney, glomerular endothelial cells, Complement system

Classification system and/or index termes (if any):

Supplementary bibliographical information:	Language English
ISSN and key title: 1652-8220	ISBN 978-91-87189-87-6
Recipient's notes	Number of pages 169 / Price
	Security classification

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LUND
UNIVERSITY

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Coverpicture by Venkata Veera Ramesh Tati

ISSN 1652-8220
ISBN 978-91-87189-87-6
Lund University, Faculty of Medicine Doctoral Dissertation Series 2013:18

Printed in Sweden by Media-Tryck, Lund University

To my family

कर्मणयेवाधिकारस्ते मा फलेषु कदाचन।
मा कर्मफलहेतुर्भूर्मा ते सङ्गोऽस्त्वकर्मणि।

श्रीमद् भगवद् गीता 2.47

"You have a right to perform your prescribed action, but you are not entitled to the fruits of your action. Never consider yourself the cause of the results of your activities, and never shy away from duty."

Bhagavad Gita 2.47

Contents

List of papers

This thesis is based on the following papers, referred to in the text by their Roman numerals:

I. Manea M, **Tati R**, Karlsson J, Bekassy ZD, Karpman D. Biologically active ADAMTS13 is expressed in renal tubular epithelial cells. *Pediatr Nephrol*. 2010;25(1):87-96.

II. **Tati R**, Kristoffersson AC, Ståhl AL, Mörgelin M, Motto D, Satchell S, Mathieson P, Manea-Hedström M, Karpman D. Phenotypic expression of ADAMTS13 in glomerular endothelial cells. *PLoS One*. 2011;6(6):e21587.

III. **Tati R**, Kristoffersson AC, Manea-Hedström M, Wieslander J, Karpman D. Von Willebrand factor cleavage in the glomerular basement membrane is multifactorial. *Manuscript.*

IV. **Tati R**, Kristoffersson AC, Ståhl AL, Rebetz J, Wang L, Licht C, Motto D, Karpman D. Complement activation associated with ADAMTS13 deficiency. *Submitted.*

Permission to reprint the previously published articles has been granted by the respective publisher.

The following papers have been published during the same period but not included in this thesis:

1. Brackman D, Sartz L, Leh S, Kristoffersson AC, Bjerre A, **Tati R**, Frémeaux-Bacchi V, Karpman D. Thrombotic microangiopathy mimicking membranoproliferative glomerulonephritis. *Nephrol Dial Transplant.* 2011;26: 3399-3403.
2. Calderon Toledo C, Rogers TJ, Svensson M, **Tati R**, Fischer H, Svanborg C, Karpman D. Shiga toxin-mediated disease in MyD88-deficient mice infected with *Escherichia coli* O157:H7. *Am J Pathol.* 2008;173: 1428-1439.
3. Karpman D, **Tati R**. Complement activation in thrombotic microangiopathy. *Hämostaseologie.* 2013 In press.

Abbreviations

VWF	von Willebrand factor
ULVWF	Ultra-large VWF
VWF-CP	VWF cleaving protease
ADAMTS13	A disintegrin-like and metalloprotease with thrombospondin type-1 motif, 13
rADAMTS13	Recombinant ADAMTS13
GP	Glycoprotein
TTP	Thrombotic thrombocytopenic purpura
Stx	Shiga toxin
TMA	Thrombotic microangiopathy
HUS	Hemolytic uremic syndrome
STEC-HUS	Stx-producing *E. coli*-associated HUS
GBM	Glomerular basement membrane
RBC	Red blood cell
CD	Cluster of differentiation
HUVEC	Human umbilical vein endothelial cells
VEGF	Vascular endothelial growth factor
MBL	Mannose-binding lectin
MASP	MBL-associated serine protease
MAC	Membrane attack complex
HRTEC	Human renal tubular epithelial cells
PCR	Polymerase chain reaction
CiGEnC	Conditionally immortalized glomerular endothelial cells

HMVEC	Human dermal microvascular endothelial cells
PGEC	Primary glomerular endothelial cells
EDTA	Ethylenediaminetetraacetic acid
PS	Phosphatidylserine
TF	Tissue factor
CFH	Factor H

Abstract

Von Willebrand factor (VWF) is an abundant plasma glycoprotein involved in platelet adhesion and aggregation at sites of vascular injury. ADAMTS13 is the sole physiological VWF-cleaving protease, thus regulating the size of thrombus growth. Dysfunctional ADAMTS13 leads to thrombotic thrombocytopenic purpura (TTP), which is either due to mutations (congenital TTP) or auto-antibodies (acquired TTP). The histopathological lesion is termed thrombotic microangiopathy and characterized by disseminated hyaline thrombi in the microvasculature of various organs including the kidney. The present study aimed to investigate ADAMTS13 expression and activity in the kidney.

Cultured renal tubular epithelial cells were shown to express biologically active ADAMTS13. Renal tissues from patients with tubulopathy exhibited an altered ADAMTS13 expression pattern in comparison to controls. ADAMTS13 was detected in urine from patients with tubulopathy suggesting local synthesis. Cultured glomerular endothelial cells were also shown to express biologically active ADAMTS13. ADAMTS13-deficiency in mice led to glomerular capillary vessel wall thickening and platelet deposition as demonstrated by electron microscopy. ADAMTS13 was also detected in the glomerular basement membrane (GBM), however its role in VWF-cleavage in the GBM appeared to be minimal, whereas serine protease activity accounted for most of the cleavage. ADAMTS13 deficiency led to complement deposition in kidney from TTP patients and from ADAMTS13-deficient mice. Plasma from TTP patients contained high levels of complement (C3 and C9)-coated endothelial microparticles. Experiments using plasma/serum samples from the patients with added normal platelets perfused over glomerular endothelial cells demonstrated C3 deposition on VWF-platelet strings and on the endothelial cells. Perfusion of patient plasma induced more release of C3- and C9-coated endothelial microparticles than control plasma.

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Introduction

Von Willebrand factor (VWF) is a large glycoprotein involved in platelet adhesion and aggregation [1]. It is synthesized in endothelial cells [2] and megakaryocytes [3]. Endothelial cells account for more than 95% of circulatory VWF [4] which is secreted as ultra-large multimers (ULVWF) that are biologically very potent in platelet adhesion leading to thrombus formation [5].

ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type-1 motifs) is the VWF cleaving protease which regulates the size of VWF into smaller multimers thereby regulating thrombus growth. ADAMTS13 cleaves the 1605Tyr-1606Met peptide bond in the A2 domain of VWF thus releasing 140kD and 176kD VWF fragments [6,7,8]. ADAMTS13 has been identified to be synthesized by hepatic stellate cells [9], endothelial cells [10,11] and megakaryocytes [12,13].

Deficient ADAMTS13 activity leads to thrombotic thrombocytopenic purpura (TTP), which may be due to mutations in the ADAMTS13 gene (congenital TTP) [14] or due to the presence of auto-antibodies against ADAMTS13 (acquired TTP) [15,16]. TTP is characterized by thrombocytopenia, microangiopathic hemolytic anemia, fever, renal and neurological manifestations. Dysfunction of ADAMTS13 results in impairment of ULVWF degradation which in turn contributes to the formation of disseminated platelet thrombi, a characteristic feature of TTP, in various organs including the brain and the kidney [17,18]. As kidneys are the main target organ in this disease the general aim of this study was to investigate the ADAMTS13 expression and its activity in renal cells.

This thesis will provide a comprehensive overview regarding VWF, ADAMTS13 and TTP as well as a description of the complement system and the kidney. A short summary of the investigations carried out and their contribution to the field is given and followed by the papers included in the thesis.

Hemostasis

Hemostasis is the process of cessation of blood loss from an injured blood vessel and is coordinated by a complex of physiological systems including vascular spasm, platelets and activation of coagulation [19]. As depicted in figure 1, vascular damage leads to vascular spasm in which smooth muscle surrounding the vessel wall contracts leading to vasoconstriction thereby limiting the blood flow [20]. Due to endothelial cell damage, blood in the lumen comes in contact with the subendothelium containing collagen fibers. Thus platelets adhere and become activated. Activated platelets release ADP and thromboxane A_2, and bind fibrinogen which promotes aggregation of more platelets and results in formation of platelet thrombi. At sites of vascular injury the process of platelet adhesion is mediated by von Willebrand factor (VWF). These events are referred to as primary hemostasis. Secondary hemostasis is the process by which assembly and activation of plasma coagulation factors leads to activation of the clotting cascade resulting in the formation of a blood clot. This cascade involves two pathways: the intrinsic pathway (contact activation pathway) and the extrinsic pathway (tissue factor pathway) [21,22].

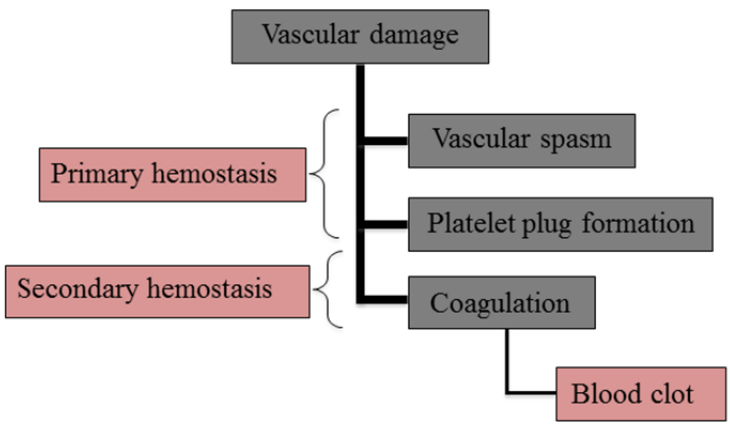

Figure 1. Hemostasis cascade

Platelets

Platelets are anucleated cells released from bone marrow megakaryocytes into the systemic circulation [23] where they are capable of surviving for up to 10 days. Normal platelet levels range from 150-350x10^9/L. Platelets contain three major storage granules: α-granules storing proteins for hemostasis, dense granules containing molecules for cell activation, and lysosomes that dissolve phagocytosed debris and secrete hydrolases. These granules account for the secretion of proteins such as VWF, platelet factor 4, fibrinogen, vitronectin, thromboxane A2, thrombin, P-selectin (membrane protein) and mediators such as ADP, calcium and serotonin [24,25].

Upon vessel wall injury, platelets play a crucial role in primary hemostasis which includes a sequence of events: platelet adhesion, activation and aggregation. Following injury the subendothelium, containing VWF, collagen and fibrinogen, is exposed. VWF immobilizes onto collagen and starts interacting with platelets. The glycoprotein (GP) Ib-IX-V complex, a constitutively expressed receptor on resting platelets, initiates platelet adhesion by binding to immobilized VWF. This interaction slows down platelets in the circulation allowing them to interact with collagen using the GPVI and $\alpha_2\beta_1$ (GPIa-IIa) receptors [25,26]. Upon activation platelets change from a normal discoid shape to a compact spherical shape with long dendritic extensions [25] (Figure 2). Activated platelets release mediators such as ADP, thromboxane A_2, and thrombin leading to platelet aggregation. Activation leads to expression of the normally cryptic GPIIb/IIIa receptor which in turn binds to VWF and fibrinogen leading to platelet aggregation and finally to thrombus formation [27].

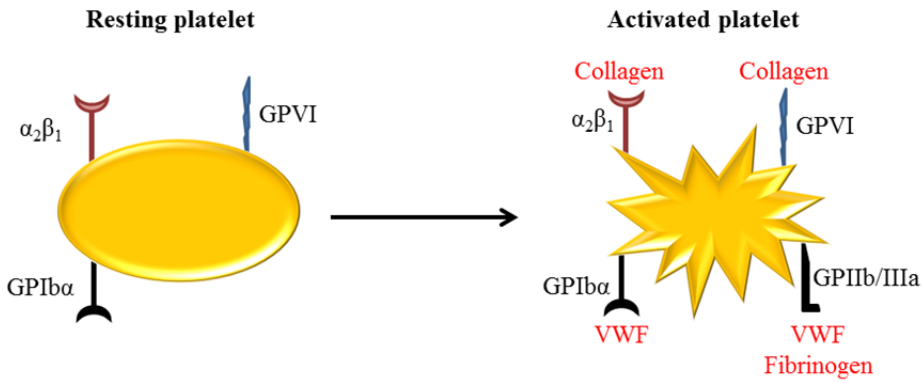

Figure 2. Schematic representation of a platelet in resting state and during activation.

von Willebrand factor

VWF, a large glycoprotein circulating in plasma, is involved in shear stress-associated platelet adhesion and aggregation during primary hemostasis [1]. Apart from platelet plug formation, VWF also stabilizes the carrier protein Factor VIII (FVIII) in the circulation [28].

History

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 hemophilia". This condition was later renamed von Willebrand disease (VWD). In 1957 it was determined that lack of a plasma factor caused this disease [29]. The first immunological detection of what is presently known as VWF was done in 1971 and initially named FVIII-related antigen [30]. VWF cloning experiments were done in 1985 [31,32,33] and a year later, the amino acid sequence was also determined [34]. Later the cause of VWD was found to be either decreased synthesis or dysfunction of VWF [35].

Structure and binding sites

The *VWF* gene is located on chromosome 12 [36] and contains 52 exons with 180kb of DNA encoding 8.2kb mRNA [33,37]. The translated product precursor protein is initially synthesized as a monomer, which is also referred to as pre-pro-VWF (Figure 3A), consisting of 2813 amino acids with a molecular weight of 380kDa [36,38]. Pre-pro-VWF contains a signal peptide (1-22), a large propeptide (23-763, also termed VWF antigen II) and a mature subunit consisting of 2050 amino acids with a molecular weight of 275kDa [1]. The pro-peptide and mature subunit are termed pro-VWF. Pre-pro-VWF contains five distinct repeated domains (A-D) in the order: D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK, which mediate interactions with other molecules [39,40].

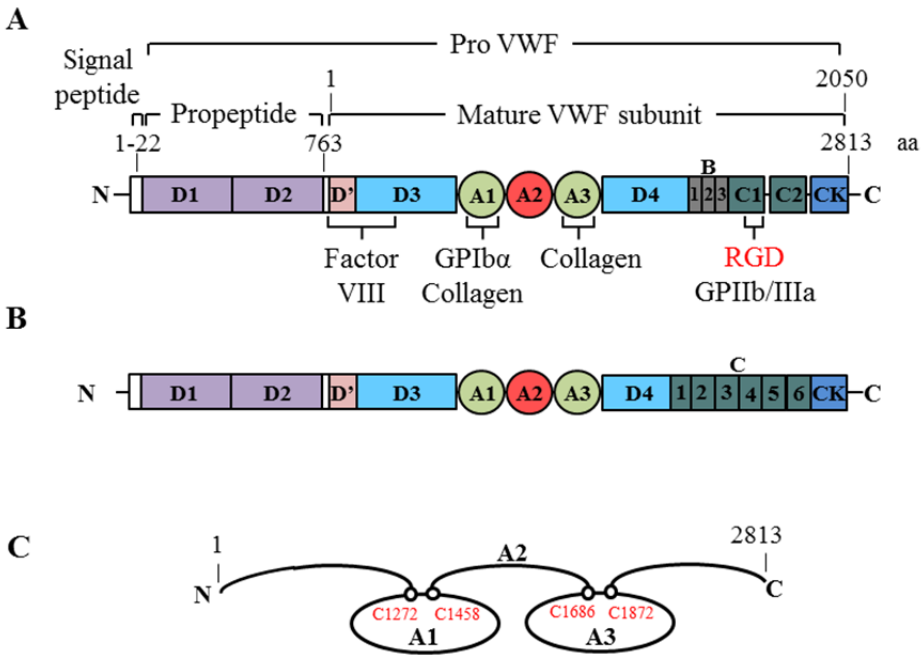

Figure 3. A. Pre-pro-VWF structure with indication of binding sites for factor VIII, collagen and platelet receptors. Amino acid (aa) numbers are depicted. B. Re-evaluated structure of VWF. C. Schematic depiction of VWF showing two loops formed by intramolecular disulfide bonds in the A1 and A3 domains.

The D'-D3 domain contains binding sites for factor VIII [41] and heparin [42]. The A1 domain contains a binding site for platelet GPIbα [43,44] in the GPIb-IX-V complex and collagen VI. The A3 domain contains binding sites for collagen I and III [45,46] (Figure 3A). The C1 domain contains a Arg-Gly-Asp (RGD) sequence and is the binding site for the platelet integrin receptor $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) [47].

A recent study has re-evaluated and updated the VWF domain structure based on the structure of homologous domains and electron microscopy. According to the re-design, the D4-CK domains comprise 6 C domains and the RGD sequence is located in the C4 domain [48] (Figure 3B). The A1 and A3 domains contain intramolecular disulfide bonds between Cys1272-Cys1458 and Cys1686-Cys1872 thereby generating two loops with identical amino acid length [49] (Figure 3C).

Biosynthesis and maturation

VWF is synthesized exclusively in endothelial cells [2] and megakaryocytes [3,50] as a precursor protein (pre-pro-VWF). During the maturation process (Figure 4) in the endoplasmic reticulum, the signal peptide is cleaved off and 12 N-linked oligosaccharide chains are added. Following glycosylation pro-VWF forms dimers via intermolecular disulfide bonds at the C-terminus.

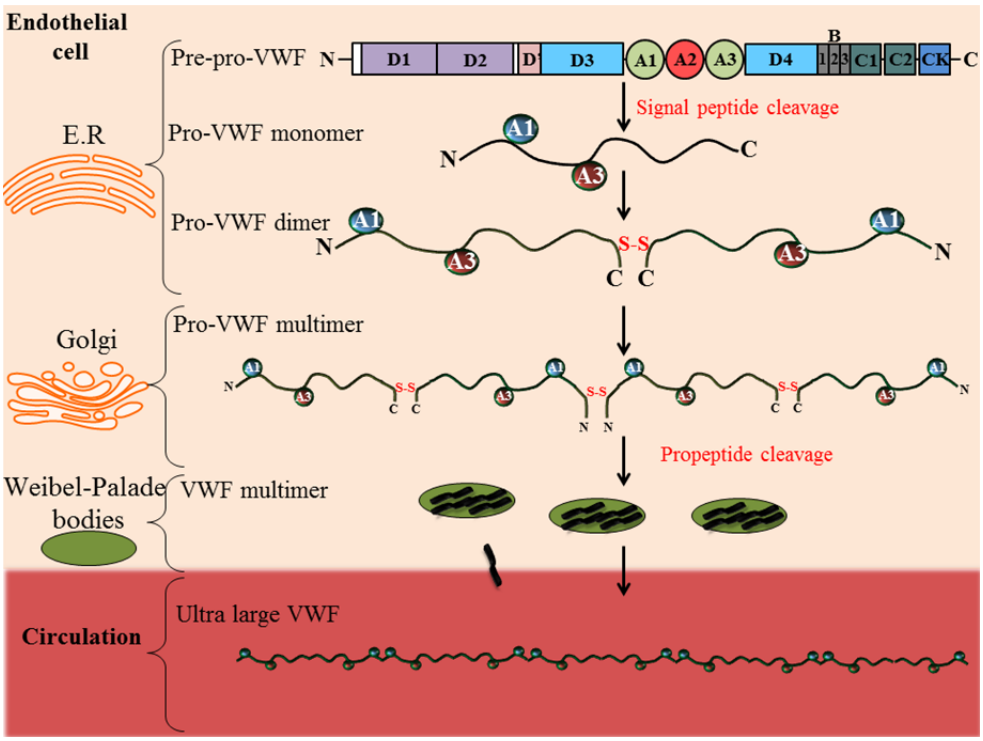

Figure 4. Schematic representation of VWF synthesis, maturation and storage. S-S represents the locations of intersubunit disulfide bonds. E.R: endoplasmic reticulum.

Upon transportation to the Golgi apparatus, 10 O-linked oligosaccharide chains are also added. The processed pro-VWF dimers, during the transportation through the trans Golgi network, form multimers via interdimer disulfide bonds at the N-termini. During the process of multimerization the propeptide, required for trafficking of the multimers to storage, is cleaved off [51,52,53].

Secretion

From endothelial cells a fraction of VWF multimers is released constitutively into the circulation and the remaining multimers are stored in Weibel-Palade bodies, which are released in a regulated fashion [54,55,56] (Figure 4). The stored components from Weibel-Palade bodies are released as ultra-large (ULVWF) multimers in response to a variety of physiological stimuli such as thrombin, histamine, fibrin, complement C5b-9 or *in vitro* by calcium ionophore or phorbol myristate acetate (PMA) [51]. Histamine induces VWF release from endothelial cells by interacting with the H1 receptor [57]. Upon release these ultra-large multimers form long string-like structures (Figure 4) by binding to endothelial cells via interaction with P-selectin or the integrin receptor $\alpha_v\beta_3$ [58,59]. In megakaryocytes VWF is stored in α-granules and is released as ULVWF multimers only upon activation [60]. As α-granular components are only released from activated platelets, circulating VWF is considered to be of endothelial origin [61] accounting for > 95% of plasma VWF [4]. VWF multimers have a half-life of about 12 hrs and a plasma concentration of approximately 10 μg/ml [62]. The mature VWF multimers range in size from 500 kDa (dimer) to 20,000 kDa (ULVWF multimers) in the circulation [63].

VWF interaction with platelets

VWF is an adhesive glycoprotein and performs its hemostatic function by linking platelets and the subendothelium. During vascular injury, subendothelial collagen is exposed and then subendothelial or circulatory VWF immobilizes onto collagen via the A3 domain [64] (Figure 5A). VWF was shown to bind collagen type I, III and VI in the subendothelial matrix [40,65].

Under conditions of high shear stress immobilized VWF unfolds from the native globular state to an extended chain conformation thereby exposing platelet binding sites [66]. The A1 domain of immobilized VWF interacts with platelet GPIbα and initiates the tethering of circulating platelets. This interaction can only mediate platelet rolling but not firm attachment, and leads in turn to the expression of GPIIb/IIIa on the platelet membrane [67]. An additional binding between the RGD sequence in VWF and GPIIb/IIIa promotes platelet aggregation and thrombus formation [68,69]. Generally, circulating VWF does not interact with platelet GPIbα but surface bound VWF is able to bind even when there is no shear [70] whereas at high shear VWF interacts with both GPIbα and GPIIb/IIIa [71].

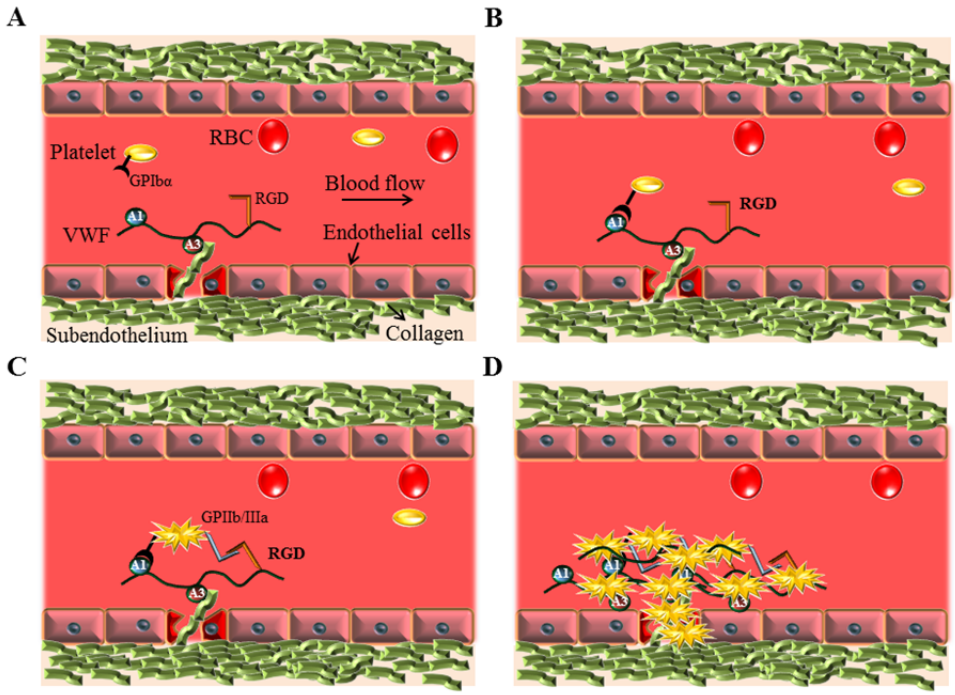

Figure 5. Schematic representation of VWF platelet interaction on a damaged blood vessel. A. Following endothelial damage collagen is exposed to the circulation and consequently VWF immobilized onto collagen. B. Platelet adhesion. C. Platelet activation. D. Platelet aggregation, VWF self-association and thrombus growth.

In comparison to the largest VWF multimers found in normal plasma, ULVWF synthesized by the regulated pathway is more effective in platelet aggregation under shear stress [5]. Due to a higher number of binding sites ULVWF exhibits more platelet adhesion by forming high strength bonds [72]. In addition, fibrinogen, a bivalent ligand, binds to GPIIb/IIIa on two activated platelets thereby tethering platelets leading to thrombus formation [26]. Following platelet aggregation circulating VWF interacts with activated platelets that leads to adhesion of more platelets resulting in thrombus growth [68]. In addition, self-association of VWF multimers may further promote thrombus growth [73].

To prevent excessive thrombus formation, ULVWF multimers are cleared from the circulation by breakdown to smaller multimers [74]. Proteolytic cleavage by the metalloprotease ADAMTS13 is the physiologically most important mechanism inhibiting excessive thrombus formation due to the presence of ULVWF multimers.

ADAMTS13

In 1982 Moake et al provided the first description of defective proteolytic processing of VWF, a multimeric glycoprotein that plays an essential role in platelet-mediated primary hemostasis [74]. VWF was later shown to be cleaved at the Tyr1605-Met1606 bond in the A2 domain resulting in the formation of 140 and 176 kDa fragments [6]. In 1996, two groups partially purified a metalloprotease in normal plasma that specifically cleaves at the aforementioned peptide bond and named it VWF-cleaving protease (VWF-CP) [75,76]. The protease requires VWF in an open conformation, which may be achieved by denaturing agents such as urea [75] or guanidine HCl, or by high shear stress [76]. VWF-CP requires divalent cations such as Zn^{2+}, Ca^{2+}, Ba^{2+} or Co^{2+} for its proteolytic activity [76]. In 2001 the VWF-CP was purified, cloned and characterized, and identified as a new member of the super family ADAMTS designated ADAMTS13 [7,8,14,77,78]. Later studies using recombinant ADAMTS13 (rADAMTS13) confirmed the physiological VWF cleavage pattern [79].

Molecular biology of ADAMTS13

Gene structure

The *ADAMTS13* gene is located on chromosome 9 at region 9q34 and contains 29 exons spanning 37kb in length (Figure 6). It transcribes into full-length 4.7kb mRNA which has been detected by northern blot in human liver [8,14,78]. Using reverse transcription polymerase chain reaction (RT-PCR) ADAMTS13 mRNA has been shown in various other human tissues including the heart, lungs, brain, kidneys, pancreas, adrenal glands, placenta, uterus, ovaries and prostate. Several splice variants were found during cloning and sequencing and a 2.4kb transcript has been found in the placenta, skeletal muscle and some tumor cell lines but their physiological significance remains unknown [8,14,78,79,80].

Protein structure

The full-length transcript translates into a polypeptide of 1427 amino acid residues (Figure 6). The sequence contains 10 consensus sites for N-linked glycosylation, many sites for O-linked glycosylation and one consensus site for C-mannosylation [8]. In consistency with other secretory proteins, ADAMTS13 is intracellularly localized in the ER and Golgi apparatus [81].

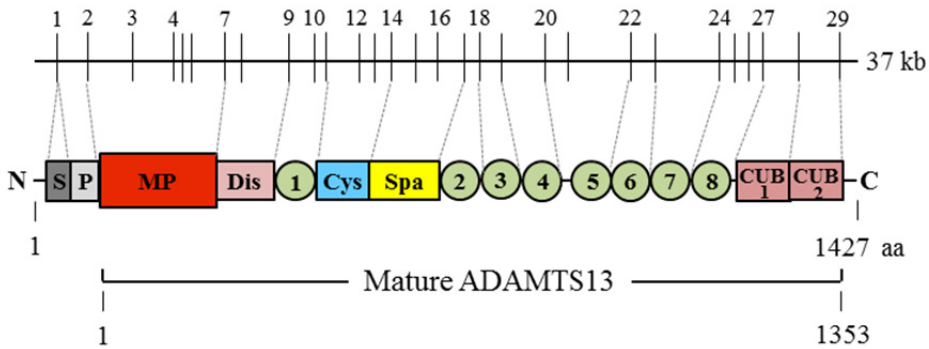

Figure 6. The ADAMTS13 gene (depicted on the top of the figure) contains 29 exons and is 37kb in length. The protein contains 1427 amino acid (aa) residues with specified domains [8,14]: the signal peptide (S, 1-33 residues), propeptide (P, 34-74 residues), metalloprotease domain (MP, 75-289 residues), disintegrin-like domain (Dis, 290-385 residues), first thrombospondin-1 repeat (TSP-1, denoted as "1", 386-439 residues), cysteine-rich domain (Cys, 440-555 residues), spacer domain (Spa, 556-685 residues), seven additional TSP-1 repeats (2-8, 686-1131 residues) and two CUB domains (CUB1, CUB2, 1192-1408 residues) standing for Complement components C1r and C1s, sea urchin protein Uegf and Bone morphogenetic protein-1 [82].

ADAMTS13 is the 13th member of the ADAMTS superfamily which has 19 distinct members, differing in number and types of domains. ADAMTS13 exhibits a multi-domain structure (Figure 6). 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 [8]. Gel electrophoresis analysis reveals, however, a molecular weight of 150 kDa and 190 kDa under non-reducing and reducing conditions, respectively [7]. The discrepancy is due to extensive glycosylation and other post-translational modifications [8].

Secretion

A major source of plasma ADAMTS13 was previously attributed to hepatic stellate cells. Synthesis in these cells was demonstrated by *in situ* hybridization and immunohistochemistry [9,83]. Cultured primary rodent hepatic stellate cells secreted full-length ADAMTS13 capable of cleaving VWF. Similar results were obtained using cell lines derived from human and rat hepatic stellate cells [83,84]. ADAMTS13 is secreted from these cells upon activation [9]. Activation of hepatic stellate cells occurs *in vivo* during liver fibrosis and cirrhosis. *In vitro* and *in vivo* studies in which rat liver cells were injured by injection of carbon tetrachloride showed hepatic increase of ADAMTS13 but a minimal effect on plasma levels, suggesting that secretion from hepatic stellate cells marginally affects plasma levels [84].

Following these studies ADAMTS13 expression was also demonstrated in endothelial cells [10,11,85] and in megakaryocytes or platelets [12,13] from which it is secreted into the plasma as an active enzyme. Cell culture experiments using primary human umbilical vein endothelial cells (HUVECs), primary human umbilical artery endothelial cells (HUAECs), primary human aortic endothelial cells (HAECs) and microvascular endothelial cell lines (EVC304) all exhibited secretion of ADAMTS13 with VWF cleaving capacity *in vitro* [10,11,85]. Considering the large surface area of the endothelium, plasma ADAMTS13 may thus be derived from endothelial cells [10,11]. The concentration of ADAMTS13 in plasma is approximately 1 μg/ml [77] and its half-life is of 2-3 days [86].

ADAMTS13-VWF interaction

The process by which ADAMTS interacts with VWF and cleaves the scissile bond has been proposed to occur according to the following steps (Figure 7) [87]:

Step 1: Circulating VWF is in globular form and ADAMTS13 can bind via the TSP5-CUB domain to the D4-CK domains of VWF.

Step 2: Shear-dependent unfolding of VWF leads to exposure of additional binding sites.

Step 3: The Arg660/Tyr661/Tyr665 sequence in the spacer domain of ADAMTS13 interacts with the Glu1660-Arg1668 in A2 domain of VWF.

Step 4: Arg349 in the disintegrin-like domain of ADAMTS13 binds to Asp1614 in the A2 domain of VWF.

Step 5: The S3 subsite (Leu198, Leu232 and Leu274) in the ADAMTS13 metalloprotease domain binds to Leu1603 in the VWF A2 domain.

Steps 3-5 bring the Tyr1605-Met1606 peptide bond in VWF closer to the ADAMTS13 active site.

Step 6: S1 (Leu151/Val195) and S1´ (Asp252-Pro256) subsites in ADAMTS13 bind to Tyr1605 and Met1606 residues, respectively.

Step 7: Proteolysis.

Figure 7. ADAMTS13 interaction and cleavage of VWF. Ca^{2+} and Zn^{2+} binding motifs in the metalloprotease domain and the location of the RGD sequence in the cysteine-rich domain are shown. The metalloprotease domain possesses the catalytic site that cleaves VWF and the regions between the disintegrin domain and the spacer domain interact with the A2 domain. Carboxy-terminal regions interact with VWF under flow. Black arrows indicate various binding sites. ADAMTS13 cleaves mature VWF at the Tyr842-Met843 (Tyr1605-Met1606 in pre-pro-VWF) peptide bond (red arrow) generating two cleavage fragments of 140 kDa and 176 kDa size.

Cleavage of VWF

The only known substrate for ADAMTS13 is VWF [75], of which it cleaves the peptidyl bond between Tyr1605-Met1606 (Tyr842-Met843 in the mature VWF subunit) in the A2 domain. Cleavage generates 140kDa and 176kDa fragments from the N-terminal and the C-terminal regions, respectively [6] (Figure 7). A functional substrate, VWF73 with 73 amino-acid residues corresponding to Asp1596-Arg1668 in the A2 domain, has been shown to be the minimal substrate

region for ADAMTS13 [88]. Apart from ADAMTS13, four leukocyte proteases: elastase, proteinase 3 (PR3), cathepsin G and matrix metalloprotease 9 (MMP9), have been revealed to cleave VWF at or near the ADAMTS13 cleavage site but the physiological contribution of this cleavage is, as yet, unknown [89]. Thus ADAMTS13 is considered to be the physiological VWF cleaving protease.

Functional importance of domains

The propeptide of ADAMTS13 lacks "cysteine-switch" motifs and hence the secreted protease is in active form before propeptide cleavage, which is generally an activation step in other metalloproteases [90]. Studies in which the propeptide was deleted did not show any effect on secretion or activity suggesting that the propeptide has no role in folding or secretion of ADAMTS13 [91]. The metalloprotease domain is the catalytic domain containing binding sites for Zn^{2+}, Ca^{2+} based on inhibitory assays with chelating agents such as ethylenediaminetetraacetic acid (EDTA). The requirement of metal ions for ADAMTS13 activity has thus been demonstrated [75,76]. Various studies using C-terminal truncation of recombinant ADAMTS13 have revealed that the metalloprotease domain alone is not sufficient for cleavage of VWF [81,92].

Based on studies using truncated ADAMTS13, the disintegrin-like domain was shown to be necessary for enzyme activity and specificity [93]. The thrombospondin type-1 repeats play a role in binding glycosaminoglycans and/or CD36 (cluster of differentiation), a cell surface receptor also known as the thrombospondin receptor [94]. The spacer domain is essential for effective VWF cleavage activity [81]. The two CUB domains were shown to be important for apical secretion of ADAMTS13 in endothelial cell cultures [10]. Furthermore, perfusion studies under flow have suggested that there is joint activity between the C-terminal TSP and CUB domains important for proteolytic activity of ADAMTS13 [95]. Taken together, these findings indicate that the metalloprotease is responsible for the catalytic activity of ADAMTS13, and the remaining domains between the metalloprotease domain and the spacer domain are crucial for substrate recognition and cleavage. The TSP-1 and distal CUB domains may be crucial in recognition of VWF under flow shear stress.

Regulation of ADAMTS13 activity

ADAMTS13 cannot cleave VWF in the globular form as the Tyr1605-Met1606 peptide bond is cryptic in the A2 domain. In order to unfold the A2 domain under static conditions denaturing agents such as urea or guanidine HCl are required, whereas under high shear stress it occurs rapidly in the absence of these agents [75,76]. VWF cleavage activity may be positively or negatively regulated by heparan sulfate, platelet GPIbα, sodium chloride and inflammatory cytokines such as interleukin-6 [96,97,98]. Thrombin, plasmin, factor Xa and elastase can regulate ADAMTS13 activity either by cleaving or inactivating the protease [99,100,101].

ADAMTS13 regulates thrombus growth

UL-VWF multimers released from storage granules bind to endothelial cells via P-selectin or integrin $\alpha_v\beta_3$ [58,59] or to exposed collagen via the A3 domain at sites of vascular injury [64]. From perfusion experiments ADAMTS13 has been shown to enhance the cleavage of VWF specifically when bound to endothelial cells rather than in fluid phase [102]. Under conditions of high shear force immobilized VWF unfolds thus exposing binding sites, and the A1 domain interacts with platelet GPIbα that initiates platelet adhesion. An additional binding between the RGD sequence in VWF and the GPIIb/IIIa receptor on platelets promotes ULVWF-platelet aggregation and thrombus formation [68,69]. Shear-induced unfolding exposes the ADAMTS13 binding sites on VWF and also the scissile peptide bond. ADAMTS13 cleaves VWF into smaller multimers by proteolysis at the A2 domain, thereby regulating the size of thrombi [87]. Dysfunctional ADAMTS13 leads to accumulation of long ULVWF-platelet strings leading to disseminated platelet thrombi in various organs [17,18] and this pathological lesion is known as thrombotic microangiopathy (TMA) [103]. In the context of ADAMTS13 deficiency or dysfunction it is related to the clinical condition known as thrombotic thrombocytopenic purpura (TTP) [14,15,16]. TTP is a subtype of thrombotic microangiopathy.

Thrombotic microangiopathy

Thrombotic microangiopathy (TMA) is a pathological lesion characterized by microvascular thrombosis leading to thrombocytopenia and microangiopathic hemolytic anemia with fragmented red blood cells (RBCs) also known as schistocytes [104]. Vessel walls are thickened mainly in arterioles and capillaries due to swelling and detachment of endothelial cells from the basement membrane and accumulation of hyaline amorphous material in the subendothelial space. Intraluminal thrombosis results in partial or complete occlusion of the vessel lumen [103]. TMA is a feature of several clinical disorders including TTP and hemolytic uremic syndrome (HUS).

Thrombotic thrombocytopenic purpura

History

TTP was first reported by Eli Moschcowitz in 1924 in a 16-year old girl with hemolytic anemia, acute fever, heart failure, paralysis, coma and death within 2 weeks. Autopsy revealed widespread hyaline thrombi in terminal arterioles and capillaries of the heart and kidney [18]. In 1960, Schulman reported a case of an 8-year old girl with repeated episodes of thrombocytopenia and hemolytic anemia who was treated with plasma infusions [105]. The disease was clinically established in 1966 as a pentad of symptoms: thrombocytopenia, hemolytic anemia, renal dysfunction, neurological symptoms and fever [106]. Later in 1978, Upshaw reported a 29-year old woman with similar symptoms since infancy. This patient responded to plasma infusion, which was suggested to replace a proposed deficient plasma factor [105]. The disease was therefore named the Upshaw-Schulman syndrome. In 1982, Moake described patients with chronic relapsing TTP with circulating ULVWF, which was proposed to be due to deficiency of a VWF depolymerase [74], later identified as VWF-CP [75,76]. VWF-CP was later purified, cloned and designated as ADAMTS13 [7,8].

Classification and disease manifestation

TTP is classified into two subtypes: congenital and acquired TTP. Congenital TTP, also referred to as Upshaw-Schulman syndrome, is associated with severe deficiency of ADAMTS13 due to *Adamts13* gene mutations [14]. Acquired TTP is associated with inhibiting auto-antibodies against ADAMTS13 [15,16]. As depicted in figure 8, in healthy individuals immobilized ULVWF multimers are cleaved by ADAMTS13 into smaller and less thrombotic forms thereby preventing spontaneous platelet activation and limiting thrombus growth. Conversely, in patients with TTP, either due to deficiency (congenital TTP) or due to inhibitory antibodies (acquired TTP), VWF-dependent platelet accumulation persists [87].

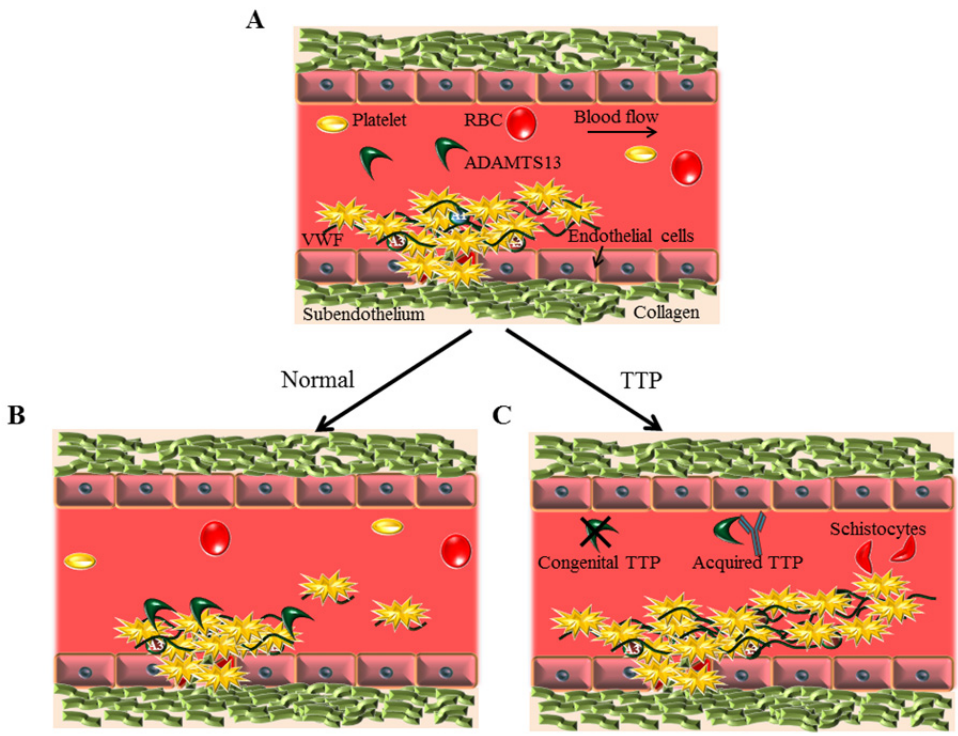

Figure 8. ADAMTS13 dysfunction leads to TTP. A. Upon endothelial damage, VWF immobilizes onto exposed collagen and mediates platelet adhesion, activation and aggregation leading to thrombus growth. B. In healthy individuals, ADAMTS13 cleaves the unfolded VWF thereby limiting thrombus growth. C. In TTP either due to deficiency (congenital TTP) or due to inhibitory auto-antibodies (acquired TTP) thrombus growth proceeds leading to thrombocytopenia due to platelet consumption and hemolytic anemia with fragmented red blood cells RBCs or schistocytes.

This uncontrolled thrombus growth leads to platelet consumption and thrombocytopenia as well as fragmentation of RBCs (schistocytes) resulting in hemolytic anemia. Renal manifestations include proteinuria, hemoglobinuria and renal dysfunction which may lead to acute renal failure. Neurological manifestations may be mild to severe and include seizures and coma [107]. The histopathological hallmark of the disease is disseminated hyaline thrombi in the microvasculature of various organs including the heart, kidneys, brain, liver, spleen and adrenal glands [17]. The disseminated thrombi are rich in VWF and platelets and obstruct the blood flow thereby leading to ischemia [108].

Depending on the location, mutations in the *Adamts13* gene result in impaired protein synthesis, secretion or activity. By 2011, 77 mutations were recognized to be associated with congenital TTP including 7 splice mutations, 10 frameshift deletions, 4 frameshift insertions, 11 nonsense mutations and 45 missense mutations [90]. Single nucleotide polymorphisms (SNPs) have been identified of which 8 have been expressed and shown to affect expression, secretion and activity of ADAMTS13 while 18 are silent [90]. Half of the patients with congenital TTP present with the first acute episode during childhood and the remainder present as adults [109].

Auto-antibodies against ADAMTS13 have been shown to inhibit the proteolytic activity or promote rapid clearance from the circulation. In patients with acquired TTP, IgG antibodies are the most frequent inhibitors [16,110] followed by IgM in 11% of patients [111].

Treatment

Severe deficient ADAMTS13 activity (<5% of normal plasma) is considered to be sufficient to cause TTP [112] and plasma replacement has been shown to be an effective treatment. Congenital TTP is treated at acute episodes by infusion of fresh frozen plasma (FFP) containing donor ADAMTS13 to compensate for the deficiency. Prophylactic treatment with FFP every 2-3 weeks is used to prevent relapses [113]. Acquired TTP is treated by plasma exchange as it removes the inhibitory antibodies and also replenishes the proteolytic activity. In addition to plasma exchange, corticosteroids have also been used [114]. For those patients who do not achieve sustained remission despite treatments, splenectomy can be an effective treatment [115]. Rituximab, a monoclonal antibody against the B cell receptor CD20 has been used to remove antibody-producing B cells [116].

ADAMTS13 deficiency alone may not be sufficient to induce TTP

Generally ADAMTS13 dysfunction leads to disseminated platelet thrombi in various organs culminating in symptomatic TTP. Patients may have repeated episodes of TTP and some episodes may occur without any clinical signs. Apart from ADAMTS13 deficiency, additional genetic or environmental triggers have been shown to initiate acute episodes of TTP. For example, conditions such as pregnancy [117], infection [118] and surgery [119] may trigger illness. Certain patients present in infancy while their family members, bearing the same genetic abnormalities, may develop symptoms at an adult age or may never develop symptoms [109].

Adamts13-deficient mouse models (on a mixed genetic background C57BL/6 and 129x1/Sv) are symptom-free. By introducing genetic backgrounds such as CASA/Rk [120] or CAST/Ei [121], which have higher endogenous VWF, the mice developed a spontaneous TTP-like phenotype. However, mouse plasma VWF levels did not correlate to the development of TTP, suggesting that higher VWF levels were not a risk factor [120]. In addition, Shiga toxin (Stx) injection (to induce endothelial cell injury) has been shown to trigger a TTP-like syndrome in mice with a susceptible genetic background [120,121,122]. Though Stx [123] or Stx B (binding) subunit alone [121] have been shown to induce secretion of ULVWF from cultured human endothelial cells *in vitro*, there is no typical exposure to Stx in TTP patients (except in exceptional cases) and the toxin was only used in order to trigger endothelial cell damage and study its effect on vulnerability to the TTP phenotype. Another study with *Adamts13*-deficient mice showed that lack of ADAMTS13 was sufficient to generate ULVWF but spontaneous TTP developed first after injection of collagen and epinephrine [124]. A recently developed *Adamts13*-deficient mouse model was shown to develop TTP-like symptoms, which were triggered by injecting human recombinant VWF including ULVWF multimers. These mice were protected prophylactically and therapeutically by administration of rADAMTS13 [125].

In a baboon (*Papio ursinus*) model ADAMTS13 deficiency, achieved by injection of an inhibitory anti-ADAMTS13 antibody, was in itself sufficient to induce TTP without any additional triggers. In spite of developing TTP with all the clinical features, the baboons survived. The reason for this is unclear, possibly due to distinct differences between humans and baboons or the lack of additional trigger factors [126].

Hemolytic uremic syndrome

HUS is characterized by a triad of symptoms: thrombocytopenia, microangiopathic hemolytic anemia and acute renal failure [127]. Based on etiology, HUS is divided into two major subtypes: diarrhea-associated (D+) or typical HUS, the most common form (> 90% of HUS), caused by Stx-producing bacteria, mostly serotypes of *Escherichia coli* (STEC) [128,129] and diarrhea-negative (D-) or atypical HUS caused by dysregulation of the alternative pathway of the complement system [130].

D+ HUS: Shiga toxin

Stx is a bacterial toxin initially identified in *Shigella dysenteriae*. D+HUS is caused by Stx-producing bacteria, usually enterohemorrhagic *E. coli* (EHEC) also called Stx producing *E. coli*. The most frequently isolated *E. coli* serotype in cases of D+ HUS is *E. coli* 0157:H7 [131,132]. Stx is divided into two major subtypes: Stx1, which is almost identical to Stx from *S. dysenteriae* and Stx2, which has approximately 50% homology with Stx1. Stx is composed of one 32 kDa A subunit, an enzymatically active part, linked to 5 B subunits (7.7 kDa each) possessing cell surface binding properties [131,133]. Toxin binds to the cell surface globotriaosylceramide (Gb3) receptor and thus undergoes endocytosis. The A subunit is proteolytically cleaved resulting an active A1-subunit. The A1-subunit binds and cleaves ribosomal RNA 28S of the 60S subunit and inhibits protein synthesis thereby mediating cell death [134].

Atypical HUS

Atypical HUS is associated with defective regulation and/or increased activation of the alternative complement pathway caused by mutations in factor H (CFH), factor I, membrane cofactor protein (MCP), clusterin, thrombomodulin, C3 or factor B due to mutations or risk-associated polymorphisms of the genes [130,135,136]. The mutations may cause loss-of-function in regulators or hyperfunctional complement factors (C3 and factor B) thus leading to uncontrolled complement activation via the alternative pathway. The complement system is described below followed by a description of complement activation in TMA.

The complement system

The complement system was discovered in the late 19th century as heat-labile components of plasma that enhances the opsonization and killing of bacteria. It has a primary function in host defense and also in disposal of foreign cells, microorganisms and cell debris either by direct lysis or by recruiting leukocytes, stimulating phagocytosis and cytotoxicity [137]. It includes more than 40 plasma and cellular proteins including activators, receptors and regulators [138]. The complement system is composed of three pathways: the classical, lectin and alternative pathways (Figure 9). The common central reaction in all three pathways is the cleavage of C3 to C3a and C3b by C3 convertases, generated by three different recognition and activation pathways.

The classical pathway can be triggered by antigen-antibody (IgG or IgM) complexes (immune complexes), which bind to the Ca^{2+} dependent C1 complex ($C1qr_2s_2$): C1q activates C1r, which activates C1s. C1s binds and cleaves C4 to C4a and C4b, which binds to the cell surface. C4b binds to C2 in the presence of Mg^{2+}. C2 is cleaved by C1s to form C2b and C2a. The latter binds C4b to form the cell bound C3 convertase C4b2a [139,140].

The lectin pathway is activated by the binding of mannose-binding lectins (MBL) or ficolins to mannose or other pathogen-associated molecular patterns (PAMPs) via interaction with MBL-associated serine proteases (MASPs) [141]. This binding cleaves and activates MASPs. The MASPs, in similarity to C1r and C1s (from the classical pathway), cleave C4 and C2 to generate the C3 convertase C4b2a. The C3 convertase cleaves C3 to C3a and C3b. C3b binds to formed C3 convertase and forms C4b2a3b, a C5 convertase that cleaves C5 to C5a and C5b.

The alternative pathway may be triggered on the surface of pathogens or on altered host cells. C3 is spontaneously hydrolyzed to form $C3(H_2O)$, which binds to factor B (CFB) in the presence of Mg^{2+}. Factor D (FD) cleaves CFB generating $C3(H_2O)Bb$. This initial C3 convertase cleaves C3 to C3a and C3b. C3b may bind to cell surfaces [142] and also binds to CFB, which is cleaved by FD generating C3bBb (C3 convertase) to further cleave more C3. Properdin (P) binds to and stabilizes this complex. By more cleavage of C3 and binding of CFB to C3b the reaction is further amplified in the alternative pathway amplication loop [143]. Thus all pathways of complement, regardless of the initiating factor, may be amplified via this amplification loop.

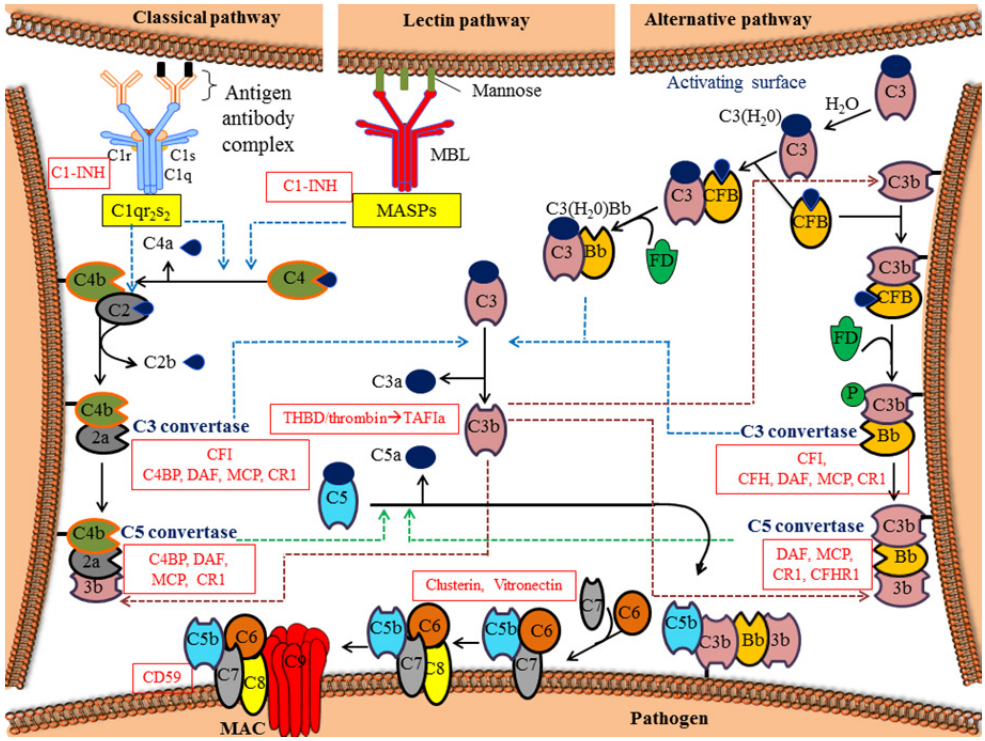

Figure 9. Schematic representation of complement activation pathways: classical, lectin, alternative pathways and the common terminal pathway. Complement activation occurs on the surface of pathogenic cells and altered host cells. Regulators are marked in red rectangles. C1NH: C1 inhibitor, MBL: Mannose-binding lectin, MASPs: MBL-associated serine proteases, CFB: factor B, FD: factor D, P: properdin, THBD: thrombomodulin (in complex with thrombin), TAFIa: thrombin-activatable fibrinolysis inhibitor, CFI: factor I, CFH: factor H, C4BP: C4 binding protein, DAF: decay accelerating factor, MCP: membrane cofactor protein, CR1: complement receptor 1, MAC: membrane attack complex. This figure was published in [144].

C3b binding to C3 convertase (C3bBb) forms the C5 convertase (C3bBb3b). The C5 convertase cleaves C5 to C5a and C5b. C5b formed by all the pathways is initially bound to the C5 convertase and further binds to C6 and C7 thereby forming a hydrophobic complex. This complex releases from the convertase and attaches onto the cell surface. C8 binds to this complex and thereafter multiple C9 molecules thus forming a pore-shaped structure on the cell surface. This structure is the C5b-9 complex also known as the membrane attack complex (MAC) causing the cell lysis [145].

In addition to cell lysis, complement components such as C3a, C4a and C5a, possess antimicrobial and/or anaphylatoxic and chemotactic properties.

Furthermore, C3b, iC3b and C4b function as opsonins by binding onto cells and allowing them to undergo phagocytosis. Excessive complement activation may lead to severe effects on host cells and tissues. A set of fluid phase and membrane bound regulators control the complement system.

Complement regulators

The regulators of complement are shown in Figure 9 and Table 1. Most of the regulators belong to the 'regulators of complement' (RCA) superfamily. This family includes fluid phase regulators: CFH and C4b-binding protein (C4BP) and membrane bound regulators: decay acceleration factor DAF (CD55), complement receptor 1 CR1 (CD35), membrane cofactor protein MCP (CD46). These regulators function either as cofactors for plasma protease factor I and/or by accelerating decay of C3/C5 convertases. In addition, C1inhibitor regulates the C1 complex and MASPs and CD59 regulates formation of the C5b-9 complex [138].

Table 1: Inhibitors of the complement system

Complement regulator	Complement pathway	Fluid-phase or membrane-bound	Mechanism of inhibition	Reference
Factor H	Alternative	Fluid phase	Co-factor for factor I in cleavage of C3b; accelerates decay of the C3 convertase and preferentially recognizes host cells	[140]
Factor H-related protein 1	Terminal	Fluid phase	Inhibits the C5 convertase	[146]
Factor I	Alternative and classical	Fluid phase	Cleaves C3b or C4b to the inactive forms in presence of cofactors factor H, C4 binding protein, MCP or CR1	[147]
CD46 (MCP)	Alternative, classical and terminal	Membrane-bound	Cofactor for factor I in cleavage of C3b	[148,149]
Thrombomodulin	Alternative, classical and terminal	Membrane-bound	Enhances factor I-mediated inactivation of C3b in the presence of factor H. The lectin-like domain inhibits the classical and lectin pathways. Generation of TAFIa[1], which inactivates C3a and C5a	[150]
C1-inhibitor	Classical and lectin	Fluid phase	Binds to C1r and C1s removing them from C1q, or binds to MASPs	[151]
C4 binding protein	Classical, lectin and terminal	Fluid phase	Decay accelerating activity (C3 and C5 convertases) and cofactor for factor I	[152]
Complement receptor 1 (CR1, CD35)	Alternative, classical and terminal	Membrane-bound	Decay accelerating activity and cofactor for factor I	[153]
CD55 (DAF)	Alternative, classical and terminal	Membrane-bound	Inhibits assembly and promotes decay of C3 and C5 convertases	[154]
Clusterin	Terminal	Fluid phase	Inhibits MAC formation	[155]
Vitronectin	Terminal	Fluid phase	Inhibits MAC formation	[156]
CD59	Terminal	Membrane-bound	Inhibits MAC formation	[157]
Carboxypeptidase N	Alternative and terminal	Fluid phase	Cleavage and partial inactivation of C3a and C5a	[158]

MCP: membrane co-factor protein; DAF: decay accelerating factor; MAC: membrane attack complex; MASP: Mannose-binding lectin- associated serine protease; TAFI: thrombin-activatable fibrinolysis inhibitor. [1] Thrombomodulin is a cofactor for thrombin-mediated activation of TAFI to TAFIa. TAFIa is a plasma procarboxypeptidase B that inactivates C3a and C5a by removal of an arginine. This table was published in [144].

Excessive complement activation plays a role in the pathogenesis of several inflammatory or auto-immune diseases [138]. Dysregulation leads to an inflammatory process including generation of anaphylatoxins, enhancing and maintaining endothelial injury by upregulating adhesion proteins, recruiting and extravasation of PMNs and platelet activation. These events may contribute to the endothelial injury and thrombus formation occurring in TMA [138,159]. Thus complement activation may contribute to formation of the TMA lesion.

Complement activation in TMA

ADAMTS13 deficiency leads to accumulation of ULVWF inducing endothelial and platelet activation resulting in the TMA lesion. Complement has no primary role in this interaction but there is evidence for complement activation in TTP. Reduced C3 levels were demonstrated in patients with TTP [160,161,162]. During acute episodes of TTP, elevated levels of circulating C3a and soluble C5b-9 were demonstrated normalizing after remission [162]. Complement C3d, C4d and C5b-9 were demonstrated in a skin biopsy from a patient with acquired TTP. This patient was successfully treated with the anti-human C5 antibody eculizumab, suggesting a role for complement activation in TTP [163]. Sera from TTP patients have caused C3 and C5b-9 deposition on human microvascular endothelial cells (HMEC-1) and promoted neutrophil-mediated endothelial cytotoxicity. Upon heat-inactivation of complement these effects were abolished suggesting the role of complement activation in the process [164]. Complement activation on endothelial cells and platelets [160] may contribute to microvascular thrombosis and promote endothelial cell injury

Also other forms of TMA have evidence of complement activation. In atypical HUS complement activation is a primary event initiated by a hyperfunctional alternative pathway as described above. STEC-HUS is primarily a bacterial toxin-mediated disease in which bacterial virulence factors bind to and injure cells. However even in this form of HUS there is evidence for complement activation as demonstrated by reduced serum/plasma levels of C3 [165,166,167] and increased levels of complement breakdown products C3a, C3d, factor Bb and C5b-9 [166,168,169]. In addition, Stx has been found to induce formation of platelet-leukocyte complexes with C3 and C9 deposits [168]. Also, three pediatric patients with STEC-HUS and severe neurological symptoms were successfully treated with eculizumab, thus inhibiting the terminal complement pathway [166].

Excessive complement activation on host cells will lead to endothelial injury and platelet activation as observed in patients with atypical HUS and *in vitro* studies [170,171,172]. In STEC-HUS Stx induces the initial endothelial injury and platelet

activation and then complement activation is a secondary event [168,173]. Similarly in TTP also complement activation is a secondary event followed by activation of endothelium and platelets due to dysfunctional ADAMTS13 [163,164]. Regardless of the triggering factor the initiation of complement activation on host cells can lead to undesirable consequences and tissue injury promoting TMA.

Kidney

Structure and function

Kidneys perform essential roles in removal of blood fluid volume and controlling the body fluid waste thereby regulating the balance of electrolytes. The kidney is comprised of an outer cortex and an inner medulla (Figure 10). The nephron is the basic structural and functional unit of the kidney and it has two parts: the renal corpuscle or glomerulus, where blood filtration occurs, and renal tubules, where filtrate is drained into the collecting duct. The glomerulus is a network of small blood capillaries, and the Bowman's capsule, the double-walled epithelial cup surrounding the glomerulus. Tubules include the proximal convoluted tubule, the loop of Henle and the distal convoluted tubule [174,175].

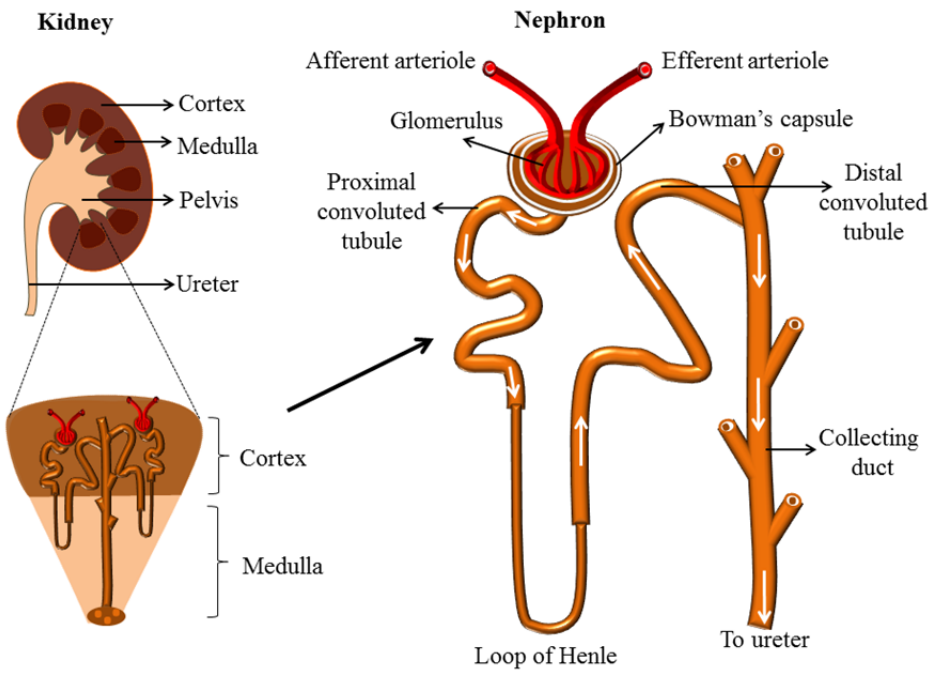

Figure 10. Schematic representation of the kidney and a nephron.

Blood flows into the glomerulus through an afferent arteriole and exits through an efferent arteriole during which it is filtered in the glomerular capillaries. The ultrafiltrate passes through to the renal tubules. In the proximal convoluted tubules reabsorption of important molecules and ions occur, in the loop of Henle urine is concentrated, in the distal convoluted tubule secretion of waste materials occurs and is finally excreted as urine through the ureter [174].

Glomerular filtration

The glomerular filtration barrier consists of three layers: the glomerular endothelium, the glomerular basement membrane and podocytes [176] (Figure 11).

Glomerular endothelium

The glomerular endothelium consists of a single layer of flattened endothelial cells perforated with large pores called fenestrae, which allow the water and smaller molecules to go through. On the luminal side endothelial cells are covered by the endothelial surface layer (ESL) consisting of the glycocalyx and endothelial cell coat [177]. The glycocalyx is composed of glycosaminoglycans and proteoglycans that together with adsorbed plasma proteins form a negatively charged layer covering endothelial cells and their fenestrae. The cell coat is more loosely composed on the top of the glycocalyx. The ESL modulates the transport of proteins across fenestrae based on size and charge [178].

The glomerular basement membrane

The glomerular basement membrane (GBM) is an acellular gelatinous layer consisting of collagen, which provides the structural strength, and glycoproteins, which restrict the filtration of small proteins [179]. The constituents of the GBM are mainly laminin, type IV collagen, nidogen and heparan sulphate proteoglycans [180]. Capillary pores restrict the proteins based on size. Even though some proteins pass the capillary pores, negatively charged glycoproteins within the basement membrane may restrict passage of negatively charged plasma proteins [179].

Podocytes

Podocytes are specialized epithelial cells with extended cytoplasmic foot processes on the outer side of the GBM. The space between the foot processes is termed the filtration slit joined by a diaphragm, in which nephrin is the key component. The glomerular filtration occurs via fenestrae of endothelial cells, the GBM and the slit diaphragm made up of nephrin molecules. Mutated nephrin leads to massive leakage of plasma proteins a condition observed in patients with congenital nephrotic syndrome [176].

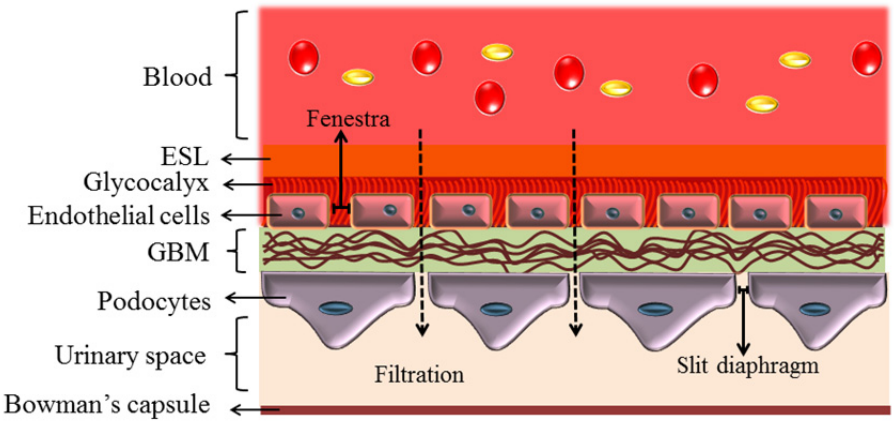

Figure 11. Schematic representation of the glomerular filtration barrier with three layers: the fenestrated endothelium, GBM: glomerular basement membrane and podocytes. The path of filtration is represented by dotted arrows. ESL: endothelial surface layer.

Glomerular filtration barrier and crosstalk

The GBM is a specialized extracellular matrix located between the endothelium and podocytes. These cells account for the synthesis and secretion of components of the GBM. The essential interaction between these three components has been highlighted by the role of vascular endothelial growth factor (VEGF) [181]. Podocytes produce growth factors such as VEGF and angiopoietin-1, which have been shown to modulate glomerular endothelial cells [177]. For example, *in vitro* cell culture experiments with conditionally immortalized human glomerular endothelial cells (CiGEnC) have been shown to express fenestrations in response to VEGF [182]. An experimental study showed that local inhibition of VEGF resulted in an altered phenotype of endothelium. This study provided evidence for transport of VEGF, produced in podocytes, via the GBM counter to the direction of urinary flow. Such a molecular impact, from podocytes to the glomerular

endothelium, was also demonstrated for angiopoietin-1 [177]. These studies indicate that factors produced by podocytes may reach and affect the glomerular endothelium. Along these lines, one of the aims of this thesis was to study if ADAMTS13, produced by podocytes and glomerular endothelial cells is present and active in the glomerular basement membrane.

ADAMTS13 in kidney

The kidney is a target organ in TTP. Thus the presence and activity of ADAMTS13 in native renal cells has been investigated. ADAMTS13 mRNA has been detected in the kidney [14,79,183]. Immunohistochemistry has demonstrated ADAMTS13 expression in normal renal tissue particularly in glomeruli and tubuli. ADAMTS13 mRNA and biologically active protein were also specifically demonstrated in cultured human podocytes [183] but the expression of biological active ADAMTS13 in other resident renal cells was not described before this thesis was commenced. Thus one of the aims of this thesis was to investigate the presence and biological activity of ADAMTS13 in tubular epithelial cells and glomerular endothelial cells.

The importance of ADAMTS13 release from endothelial cells has been previously described [11] and is ascribed to a protective mechanism by which the protease prevents thrombus formation on the endothelial surface. However, tubular cells are not in direct contact with the circulation and VWF. They have, nonetheless, been shown to express regulators of thrombosis, coagulation and fibrinolysis such as tissue factor and its inhibitor [184,185], protein C and its inhibitor [186,187], and urokinase-type plasminogen activator [188]. Thus the assumption on which our investigation was based was that ADAMTS13, if expressed and secreted from tubular cells, may play a role in hemostasis.

Complement activation in the kidney during TTP

In TTP the kidney is affected with typical TMA lesions. Dysfunctional ADAMTS13 allows formation of ULVWF multimers upon endothelial cells followed by platelet aggregation and thrombus growth [87]. Though this process is not initiated by complement, there is limited evidence of complement activation in the kidney. Autopsies performed in the 1970s showed complement deposition on renal and myocardial tissues from two separate TTP cases [189,190] but

ADAMTS13 had not yet been described at that time and thus the patients' definite diagnosis was unclear. Apart from these results, little is known about complement activation in renal tissue and on renal cells during TTP associated with ADAMTS13 deficiency. Thus one of the aims of this thesis was to demonstrate complement activation in renal tissue from TTP patients and study the mechanism by which this occurs.

Renal manifestations in TTP

Hematuria, hemoglobinuria and proteinuria are common manifestations of renal involvement in TTP patients. In most of the cases mild impairment of renal function occurs but if untreated, it may lead to more severe renal involvement [191]. Immunohistochemistry has demonstrated the expression of VWF in normal renal tissue specifically in glomerular capillaries and endothelial vessel wall whereas renal tissue from patients with TTP exhibits pronounced VWF-rich thrombi in the same locations [192].

Glomerular endothelial damage in TTP

The glomerular endothelium is highly specialized in filtration of massive volumes of plasma. Various mechanisms that lead to loss of glomerular endothelial function include alterations in the glycocalyx, changes in fenestrae, swelling of endothelial cells, encroachment and occlusion of the capillary lumen and increase in shear forces [193]. Upon injury, the endothelium loses its anticoagulant properties and become procoagulant [194]. Apoptotic endothelial cells express decreased thrombomodulin, tissue factor pathway inhibitor and heparan sulfate whereby the endothelium becomes procoagulant [195]. Endothelial cells may undergo apoptosis in TTP as plasma samples from patients with TTP have been shown to induce apoptosis in cultured renal microvascular endothelial cells but not in endothelial cells derived from large vessels [196,197].

Thrombomodulin is a cofactor for thrombin, expressed on the surface of endothelial cells with anticoagulant properties [198]. An increase in the plasma level of thrombomodulin is attributed to endothelial cell injury [199]. Patients with TTP were shown to have elevated markers of vascular endothelial cell injury such as thrombomodulin, antithrombin and protein C [199,200,201]. Supporting the hypothesis that vascular endothelial injury may also be immune-mediated, some patients with TTP have been shown to have antibodies against GPIV (CD36), a cell surface glycoprotein receptor (the thrombospondin receptor) on platelet and endothelial cell surfaces [202,203]. Another marker of endothelial activation or

injury is circulating microparticles released from injured endothelial cells [204], which will be discussed below.

Microparticles

Microparticles or microvesicles are irregularly shaped submicron vesicles (ranging in size from 0.1 to 1 μm in diameter) derived from activated, injured or apoptotic cells. One important characteristic feature of microparticles is surface expression of negatively-charged phosphatidylserine (PS) [205], although not all microparticles express PS. Microparticles lack nuclei but consist of a plasma membrane with a small amount of cytosol and surface antigens from the parent cell. They are present in the circulation of healthy individuals and are increased in number during various pathophysiological processes including cell adhesion, angiogenesis, immune responses, infections, vascular remodeling, thrombosis, hemostasis, cancer, vascular diseases, diabetes, sepsis, inflammation and other conditions [206].

Microparticles mediate cell-cell communication by transporting cell surface receptors, mRNAs, microRNAs, proinflammatory cytokines and signaling molecules [207]. They may also bind or fuse with the target cell membrane or be engulfed by the target cell [207]. Microparticles may be derived from various cells including endothelial cells, platelets, monocytes, leukocytes and RBCs [206]. Endothelial microparticles have been shown to express markers (Figure 12) such as tissue factor (TF), CD62E (E-selectin), CD62P (P-selectin), CD54 (ICAM-1: intercellular adhesion molecule-1), integrin $\alpha_v\beta_3$, CD31 (PECAM-1: platelet endothelial cell adhesion molecule-1), CD105 (endoglin), CD144 (VE-cadherin), VWF and CD146 (S-endo-1) [204,208].

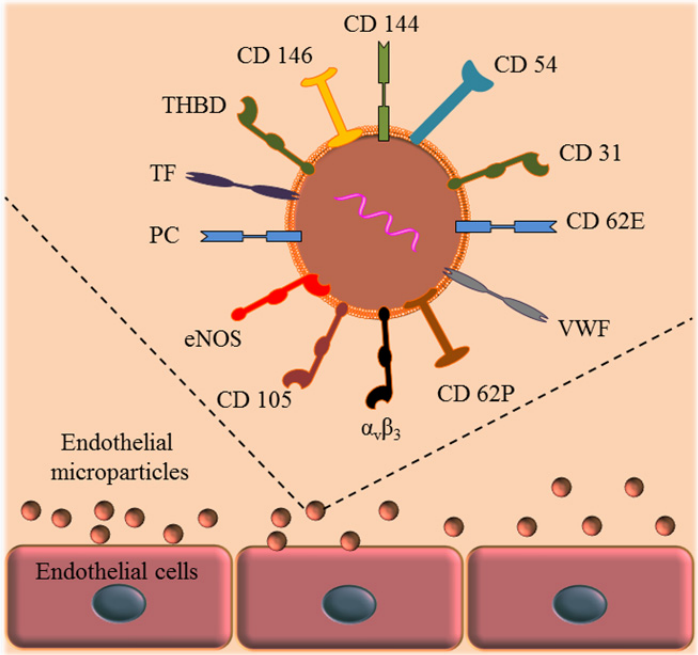

Figure 12. Schematic representation of endothelial microparticles with surface expression of TF: tissue factor, THBD: Thrombomodulin, PC: Protein C, CD62E: E-selectin, CD62P: P-selectin, CD54: ICAM-1(intercellular adhesion molecule-1), $\alpha_v\beta_3$: integrin, CD31: PECAM-1 (platelet endothelial cell adhesion molecule-1), CD105: endoglin, CD144: VE-cadherin, VWF: von Willebrand factor, eNOS: endothelial NO synthase and CD146: S-endo-1 [208].

Endothelial and platelet microparticles in TTP

Microparticles increase in number during pathological conditions and can be strongly procoagulant in the presence of PS and TF, mediating thrombosis and hemostasis [209]. Platelet microparticles derived from activation of platelets were shown to activate platelets and endothelial cells [210].

TTP plasma from the acute phase was shown to induce the release of endothelial microparticles from microvascular endothelial cells normalizing during remission [211,212]. Levels of circulating endothelial microparticles increase before the onset of thrombocytopenia [211] and hence can be used as an early prognostic marker in TTP. In STEC-HUS patients, platelet and leukocyte derived microparticles have been shown to have C3 and C5b-9 deposition [168]. Complement deposition may occur before membrane budding or after. In either

case, it would facilitate phagocytosis of the microparticle. Complement labeling of microparticles may also indicate that complement deposition occurred on the parent cell during the disease process as part of cell activation and injury.

The present investigation

Aims

The overall aim of the present study was to examine ADAMTS13 expression and activity in renal cells and tissue and to investigate the contribution of ADAMTS13-deficiency to complement deposition in TTP.

Specific aims

Paper I

- To investigate ADAMTS13 expression and biological activity in human renal tubular epithelial cells as well as its expression in renal tissues and urine from patients with tubular damage.

Paper II

- To examine ADAMTS13 expression and phenotype in glomeruli from wild-type and ADAMTS13-deficient mice.
- To investigate ADAMTS13 expression and biological activity in human glomerular endothelial cells.

Paper III

- To examine if VWF is cleaved by components of the GBM and if ADAMTS13 and/or another protease is involved in this cleavage.

Paper IV

- To define if ADAMTS13 deficiency contributes to complement activation in TTP.
- To examine TTP patients' renal tissues and circulating microparticles for the presence of complement deposition.
- To investigate if TTP plasma/serum induces complement deposition on VWF-platelet strings and on glomerular endothelial cells under shear and if complement-coated endothelial-derived microparticles are released.

Methods, results and discussion

Paper I: Biologically active ADAMTS13 is expressed in renal tubular epithelial cells

A previous study demonstrated ADAMTS13 expression in renal cortical tissue including tubular structures [183]. The present study aimed to investigate if biologically active ADAMTS13 is expressed in renal tubular epithelial cells as well as in renal tissues and urine samples from patients with tubular damage.

Materials and methods

Primary human renal tubular epithelial cells (HRTEC) and human renal epithelial carcinoma cell line of tubular origin A498 were cultured [213,214]. ADAMTS13 expression at the mRNA level was examined by real-time PCR with a probe against exons 28-29 [183]. Protein expression was examined by immunofluorescence using monoclonal mouse anti-human ADAMTS13 antibody (A10) and polyclonal rabbit anti-human ADAMTS13 antibody (SU19) [183] and in medium and lysate by immunoblotting under reducing conditions using polyclonal rabbit anti-human ADAMTS13 antibody (SNO357). VWF cleavage activity was measured using fluorescence resonance energy transfer substrate FRETS-VWF73 assay [215]. In patients with tubulopathy ADAMTS13 expression was detected using the A10 monoclonal antibody in renal tissues by immunohistochemistry and in urine samples by immunoblotting under non-reducing conditions.

Results

ADAMTS13 expression was detected in cultured HRTEC and A498 cells at the mRNA level by real-time PCR and at the protein level by immunofluorescence. Protein expression was further detected by immunoblotting in HRTEC cell lysates and media exhibiting 170 and 150 kDa bands, respectively. VWF cleavage activity was exhibited by cell lysates from both cell types. ADAMTS13 expression was demonstrated by immunohistochemistry in tubuli where it was diffusely stained in normal tissue whereas it was distributed diffusely or apically in patients with tubular damage. Urine samples from patients with tubular damage showed a band at 150kDa corresponding to the full-length ADAMTS13 band in normal plasma. Urine samples from healthy individuals did not exhibit an ADAMTS13 band.

Discussion

This study demonstrated the expression of biologically active ADAMTS13 in renal tubular epithelial cells. ADAMTS13 expression was further detected *in situ* in renal tissues with an altered pattern in patients with tubular disease. ADAMTS13 detection in urine from patients with tubulopathy revealed the

presence of full-length protease. This suggested that the protease was synthesized in these cells as such large proteins (150 kDa) cannot pass the glomerular filtration barrier in healthy glomeruli. The results indicate that tubular cells may play a role in primary hemostasis by releasing ADAMTS13. Although not addressed the protease may gain access to the circulation via peritubular capillaries.

Paper II: Phenotypic expression of ADAMTS13 in glomerular endothelial cells

The present study aimed to investigate if biologically active ADAMTS13 is expressed in the glomerular endothelium. To this end, studies were performed using renal tissues from wild-type and ADAMTS13-deficient mice as well as human glomerular endothelial cells.

Materials and methods

ADAMTS13 expression in mouse renal tissues was detected by immunohistochemistry using polyclonal chicken-415 anti-mouse ADAMTS13 antibody. Using scanning electron microscopy [216], the vessel wall phenotype in normal and ADAMTS13-deficient mice [120] was studied. Platelet deposition on the capillary wall was examined using polyclonal goat anti-human integrin-β3 antibody that exhibited cross-reactivity with the mouse protein [217]. Conditionally immortalized glomerular endothelial cells (CiGEnC) [182] and human dermal microvascular endothelial cells (HMVEC) were cultured. ADAMTS13 expression at the mRNA level was examined by real-time PCR with a probe against exons 28-29 [183] and at the protein level by flow cytometry and immunofluorescence using the A10 and SU19 antibodies. ADAMTS13 in CiGEnC culture media was investigated by immunoblotting using goat anti-human ADAMTS13 antibody (BL156). VWF cleavage activity was analyzed by VWF multimer structure analysis as previously described [183] and cleavage products were identified by immunoblotting using a combination of pooled mouse anti-human VWF antibody (for the 176 kDa fragment) and mouse anti-human VWF M13 antibody (for the 140 kDa fragment) [6].

Results

Renal tissue from wild-type mice exhibited ADAMTS13 expression in glomerular endothelial cells as well as in podocytes and tubuli whereas tissue from ADAMTS13-deficient mice did not stain for ADAMTS13, as expected. Ultramorphology showed thickened and irregular glomerular capillary walls in the latter mice in comparison to the wild-type mice. Significantly, more platelet deposits were exhibited on the capillary walls of ADAMTS13-deficient mice. Cultured glomerular endothelial cells CiGEnC exhibited ADAMTS13 expression at the mRNA level and at the protein level, as was also shown for HMVEC. ADAMTS13 was also detected in media from both endothelial cells. VWF

proteolytic activity was observed in cell lysates from both endothelial cells and specific cleavage products corresponding to 176 kDa and 140 kDa fragments were observed. Blocking assays showed the specificity of ADAMTS13-mediated VWF cleavage.

Discussion

ADAMTS13 expression was demonstrated in glomerular endothelial cells of murine and human origin. The observation that ADAMTS13-deficient mice exhibit a thickened vessel wall with platelet deposits suggests that ADAMTS13 prevents platelet deposition on the capillary wall by cleaving ULVWF multimers. As discussed above, ADAMTS13 alone is not sufficient for the development of TTP. The ADAMTS13-deficient mouse exhibited platelet deposition on the irregular vascular surface of glomerular endothelial cells, and, in the setting of endothelial injury, would thus be more prone to develop thrombi during endothelial injury than the wild-type mouse. Furthermore, the ADAMTS13 was shown to be biologically active in human glomerular endothelial cells. In addition to circulatory ADAMTS13, presumably released from the endothelial cell lining of the systemic vasculature, the high shear rate in the glomerular vasculature may require an extra source of ADAMTS13 and thus secretion of biologically active ADAMTS13 in glomerular endothelial cells may contribute locally. ADAMTS13 expression has been previously demonstrated in other endothelial cells including HUVECs, primary human umbilical artery endothelial cells and microvascular endothelial cell lines (EVC304) with VWF cleaving capacity *in vitro* [10,11,85]. Along with the results presented in the current study and considering the large surface area of the endothelium, plasma ADAMTS13 may thus be contributed from endothelial cells.

Paper III: Von Willebrand factor cleavage in the glomerular basement membrane is multifactorial

ADAMTS13 was previously demonstrated in the glomerular basement membrane by immunogold labeling and transmission electron microscopy [183]. The present study aimed to investigate if VWF could be cleaved by components of the GBM and if ADAMTS13 and/or other proteases in the GBM could cleave VWF.

Materials and methods

A previously described GBM preparation pooled from several individuals [218,219] was used in this study. Immunoblotting was performed on the GBM sample for the detection of ADAMTS13 using the BL156 antibody (also used in Paper II). The presence of VWF was examined by immunoblotting using a combination of pooled mouse anti-human VWF antibodies (used in paper II). The presence of the VWF antigen was also examined by enzyme-linked

immunosorbent assay (ELISA) [220]. Immunoblotting was also performed on the GBM sample for the detection of leukocyte proteases: elastase, PR3, cathepsin G and MMP9 that were previously shown to cleave VWF [89]. VWF cleavage activity was analyzed by VWF multimer structure analysis as previously described [183] using single or combined protease inhibitors and a specific anti-ADAMTS13 antibody to block activity. The cleavage specificity was also investigated by immunoblotting using the above-mentioned combination of pooled mouse anti-human VWF antibodies [6].

Results

Immunoblotting of the GBM sample exhibited a band at 190 kDa corresponding to ADAMTS13. In addition, the GBM sample showed two VWF cleavage fragments at approximately 170 and 135 kDa. The presence of VWF in the GBM was confirmed by ELISA at a concentration of 0.5µg/ml. Leukocyte proteases were not detected in the GBM. Based on the inhibitions assays with specific protease inhibitors, the VWF cleavage activity in the GBM was mostly attributed to serine proteases in combination with a metalloprotease. Partial inhibition of VWF-cleaving activity by the anti-ADAMTS13 antibody suggested that ADAMTS13 had a minor role in cleavage.

Discussion

The present study provided evidence for non-ADAMTS13 mediated VWF cleavage in the GBM. Biologically active ADAMTS13 has been previously demonstrated in podocytes [183] and in glomerular endothelial cells (Paper II). Hence ADAMTS13 is also expected to be present in the GBM, which is located between these two cell types. We could demonstrate its presence in the GBM but it made only a partial contribution to VWF cleavage. VWF cleavage in the GBM was mostly induced by a serine protease and only partly by ADAMTS13. Though the known VWF cleaving leukocyte proteases were undetectable in the GBM, their contribution cannot be excluded. VWF-cleavage by proteinase 3 could, however, be ruled out. Future studies will address the specific VWF cleaving protease in the GBM.

Paper IV: Complement activation associated with ADAMTS13 deficiency

In TTP dysfunctional ADAMTS13 leads to ULVWF release followed by endothelial injury and platelet activation leading to thrombosis. Complement may be involved in enhancing and maintaining the endothelial injury and platelet activation. The present study aimed to demonstrate the contribution of ADAMTS13 deficiency to complement activation in TTP.

Materials and methods

Renal tissues, plasma or serum samples were available from patients (n= 12) with TTP, of which 9 had congenital TTP and 3 had acquired TTP (the diagnostic work-up of one patient in the last category is, as yet, incomplete). Renal tissues from Stx2-treated or vehicle-treated ADAMTS13-deficient and wild-type mice were also available [120]. Complement activation was examined on renal tissues from patients with TTP by immunofluorescence or immunohistochemistry using rabbit anti-human C3c antibody or mouse anti-human C5b-9 antibody. C3 deposition in murine renal tissues was studied using rabbit anti-mouse C3 antibody. Flow cytometry was performed to detect endothelial microparticles in plasma from TTP patients using mouse anti-human CD105 antibody. C3 and C9 deposition on CD105-positive microparticles were analyzed using chicken anti-human C3 or mouse anti-human C9 antibodies. Primary glomerular endothelial cells (PGEC) were cultured. Plasma or serum samples from TTP patients or controls were perfused together with normal platelets over histamine-stimulated PGEC using a semi-automated microfluidic platform. This was followed by detection of C3 on VWF-platelet strings. Perfused samples were analyzed by flow cytometry for the release of endothelial microparticles with surface-bound C3 and C9.

Results

C3 and C9 deposition were demonstrated in renal tissues from two TTP patients and C3 deposition was observed in renal tissue from Stx2-treated ADAMTS13-deficient mice but not in wild-type mice. Complement-coated endothelial microparticles were detected in TTP plasma samples and were significantly increased in number upon perfusion with normal platelets over PGEC. ADAMTS13 deficiency leads to the accumulation of ULVWF-platelet strings resulting in C3 deposition on the strings. Control plasma or rADAMTS13 cleaved the VWF-platelet strings and no complement deposition was thus observed. EDTA blocked the complement deposition on the VWF-platelet strings suggested that activation occurred via the alternative pathway of complement

Discussion

This study demonstrates that ADAMTS13 deficiency leads to complement deposition in human TTP and murine renal tissues. In the mice Stx2-induced endothelial injury promoted complement deposition but this occurred only in ADAMTS13-deficient mice suggesting the role of ADAMTS13 deficiency in this process. Even one PBS-treated ADAMTS13-deficient mouse that developed spontaneous TTP exhibited C3 deposition in the kidney, thus indicating that complement activation was secondary to the microvascular process induced by ADAMTS13 deficiency and not primarily by Stx-induced injury.

Endothelial microparticles are a marker of endothelial activation [204].Complement-coated circulating endothelial microparticles were observed in TTP patients in samples taken during remission. These results suggest that ongoing complement activation occurs in the microvascular process even during remission. Perfusion experiments showed that patients' plasma samples released more C3- and C9-coated endothelial microparticles than control samples. Complement activation may initially take place on parent cells which, upon activation, release microparticles with C3 or C9 deposits. Alternatively, the activation may take place after membrane budding on the microvesicles themselves. Either way deposition of opsonins may result in phagocytosis. However, endothelial microparticles possess pro-thrombotic potential [209] and, as long as they circulate, may thus contribute to the thrombotic process.

Conclusions

Paper I

- Renal tubular epithelial cells synthesize biologically active ADAMTS13.

Paper II

- Glomerular endothelial cells synthesize biologically active ADAMTS13.
- ADAMTS13-deficiency in renal tissue leads to thickening of glomerular capillaries with platelet deposition thus predisposing to a thrombotic lesion.

Paper III

- Cleavage of VWF by proteases present in the GBM is multifactorial including a serine protease in addition to ADAMTS13.

Paper IV

- ADAMTS13-deficiency leads to complement activation in TTP.

Acknowledgements

I would like to extend my heartfelt thankfulness to my **Pediatrics** family members, especially to those who created a pleasant ambience during my PhD duration.

Dearest **Diana**, firstly thank you very much for giving me the opportunity to be a PhD student under your stupendous supervision and I am very proud of it. Extending a simple thanks to you doesn't completely show my gratitude towards you, but, please accept my ''sincere'' thanks, not just for being my supervisor but also for your excellent guidance, knowledge, care, affection, inspiration, suggestions and corrections, and I mean it ☺. Special thanks to your **words of encouragement** at all times. I always felt like you are my mother correcting me from making mistakes. I still remember the day when I came for an interview when you mentioned about the Holy (†) hour, my initial assumption was prayer time but later realized it was the lab meeting!! ☺. All these years, Friday 1pm has become very important time point for suggestions, new ideas, critics and appreciations. Special thanks for all the parties, dinners, picnics and conferences. Last but not the least, it's always on my mind to get your acceptance before I finalize my……. (You know what it is! ☺)

Lars Holmberg, you are the powerhouse of knowledge and thank you for your valuable suggestions and questions all these years.

Ann-Charlotte, writing a small paragraph for you is not sufficient; I should probably write a book. I always remember my first meeting with you the lab with a big smile. You are such an amazing and important person in my life with loads of energy and knowledge. Please accept my infinite number of thanks for your encouragement, kindness, love, teaching/learning, help with experiments, endless support, suggestions, enthusiasm and what not. You are my mom away from home. With your spontaneous singing and big laugh we never even needed a radio.

Anne-lie, thank you for endless support, help with FACS experiments, question during the meetings and for all the very good suggestions. Thank you also for your very special comment "svärmorsdröm" (You know? It's one of the best compliments I've ever got). All these years you and ACK have been my reviewers, critics, translators and good companions especially in travelling and conferences.

Minola, thank you for being my co-supervisor (Boss-2) and sincere thanks to you for all the techniques you have taught me and endless support all these years. Thank you for your time even when you were away from the lab, especially the skype sessions along with **Victoria**. Thanks to both you and **Erik** for your friendship, dinners, movies (especially animation movies, still remember your imitations….), kubb, parties, dance and general discussions.

Carla, my dear sister, you are such a nice person to have known. Please accept my special thanks for your friendship. I always remember our golden moments together during fun filled parties, lunch breaks, long dinner sessions with our other friends, dances (especially Salsa…☺.) gossips!!, conferences, and so on. I always miss you and hope to meet you again. I will always remember you as a special person in my life. Wish you a wonderful life ahead.

Milan, brother! You are my inspiration and symbol of knowledge and simplicity. Thank you for being always helpful, great suggestions, helps with statistics, good time during parties and specially conferences. I will miss you during the dissertation and the party. I wish you all the best in London.

Roland and **Robin** thank you for friendship, general discussions, support, critical questions and suggestions during the lengthy lab meetings (along with Milan). I have always looked at both of you with my head held high (with your vast knowledge) * Wink! Wink! *

Ida, thanks for the friendship, encouragement, discussions and support. Good luck with your work and hope you get at least 4 more papers by the time of your dissertation.

Lisa, thanks for friendship, encouragement and support all these years. Hope our plan of international food evening will soon be possible. Good luck with clinic.

Maria, thank you very much for your friendship and the time we spent at the lab. I wish you all the best with your research.

Zivile, thank you for being my officemate and I still remember the taste of Hungarian food. Good luck with your research and clinic.

Ingrid, thank you for your time in the lab and I wish you all the best with your education and research.

Johan, thank you for your friendship, encouragement and especially for help with paper IV. I wish you good luck with your upcoming research.

My sincere thanks to all the patients, co-authors, summer students for your contributions.

Pierre, special thanks to you my friend for your endless support with computer and other technical issues. Thank you for also teaching me several things with computers.

Thank you **Eva Sandmark** and **Eva Fredriksson** for your administrative help.

Thank you my colleagues from other Pediatrics and Gynecology lab: **Eva, Heike, Vineta, Zuzana, Vera, Irene, Nikica, Tina** for all you support, suggestion, fun, breakfast and so on. Special thanks to Eva for sharing your knowledge in immunohistochemistry and to Vera, Heike, Irene and Zuzana for sharing your knowledge in real-time PCR.

Special thanks to **Maria Baumgarten** and **Matthias Mörgelin** for your help with electron microscopy and especially for the fun discussions when we meet in the corridor. Special thanks to **Anders Olin**, **Lena Gunnarson**, **Inga-maria Frick** and **Murphy** for contribution with material and **Maria Allhorn** for help with fluorometer. Thank you **Birthe Jensen** for help with FRETS assay and **Ulla Karin** for help with the histology sections.

Thank you **Marita Cohn** for believing in me and for allowing me to your department for master's thesis. I will always be thankful to you and **Jenny Rhodin** for introducing me to research field. It's only because of you that I opened my Pubmed account ☺. I owe you a lot.

Thank you **Maria Falman** from Umeå University for excellent immunology course, for believing in me and for being a good referee.

Special thanks to my friends **Padma** akka, **Kiran** anna **(**always remember Damal!!, fun moments**), Sowji**, for your encouragement and suggestions. Thank you **Ram (**Guruji**), Parveen, Soniya, Vivek, Chinmay, Amol, Rama, Srikanth, Deepthi, Amol, Karthik** for all your friendship and fun during the parties.

Mina and **Swati (Shukla ji)** thanks to both of you for your friendship and also for making my lunch and tea breaks very interesting with deep discussions and gossips about everything under planet earth. **Swati**, special thanks to you for all funny satires, support and lunch times.

Thank you all my friends and well-wishers from Umeå **Manah, Deepa Giri, Shiva, Venky, Santhi Sisir, Vidya Ravi, Devi Maribasappa, Vivek, Munender** and **Pranuthi** for your love, support and friendship. Special thanks to **Shiva anna**, for accommodating me when I arrived in freezing Umeå. Special thanks Venky for your friendship all the way from Vijayawada. Thank you Sisir for our good conversations and your visit to Lund.

Thank you my entire batch mates from Umeå **Suresh, Vani, Karuna, Azhar, Murthy, Pramod, Kishan** and **Bindu** for all your encouragement, fun we had as roommates and support all these years. **Suribabu** thank you for all the special

memories (in Umeå, Uppsala, Malmo, Lund). I always enjoy our conversations and trips along with **Azhar** (to Copenhagen, Berlin, Falsterbo). I am happy that you found a position in USA and I wish you good luck with everything. **Karuna**, you are one of the happiest persons I've met. I enjoy your timing during conversations; especially the Nydala lake trip is unforgettable. Good luck with your PhD.

Special thanks to special friends **Vikram** and **Rajender** for your friendship, love and support. **Viks,** I always enjoy our phone conversations about life, financial problems, secrets and so on. And also thank you for the free phone during our initial time!! **Rajender**, thankyou for your friendship, I always remember our times as roommates, the special cookings, movie nights ('Paranomal activity' with **Vini** hahaha!) and our special special events during our trip to Amsterdam, Paris and Norway. Thank you **Manasa** and **Ramesh** for your help, friendship and fun during our trips (especially Ven island, Helsinki and Munich)

Thanks to my friends from B14, **Praveen, Gopi, Ravi, Tirthankar** and **Martina** for your encouragement and support.

Special thanks to **Praveen** for our friendship, movies, food, evari gola vaaride, introducing photoshop and prism. **Sravani,** I like the way you call my name Rameshuuu, no one has ever called me like that**. Gopi,** thank you anna for our friendship, dum biryani practicals and garage room with tables, bed transportation, speakers. Sister **Saritha (**dimple chin with smily face**)** and cute little **shreeya.** Thank you both families for all your support, nice trips (Ven Islands, Paris, Oslo, Helsinki) and parties and the time as roommates, funny discussions, movies, special food and so on. God bless both of your families.

Sister **Madhuri** (Rangu Rangamma), I have always enjoyed our conversations about our Vikas college, your imitations, non-stop radio, satires. **Raghu**, I have learned many positives things from you, especially the hard work and determination. I have also learnt from you the ideal qualities of how a husband should be. Thank you for your friendship and affection from little **Ananya.** I always remember our first confusing meeting. God bless your family.

Shweta, Ananth, Aarush (Up! Up! Dishkaavon dishkaavon) Sometimes, I think how close we have grown in a very short period of time. Thank you for your friendship and affection from little Aarush.

Thanks to my dear friends: **Madhu** (New peanut), **Madhu** (Budampadu), **Suri** (six pack), **Raju** (Sweets), **Prudhvi** (puthuvi) , 'Pisuku' **Susi**, **Bharath** (A-Z), **Praveen**, **Pradeep**, **Naresh** (Rice cooker), **Avinash**, **Emmi**, **Satish**, **Goutham**, **Rahul**, **Anugraha**, **Kalyani**, **Soumya**, **Shamini**, **Sruthi** (Anchor) and also other members of gully cricket. Thank you all for your help, support especially for your

wonderful teamwork during festivals, parties, cricket and so on. I wish you all the very best for your successful futures.

Negar OMG! Thank you very much for your friendship and all the fun during summer trips (Stonehenge beach, lunch at 8AM under pouring rain and parties).

Special thanks to **Veronika, Martina, Gonzalo, Nataliya** and **Galya** for your friendship, fun and for all the good memories.

Thank you **Manju, Shiva, Cherry ‘**’The Dingman’’ and **Sravya,** for your love, support and encouragement. **Shiva**, I always miss you ya! The fabulous movies you made us watch (The Mist IMDB rating: 8/10, Amma, That’s how it started and all the funnily translated movie names). I will always remember and cherish the love and affection Cherry and Sravya have shown towards me.

Special thanks to **Sridevi, Ram Kumar, Anusha, Dinesh, Anusha, Prashanth** for your friendship and all the fun times together.

Thank you **Pallavi, Pintesh and Rohan** (Police uncle, Spindle) for all the get-togethers we had. Pintesh, please convey my special greetings to your parents.

Thanks to a special person **Mukesh** anna, you are the inspiration for us and also thank you for your help with everything (papers, end note, Photoshop problems). Thank you for your encouragement, support, fun (3G, Monaco, disco, and also others which I shouldn’t mention here) and care.

Special thanks to Sister **Kavitha, Chaitanya** and **Shreyas** for your love, support and for fun time we have. I always remember the spicy chicken, parties with ‘’Dumb charades’’ (Kav—Chi--mani) and political discussions with **Chaitanya**. You are very special to me and thank you for everything. You are the most beautiful couple I have met and God bless your family. My cute nephew **Shreyu**, you are something special to me, I enjoy your cute behavior and specially dance: Gabbar sing, chammak challo.

Special thanks to Sodari **Dhana,** Baa **Sunny** and darling **Hansika** for your support, love, encouragement and so on. Special terms which always make fun: Multivitamin drink at our first meeting, chicken skin, hybrid, pilk, cooker mutton, gootalk and so on. **Sunny** I always remember your special dancing steps (Sunny’s style like gangnam style) at parties with dances, ‘’Dumb charades’’, cards, summer meet ups and all the good memories we have. Sodari I always remember our special conversations and thank you for your special preference for me. I always remember our garden works. **Hansika**, darling I enjoy playing with you and I am privileged that you only as “ maama”, hoppa hoppa, slutta maama, ledsen, aay, aay maama , nej maama and so on.

Special thanks to sister **Vini** (bujjulu, paapa) for your truthful laugh (deep from bottom), support, care, special food (--- pappu/sambar/idly, I still don’t know what

to call it as). You are such a nice and happiest person I have ever met, thank you for everything specially cooking during thesis time and classical singing. I enjoy your company especially during shopping, travelling (Munich, Helsinki, Amsterdam, Ven islands). Funny new terminologies Milkstone, sota, Kaandamu, Jilebi, A to the B to the …. so on. Still remember your dance in cruse to Helsinki. Thank you also for listening to my silly dreams specially the Garbage supervisor and for all your stories such as kaaki ka kaa… If I write a book of my dreams I will dedicate it to you.

Dhana and **Vini** my dearest gifted sisters, you have been always so special and important for me. I should not simply say thanks to you as we are one family. But still, my heartfelt wishes to you both for your care, affection, love, encouragement, valuable suggestion and discussions. I enjoy our conversations, teasing, spontaneity, satires, food, creative imaginations at D**raw something** (with strange drawings: iron man, road kill, Zeebra, mascara, kaandamu,).

Sincere thanks to **Chandana** madam, **Durga** madam, **R Venkatesan** sir and **VK Srinivas** sir from BBIL and **Saradhi** sir and **Prasad Reddy** sir for your support, encouragement and thank you for inspiring me towards PhD.

Thank you **Naidu** maam, **Murali** maam, **Durga Rao** and **U Venkateswara Rao** garu for endless support, helping and showing proper path to Sweden.

Special thanks to my close friends **Naresh, Purnima** (poori)**, Srilatha** (Srilu) **Chakry** and **JP** for your great friendship, love, care, support and suggestions. I would like to thank all my other friends and relatives especially **Dharma** annaya, **Malathi** akka, **Lakshmana**, **Raani** akka, **Moni** akka, **Srinu** maam who have always be so kind, helpful and supportive.

Thank you **Pujyasri Bikshamayya guruji**, **Surya Kumari** maathaji, **Raghu guruji, Radha** akka**, Raghu** and others for your pleasant teachings in spirituality, yoga and inspiration. Sessions with you have taught me most enlightening things in my life. **Srinu** annaya, thank you your friendship and company specially during yoga times, fun clubs, food, posters, banners and so on.

I thank you **GOD** for this life, kindness, blessings, for always being with me and guiding me through all walks of my life.

My grandparents **Naryana Rao, Venkatanarasamma, Rangaiah Naidu** and **Jaanamma**, thank you for your love, care and for all the inspirational stories.

My dearest sisters **Jamuna (Bujji)** and **Indumathi,** brother-in-laws **Srinivas** and **Vishnu,** my dearest brother **Janardhan (Nani)** and sister-in-law **Navya** you have been always very special and important. **Bujji and Indu**, though I am away from you, our regular phone conversation (flashnews, updates, gossips etc etc.,) have made us much more closer. **Nani**, you have always been my best friend, with

whom I can share anything and everything. I always enjoy our funny fights, scoldings, shoppings, bike rides, phone conversations and many more. You always inspire me in several ways. I miss you my brother-in-law **Srinivas**. Thank you for you love, care, support and encouragement. I don't know if there is next life, if so, I wish we would all be in same family again. I always remember our my nieces and nephews **Srija** (Peddulu)**, Rahithi** (budakka)**, Santhosh Karthikeya** (Shababu)**, Shreyan** (Baabba) and **Aryan Rithesh** (Bujji Baabba), you have been so special with your sweet sayings and fun moments. I refresh myself completely only when I talk to all of you. I love you all very much.♥

Naannagaru **Raghava Rao** and *Amma* **Jayasri**, ki paadabivandanaaalu. I should not say thanks to you both, but my life is yours, bless me. I feel very privileged to have you as my parents, who would always encourage me, support me at each and every point of my life. I fall short of words to thank you. You are the best parents in the world. I am what I am only because of you both. I always remember that you want to see the prefix 'Dr' before my name, I am happy that I have fulfilled your wish in this way. I dedicate this PhD for you. I will always carry both of you in my heart ♥.

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