Active Von Willebrand Factor, thrombocytopenia and thrombosis

Janine Hulstein

Active Von Willebrand Factor, thrombocytopenia and thrombosis

Janine Hulstein

ISBN-10: 90-393-4405-1

ISBN-13: 978-90-393-4405-7

Lay-out: Thessa Kockelkorn, www.doublestandard.nl

Cover: The cover shows the A1-domain of VWF (Thessa Kockelkorn)

Printed by: Febodruk B.V., Enschede/Utrecht

Active Von Willebrand Factor, thrombocytopenia and thrombosis

Actief Von Willebrand Factor, trombocytopenie en trombose
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. W.H. Gispen,
in gevolge het besluit van het college voor promoties
in het openbaar te verdedigen op donderdag 7 december 2006
des middags te 2.30 uur

door

Janine Jeriena Johanna Hulstein
geboren op 3 mei 1979 te 's-Hertogenbosch

Promotor:
Prof.dr. Ph.G. de Groot

Co-promotoren:
Dr. R. Fijnheer
Dr. P.J. Lenting

The studies described in this thesis were supported by a grant of the Landsteiner Foundation of Blood Transfusion Research (LSBR grant nr. 01.14)

Financial support by Ablynx for the publication of this thesis is gratefully acknowledged

Additional financial support is also gratefully acknowledged:
Dr. Ir. Van de Laar Stichting
J.E. Jurriaanse Stichting
DadeBehring B.V.
Bayer B.V. HealthCare

As knowledge increases, wonder deepens.

Charles Morgan

Voor Wilmer

Table of contents

Abbreviations

ADAMTS	A Disintegrin And Metalloprotease with ThromboSpondin motif
BSA	bovine serum albumin
β_2-GPI	β_2-Glycoprotein I
DDAVP	1-deamino-8-D-arginine vasopressin
DIC	disseminated intravascular coagulation
DRVVT	dilute Russell viper venom time
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FRET	fluorescence resonance energy transfer
GpIb	glycoprotein Ib
HELLP	hemolysis elevated liver enzymes and low platelets
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HIT	heparin-induced thrombocytopenia
HRP	horse radish peroxidase
HUS	hemolytic uremic syndrome
HUVEC	human umbilical vein endothelial cells
IgG	immunoglobulin G
ITP	idiopathic thrombocytopenic purpura
LAC	lupus anticoagulant
LB	Luria broth
LLP	lupus like disease
LRP	low-density lipoprotein receptor related protein
UL	ultralarge
OPD	*o*-phenylenediamine
pd	plasma derived
PNP	poly-vinyl-pyrrolidone
PPACK	H-D-Phe-Pro-Arg Chloromethylketone
NPP	normal pooled plasma
PAPS	primary antiphospholipid syndrome
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
PRP	platelet-rich plasma
PTT-LA	partial thromboplastin time-lupus anticoagulant sensitive
RES	reticuloendothelial system
SDS	sodium dodecyl sulphate
SLE	systemic lupus erythematosus
SPR	surface plasmon resonance
TBS	tris based solution
TSP-1	thrombospondin-1
TTP	thrombotic thrombocytopenic purpura
VWD	von Willebrand disease
VWF	von Willebrand factor
VWF:RCo	VWF-ristocetin cofactor activity
wt	wild type

Chapter 1

General introduction

Thrombocytopenia

Blood is composed of a fluid (plasma) and a cellular fraction. This cellular fraction contains red blood cells (erythrocytes), white blood cells (leukocytes) and anuclear cells that are known as platelets. Platelets play an important role in haemostasis, since they can adhere at sites of injury and pile up to form a plug that stops the bleeding. It is therefore not surprising that a decreased platelet count in the peripheral blood may lead to serious bleeding problems. Normal platelet counts in Caucasians vary between 150 and 350 x 10^9/L. Africans have lower platelet counts, varying between 115 and 300 x 10^9/L and in all ethnic groups platelet numbers in women are higher than in men.[1] Although thrombocytopenia is defined as a platelet count below the lower limit of 150 x 10^9 /L, most patients whose platelet count is above 50 x 10^9/L do not exhibit significant signs of haemostatic impairment. Mild thrombocytopenia (50-100 x 10^9 platelets/L) is usually associated with an increased bleeding risk after trauma. Serious spontaneous hemorrhage only occurs when platelet counts drop below 10 x 10^9/L.

Thrombocytopenia can be caused by four general mechanisms: (1) platelet underproduction; (2) platelet sequestration in the spleen; (3) haemodilution; and (4) increased platelet consumption or destruction. The latter develops when the rate of platelet consumption or destruction surpasses the ability of the bone marrow to produce platelets. This can be induced by immune mediated mechanisms, in which antibodies cause a rapid clearance of platelets or by non-immune mechanisms that lead to excessive consumption of platelets. Increased platelet consumption or destruction is thought to play a role in the pathology of clinically very distinct diseases (Table I). Four diseases, hallmarked by thrombocytopenia, were studied in more detail in this thesis. Non-immune consumptive mechanisms have been implicated in three of the diseases, Von Willebrand Disease (VWD) type 2B, Thrombotic Thrombocytopenic Purpura (TTP) and HELLP syndrome (Haemolysis, Elevated Liver enzymes and Low Platelets). The fourth, the antiphospholipid syndrome, has been suggested to be associated with immune-mediated platelet destruction.

Table I: Mechanisms of platelet consumption and destruction

Type of Thrombocytopenia	Specific examples
Immune mediated	
Autoantibody-mediated platelet destruction via reticuloendothelial system (RES)	Idiopathic Thrombocytopenic purpura (ITP) and "secondary" ITP (e.g. Immune thrombocytopenia associated with lymphoproliferative disease, collagen vascular disease, infections, such as infectious mononucleosis, HIV)
Alloantibody-mediated platelet destruction via RES	Neonatal alloimmune thrombocytopenia; post-transfusion purpura; passive alloimmune thrombocytopenia; alloimmune platelet transfusion refractoriness
Antibodies against microbial antigens adsorbed onto platelets	Malaria-associated thrombocytopenia
Drug-dependent, antibody-mediated platelet destruction via RES	Drug-induced immune Thrombocytopenic purpura (e.g. quinine, quinidine, sulfa drugs, vancomycin, etc.)
Platelet activation by binding of IgG Fc of drug-dependent IgG to platelet FcγRIIa receptor	Heparin-induced thrombocytopenia (HIT)
Platelet destruction via uncertain mechanisms (possible via platelet-glycoprotein reactive antibodies)	Antiphospholipid syndrome
Non-immune mediated	
Platelet activation via thrombin or inflammatory cytokines	Disseminated intravascular coagulation (DIC), septicemia and other systemic inflammatory response syndromes (e.g. adult respiratory distress syndrome, fat embolism syndrome, pancreatitis)
Platelet destruction via ingestion by macrophages (hemophagocytosis)	Infections, malignant lymphoproliferative disorders
Platelet destruction via uncertain mechanisms (possible consumption via platelet-VWF interaction)	Thrombotic thrombocytopenic purpura (TTP), haemolytic-uremic syndrome, Von Willebrand Disease type 2B
Platelet losses on artificial surfaces	Cardiopulmonary bypass surgery; use of intravascular catheters
Decreased platelet survival associated with cardiovascular disease	Congenital and acquired heart disease; cardiomyopathy; pulmonary embolism
Endothelial cell dysfunction and platelet activation	Syndrome of hemolysis, elevated liver enzymes and low platelets (HELLP)

Von Willebrand Factor

A connection with von Willebrand factor (VWF) has been implicated in all 4 diseases.[2-6] VWF is a multimeric plasma protein that mediates adhesion and aggregation of platelets[7] and modulates the survival and function of coagulation factor VIII. Factor VIII forms a non-covalent complex with VWF, by binding the amino-terminal (D'D3)-domain of VWF. In this way factor VIII is stabilized[8] and protected from clearance via the low-density lipoprotein receptor-related protein (LRP)[9] and proteolysis by phospholipid binding proteases, such as activated protein C and factor Xa[10,11]. Platelet adhesion and platelet-platelet interactions are also mediated by VWF. VWF binds via its A3-domain to exposed collagen at a site of injury, after which VWF captures platelets via its A1-domain (Figure 1A). In this way VWF functions as a molecular bridge between the subendothelial collagen and the GpIb-IX-V receptor complex on platelets.

Synthesis and secretion

The multimeric structure of VWF is essential for its function. VWF is synthesized by megakaryocytes and endothelial cells as a single pre-pro-polypeptide chain (Figure 1).[12,13] After removal of the signal peptide in the Endoplasmic Reticulum (ER), VWF is dimerized via the C-terminal CK-domain (Figure 1B). During further maturation in the Golgi, VWF is multimerized via the D'D3 domain (Figure 1B). This process is facilitated by the propeptide (D1-D2), which is separated from the mature VWF-multimer in the Trans-Golgi network. The newly synthesized VWF is either directly released by the endothelial cells via the constitutive pathway or stored in the Weibel-palade bodies in a 1:1 molar ratio with its propeptide.[14] VWF stored in the Weibel-palade bodies or in the α-granules of platelets is enriched in ultralarge (UL-) VWF multimers, whereas the constitutively secreted VWF consists mainly of lower multimers.[15] Release of UL-VWF multimers from the storage organelles is tightly regulated and occurs only upon stimulation of the cells with an agonist.

Regulation of the multimeric size: ADAMTS13

Newly secreted UL-VWF multimers are subjected to proteolysis upon release.[16] This is essential to prevent circulation of UL-VWF multimers, that have a higher haemostatic potential.[17] The very reactive UL-VWF multimers are converted into smaller, less adhesive forms by thrombospondin-1 (TSP-1)[18], although the exact role of TSP-1 in VWF proteolysis remains unclear. The size of VWF is also regulated

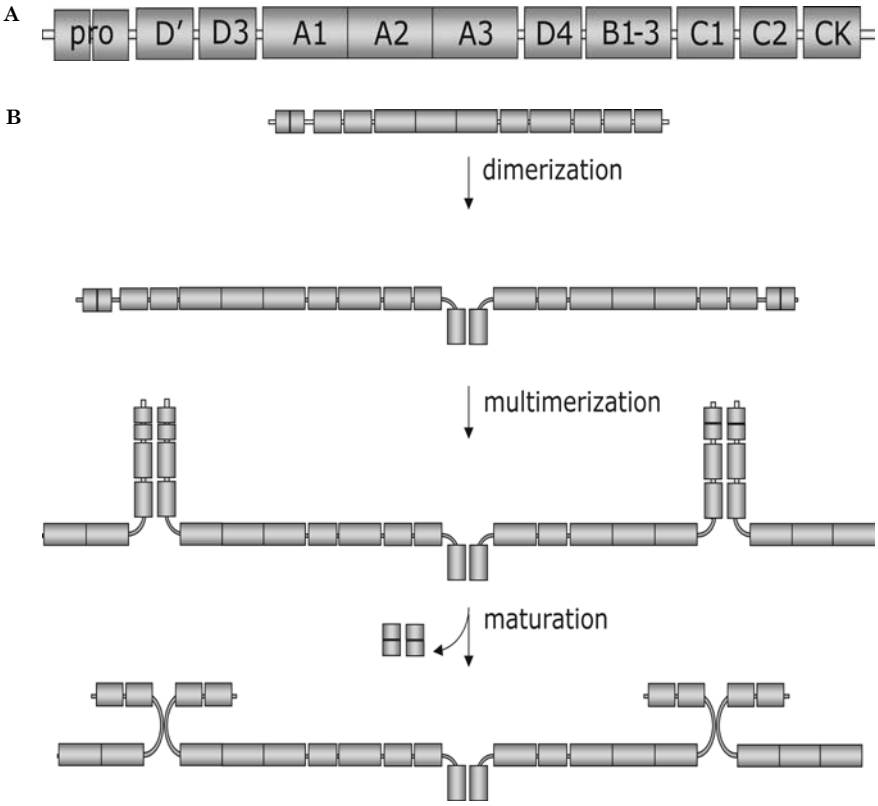

Figure 1: Synthesis of VWF. A: Structure of von Willebrand factor. **B:** Assembly of VWF multimers. In the ER, pro-VWF forms dimers via the C-terminal CK-domains. VWF further multimerizes through formation of disulfide bonds between the propeptide and D'D3-region. In the Golgi apparatus VWF-propeptide is cleaved off and mature VWF and propeptide are secreted or stored in a 1:1 stochiometry.

Figure 2: Structure of ADAMTS13. The primary translation product of ADAMTS13 consists of a signal peptide (s), a short propeptide (p), a metalloprotease domain, a Disintegrin-like domain (dis), 8 thrombospondin-1 repeats (number 1-8), a cysteine-rich domain, a spacer domain and 2 CUB domains.

by a metalloprotease designated ADAMTS13 (A Disintegrin And Metalloprotease with ThromboSpondin motif; Figure 2). ADAMTS13 was only recently identified[19], although it was observed 1991 that the heterogeneity of the VWF multimers in plasma was due to a VWF cleaving protease.[20] Proteolysis of VWF occurs at a single peptide bond between tyrosine 1605 and methionine 1606 in the A2 domain of VWF. In vivo proteolysis is probably a well regulated process, leading to the existence of multimers of different sizes in circulation, in stead of total fragmentation of VWF.

Tight regulation is required, since excessive cleavage into minimal fragments would cripple haemostasis, resulting in a bleeding disorder. This is illustrated by VWD type 2A, a bleeding diathesis caused by a lack of larger VWF-multimers, often due to excessive VWF proteolysis.[21]

Regulation of VWF proteolysis

To make VWF susceptible for cleavage by ADAMTS13, unfolding of the globular VWF molecule seems to be required. This is apparent from the fact that denaturing conditions are necessary for VWF cleavage to occur in vitro.[22] Fluid shear stress, therefore, can be considered as one of the determinants of VWF proteolysis by ADAMTS13.[23] Furthermore, proteolysis is modulated by chloride ions that bind to VWF and alter its conformation upon secretion into the circulation.[24]

Cleavage of the A2-domain of VWF is also influenced by the A1-domain. The presence of this domain was shown to decrease proteolysis of A2 when compared to the isolated A2-domain. Binding of GpIbα to the A1-domain abrogated the inhibitory effect of this domain on cleavage.[25] Ristocetin has also been suggested to enhance VWF proteolysis by ADAMTS13.[26] Botrocetin did not influence proteolysis, possibly because it does not induce a conformational change like ristocetin.[27] The overall structure and conformation of VWF also influences the rate of proteolysis as illustrated by VWD type 2A, in which VWF is more susceptible for proteolysis due to mutations.[21]

On the other hand, the efficacy of proteolysis is determined by the activity and binding of ADAMTS13. Serine proteases like plasmin or thrombin might regulate the activity of ADAMTS13 under normal conditions. In vitro, it has been shown that ADAMTS13 is proteolyzed by thrombin and plasmin and is thereby inactivated.[28] There is also evidence that TSP-1 interferes with VWF proteolysis. Although UL-VWF multimers can be processed by TSP-1 itself[18], the size of the VWF multimers in TSP-1 knock-out mice was found to be reduced.[29] This indicates that TSP-1 might compete with ADAMTS13 for binding to VWF and that ADAMTS13 is a more effective protease in the absence of TSP-1.

Inflammatory cytokine IL-6 has been suggested to inhibit ADAMTS13 activity under flow conditions, but not under static conditions.[30] The mechanism behind this inhibition remains to be investigated. A potential mechanism could be that IL-6 impairs docking of ADAMTS13 to VWF under flow. A possible effect of IL-6 on VWF

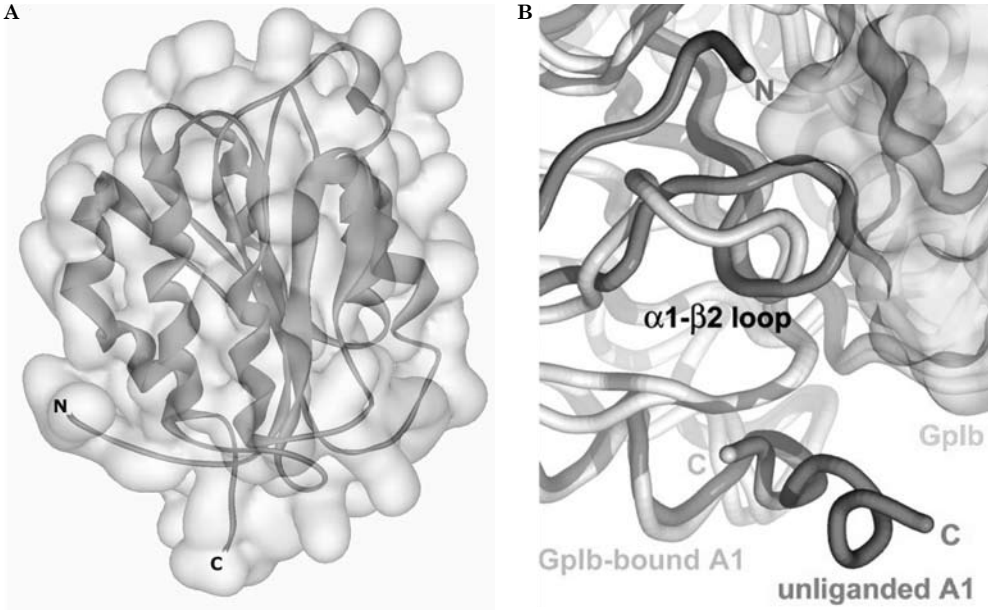

Figure 3: A1-domain of VWF. A: Solid-ribbon representation of "unliganded" wild type (wt) VWF-A1 with a semi-transparent molecular surface. **B:** Comparison of unliganded wt-A1 and GpIbα-bound A1, focused on the changes in the α1ß2-loop region. Unliganded A1 is depicted in dark grey, liganded A1 in light gray. N = N-terminal C = C-terminal (Figure B was adapted from Dumas et al. J Biol Chem. 2004)

cleavage has previously been suggested based on clinical observations. IL-6 levels are increased at the onset of TTP episodes, for instance, and high levels of IL-6 are associated with a poorer prognosis in TTP.[31]

VWF-platelet interaction

The multimeric pattern of VWF in plasma is very heterogeneic after proteolysis. Under physiological circumstances not only dimers circulate, but also multimers of more than 20.000 kDa are present. Although these multimers circulate together with platelets, VWF and its receptor on platelets do not interact under normal conditions. This indicates that multimers released after cleavage by ADAMTS13, have a latent biological activity. To induce an interaction with the GpIb-IX-V complex, a conformational change in the A1-domain of VWF is required (Figure 3).[32] This conformational change is thought to be induced by binding of the A3-domain to exposed collagen at a site of injury[33] and can be mimicked in vitro by coating VWF onto a artificial surface or by incubation of VWF with modulators, such as botrocetin or ristocetin.[34,35]

There are also pathological conditions that may lead to premature VWF-platelet complex formation, such as VWD type 2B, in which gain-of-function mutations in the A1-domain induce spontaneous interaction between VWF and GpIbα. One of the aims of our study was to develop a new assay to measure the amount of VWF that circulates in a GpIbα-binding conformation. We hypothesize that the amount of 'active' VWF in circulation determines the progress of consumptive thrombocytopenia in various diseases, such as TTP, HELLP syndrome, antiphospholipid syndrome and VWD type 2B.

Von Willebrand Disease

Classification of Von Willebrand Disease

Von Willebrand disease (VWD) is defined as a disease caused by abnormalities in the gene encoding VWF or by antibodies against VWF (acquired VWD), resulting in a qualitative or quantitative defect in VWF. The disease was first described by Erik Adolf von Willebrand in 1926.[36] However, it was only in 1971 that the so-called factor VIII-associated antigen was described, a complex that was later on discovered to be composed of two different proteins with different functions: Factor VIII and Von Willebrand Factor.[37] Until that time, classification of VWD was mainly based on the severity of the principal clinical symptoms, which are mucosal bleeding and easy bruising. We now know that the heterogeneity of VWD is largely based on the characteristics of VWF, a multifunctional protein with a complex biosynthesis, extensive posttranslational processing and distinct pathways of intracellular transport and secretion. During this complex process, errors may occur at all levels. These errors give rise to the many different manifestations of VWD.

The main classification of VWD into 3 types is based on structural and functional defects in VWF.[38] In VWD type 1, VWF antigen levels are decreased without evidence of a qualitative defect in VWF. Low levels of VWF can result from decreased synthesis, impaired secretion or increased clearance or a combination of these conditions. The most severe type of VWD, VWD type 3 is characterized by a virtually complete deficiency of VWF. This type of VWD is mostly caused by large gene deletions, transcriptional defects, frameshift mutations and stop codons.[39,40] The mutations are not confined to specific regions of the VWF gene.

VWD type 2 is hallmarked by qualitative defects, independent of the VWF antigen levels. This type can be subdivided into type 2A, 2B, 2M and 2N. VWD type 2N (Normandie) is caused by a decreased affinity of VWF for factor VIII.[41,42] Since VWF stabilizes the heterodimeric structure of factor VIII and protects it from clearance and proteolysis, the clinical manivestations in VWD type 2N mimic haemophilia A.[43]

VWD type 2M includes patients with decreased platelet-dependent VWF function, although the multimeric size of VWF is normal. Mutations causing this type of VWD are mostly clustered in the A1-domain and results in a reduced GpIb binding capacity.[44]

VWD type 2A includes all patients with a lack or decrease of high molecular weight multimers, which is accompanied by a decreased VWF function. The reduction of high molecular weight multimers in type 2A can be caused either by an impaired biosynthesis of large VWF multimers (group 1 mutations)[45] or an increased proteolysis of VWF multimers in plasma by ADAMTS13 (group 2 mutations).[46] Group 2 mutations are mainly located in the A2-domain, the domain that is susceptible for proteolysis. In some cases, such mutations are also found in the A1-domain.[47] Type 2A can also be caused by a defective post-translational processing that includes defects of dimerization at the VWF C-terminus or defects of further polymerization of VWF dimers to multimers at the N-terminus. These defects are caused by mutations in the D1 and D2 domains of the propeptide or in the D3-domain.[48,49]

Von Willebrand disease type 2B

The lack of higher multimers in VWD type 2B is due to an enhanced affinity of VWF for the GpIb-IX-V receptor complex on platelets.[5] This enhanced affinity is caused by the presence of gain-of-function mutations in the A1-domain of VWF. At least 20 mutations have been characterized in patients with VWD type 2B. One is a single amino acid insertion[50], but the majority are single amino acid substitutions.[51] Paradoxically, the gain-of-function of VWF does not induce thrombosis. Instead, it results in a specific loss of the biologically more active higher multimers that react spontaneously with circulating platelets. The combination of loss of high multimers and consumption of platelets results in a bleeding diathesis.[5] Thrombocytopenia in VWD type 2B is often moderate to mild, can be intermittent and is exacerbated by surgery, physical effort or the administration of desmopressin (1-deamino-8-D-

arginine vasopressin, DDAVP).[52-54] Pregnancy also triggers the release of VWF from the Weibel-palade bodies, resulting in higher VWF antigen levels and lower platelet number. In VWD type 2B the increased amounts of mutant VWF induces more severe thrombocytopenia. The decrease in platelet count is dependent on the length of the pregnancy and is immediately reversed upon delivery. The sudden increase after delivery suggests that thrombocytopenia in VWD type 2B might also be due to other phenomena besides higher production of VWF during pregnancy.[52]

There is a high degree of heterogeneity in thrombocytopenia in VWD type 2B, even between patients with the same molecular defects. Despite many case reports on thrombocytopenia associated with VWD type 2B, studies in larger cohorts addressing the question of heterogeneity in thrombocytopenia have not been performed yet.

Thrombotic Thrombocytopenic Purpura

Apart from VWD type 2B, VWF multimers with a high biological activity also circulate in TTP. Under physiological conditions UL-VWF multimers are cleaved by ADAMTS13 into smaller, less reactive VWF multimers (Figure 4).[16] ADAMTS13 activity is deficient in TTP, resulting in the presence of UL-VWF multimers in the circulation of patients with TTP (Figure 4). UL-VWF multimers form high strength bonds with the GpIb-IX-V receptor complex on platelets[17], which leads to formation of VWF-rich thrombi in the microvasculature of many organs, consumptive thrombocytopenia and microangiopathic hemolytic anemia.[4] TTP can also be associated with fever, renal dysfunction and neurological deficits.[4] However, the latter three symptoms vary in severity amongst patients or can even be absent, which makes it difficult to distinguish TTP form other microangiopathies.

Reduced ADAMTS13 activity in TTP can be caused by mutations in the ADAMTS13 gene (congenital TTP)[55] or by the presence of inhibitory auto-antibodies (acquired TTP; Figure 4).[56] Hereditary TTP is an extremely rare disorder, which is treated with regular plasma infusions. Until now, many different mutations have been described, mostly clustered in the N-terminal region of ADAMTS13. Approximately 75% of the described missense mutations are located in the metalloprotease, disintegrin and the cysteine-rich/spacer domains (Figure 2).[57] This emphasizes the importance of these domains for the activity of ADAMTS13. Patients with congenital TTP are compound

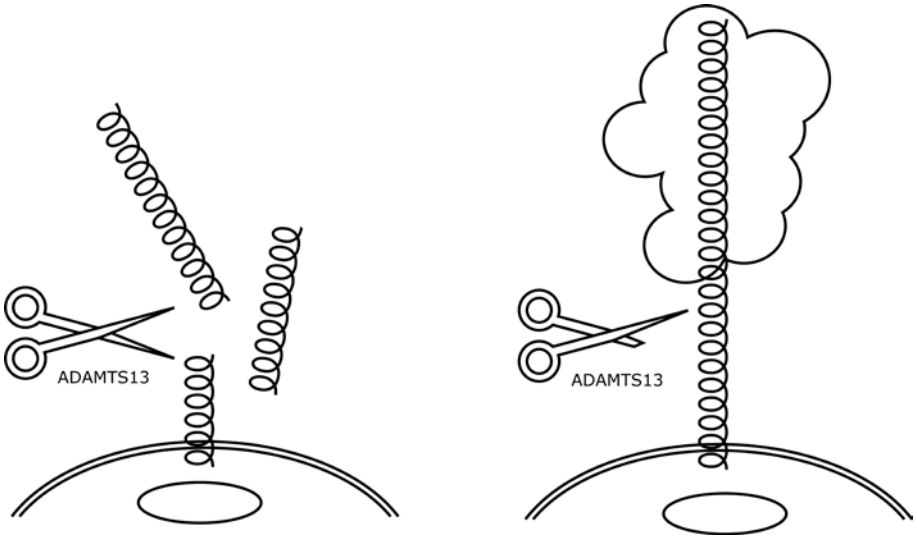

Figure 4: ADAMTS13 activity. Left: In healthy individuals ADAMTS13 cleaves UL-VWF multimers secreted from the Weibel-palade bodies of activated endothelial cells or bound to platelets in circulation. The smaller VWF multimers that circulate after cleavage are unable to bind spontaneously to platelets. **Right:** ADAMTS13 activity is absent or severely reduced in patients with TTP. UL-VWF multimers circulate and are able to induce spontaneous platelet adhesion and aggregation in flowing blood.

heterozygous or homozygous for the mutated alleles. Family members that are heterozygous are mostly asymptomatic and have only mildly reduced ADAMTS13 activity.[58] Clinical symptoms associated with congenital TTP usually present during the postnatal period or during early childhood, although several cases have been described in which the first manifestations appeared in adulthood.[59]

In the Netherlands each year 30-50 patients are diagnosed as acquired TTP. This form of TTP is associated with auto-antibodies against ADAMTS13. Treatment involves massive plasma exchange, often in combination with corticosteroids. This reduces mortality rates from 90% to approximately 20%.[60] Plasma exchange therapy is thought to be successful, since it not only replaces the inactive ADAMTS13, but also washes out the deleterious antibodies. In addition, treatment aiming at diminishing auto-antibody production can be used. Examples of such therapies are splenectomy[61] or administration of Rituximab[62,63], a humanized antibody against B-cell surface antigen CD20. The anti-ADAMTS13 antibodies often disappear spontaneously following plasma exchange.[64,65] However, in some patients the antibodies remain present, sometimes even in the absence of clinical symptoms.[64-66] Laboratory diagnosis of TTP is based on ADAMTS13 activity in plasma of patients and is usually

measured under static conditions[67], although it is generally thought that cleavage in vivo occurs under conditions of shear stress.[23] Certain inhibitory antibodies might be missed in static assays, for instance antibodies that impair docking of ADAMTS13 to VWF. Recently, new assays have become available to determine the amount of antibody and ADAMTS13 antigen levels. These assays might provide more insight in the onset of TTP and the role of the antibodies.

Besides primary TTP, TTP can also occur as a secondary manifestation in patients with other autoimmune diseases[68,69], cancer[70], infection[71], bone marrow transplantation[72], during use of certain anti-platelet drugs[73] or during pregnancy[74,75].

TTP-like diseases

Many conditions have a differential diagnosis that can mimic or overlap with TTP. Since TTP is a fatal disease when left untreated, an accurate diagnosis is critical for a good outcome. Likewise, the decision to omit plasma exchange with its potential side effects[76] when it is not TTP, is just as important. The presence of thrombocytopenia and Coombs negative hemolytic anemia without other causes is sufficient to make the diagnosis TTP.[60,77,78] However, based on these criteria it is difficult to distinguish TTP from other diseases like disseminated intravascular coagulation (DIC), hemolytic uremic syndrome (HUS), auto-immune diseases such as the antiphospholipid syndrome and during pregnancy the syndrome of hemolysis, elevated liver enzymes and low platelets (HELLP). ADAMTS13 activity has proven to be an important marker for the diagnosis of TTP. Various studies have shown that absence of ADAMTS13 activity or an activity of less than 1% is indicative for TTP and mildly reduced ADAMTS13 activity has been found in patients with other diseases.[79-81] On the other hand, normal ADAMTS13 activity has been found in otherwise typical cases of TTP.[82] Moreover, the assay to determine ADAMTS13 activity that is used in many laboratories is laborious and results are often not available soon enough to influence the acute decision-making regarding the treatment. Table II summarizes the features of the different TTP look-alikes, showing the differences and similarities between TTP and the other disorders.

Table II: Features of TTP and TTP-like diseases

Disorder	Features
Acute idiopathic TTP	TTP without underlying cause prominent microangiophathic hemolytic anemia thrombocytopenia renal function impairment neurologic symptoms strongly decreased ADAMTS13 activity (<1%) response to plasma exchange therapy
Secondary TTP	TTP with demonstrable underlying cause, such as: pregnancy, Bone marrow transplantation, cancer, medication and HIV-infection. (mildy) decreased ADAMTS13 activity treatment of underlying cause
Hemolytic uremic syndrome	presence of verocytotoxin (produced by Escherichia Coli) prominent renal function impairment more likely in younger adults and children normal ADAMTS13 activity variable response to plasma exchange therapy
Disseminated intravascular coagulation	prolonged clotting time and evident fibrinolysis increased levels of D-dimers clinical bleeding in advanced stage
Antiphospholipid syndrome	presence of antiphospholipid antibodies positive Coombs test if associated with SLE may have lupus anticoagulant and prolonged clotting time response to plasma exchange therapy has been reported
Preecclampsia	hypertension proteinuria edema response to delivery, not to plasma exchange therapy
HELLP syndrome	liver dysfunction, elevated liver enzymes microangiopathic hemolytic anemia thrombocytopenia response to delivery, not to plasma exchange therapy
Malignant hypertension	Severe hypertension (diastolic blood pressure > 130 mmHg) Medical history of uncontrolled hypertension normalizes with bloodpressure control

HELLP syndrome

Preeclampsia and HELLP syndrome are conditions in pregnancy that can strongly resemble pregnancy-related TTP. Since HELLP syndrome only resolves after delivery and TTP has to be treated with plasma exchange therapy, it is vital for both the mother and the unborn child that the appropriate diagnosis is made. HELLP

syndrome is thought to be a severe complication of preecclampsia. Both preecclampsia and HELLP syndrome are associated with endothelial injury, fibrin deposition in the vessel lumen, increased platelet activation and platelet consumption.[83] Especially in HELLP syndrome platelet counts below 100 x 10^9 /L are found. Endothelial cell activation in preecclampsia is considered to be one component of a more generalized inflammatory response to pregnancy. This response is common in all pregnancies, but is exaggerated in preecclampsia and is even further aggravated in patients with HELLP syndrome.[84] Many inflammatory markers have been reported to be increased in preecclampsia[85,86] and in HELLP syndrome[87-89]. It remains unclear how increased endothelial cell damage and the inflammatory state relate to platelet aggregation and thrombocytopenia observed in patients with HELLP syndrome. It has been suggested that depositions of thrombi in the microvasculature and in the sinusoids of the liver induce hemolytic anemia and increased liver enzymes.[90] Variable degrees of hepatic damage, microangiopathic hemolytic anemia and thrombocytopenia characterize HELLP syndrome[91]. It is still unknown why some preeclamptic women develop HELLP syndrome while others do not and what determines the severity of the symptoms.

Antiphospholipid syndrome

The antiphospholipid syndrome can also present with some of the features of TTP, including thrombocytopenia and hemolysis. However, the main symptoms are arterial and venous thrombosis and recurrent pregnancy-loss.[92,93] The syndrome is diagnosed when one of these clinical features is present in combination with the presence of antiphospholipid antibodies.[94] Actually, these antibodies recognize proteins that bind anionic phospholipids in stead of the phospholipids themselves.[95] Probably the most important antigen in the antiphospholipid syndrome is $ß_2$-glycoprotein (GP) I (Figure 5).[96] Antibodies that recognize this protein and cause a prolongation of phospholipid-dependent coagulation tests, have been described to correlate strongly with thrombosis.[97]

Despite all research that has been performed in this field, our understanding of the pathology of the syndrome is still limited. Data concerning the onset and clinical association of thrombocytopenia with the various antiphospholipid syndrome-related manifestations, for instance, are still scarce. Thrombocytopenia in the antiphospholipid syndrome is usually mild (70-120 x 10^9 /L) and rarely associated

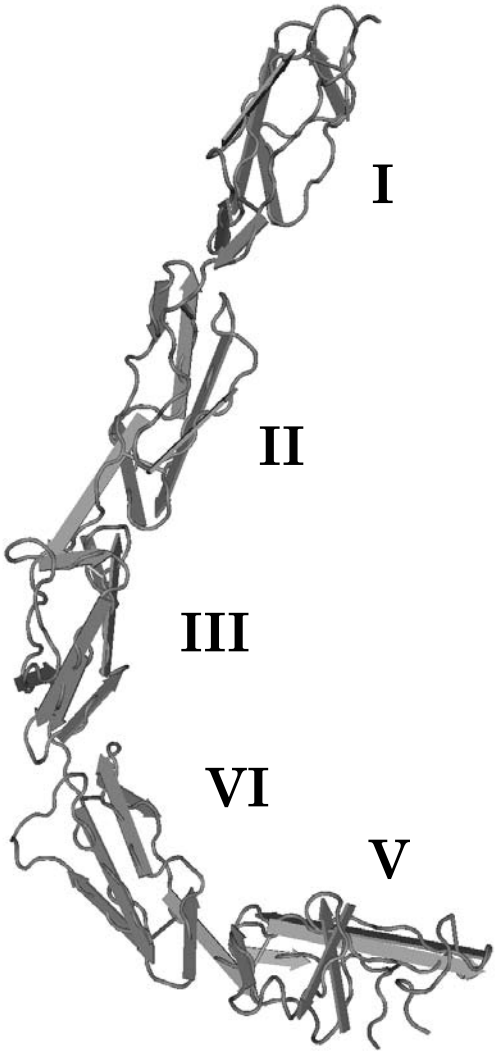

Figure 5: Crystal structure of β_2-GPI. β_2-GPI consists of 5 short consensus repeats, the so-called sushi- or Complement Control Protein-domains. Domain V contains the phospholipid binding site.

with bleeding complications.[98] There are different theories about the onset of the thrombocytopenia. The general thought is that this is a result of immune-induced platelet destruction, either induced directly by the anti-phospholipid antibodies or by the presence of other antibodies directed against platelet glycoproteins.[99,100] Other studies have questioned a direct role of the antiphospholipid antibodies in the pathogenesis of thrombocytopenia.[101,102]

Outline of this thesis

Although VWD type 2B, TTP, HELLP syndrome and the antiphospholipid syndrome are four well defined diseases with a distinct clinical entity, several reports documented the development of two of these diseases in one patient. HELLP may reveal the presence of the antiphospholipid syndrome[103] and TTP is observed in patients diagnosed with primary antiphospholipid syndrome[104]. Moreover, clinical features of pregnancy-related TTP and HELLP syndrome are very similar, which makes it difficult to distinguish between the diseases.

As described in the previous paragraphs, all four diseases are associated with a mild to severe thrombocytopenia, which is accompanied by thrombosis in TTP, HELLP syndrome and the antiphospholipid syndrome. Although VWD type 2B is also characterized by a consumptive thrombocytopenia, thrombosis has not been reported in these patients.

A connection with VWF has been implicated in all 4 diseases.[2-6] TTP is characterized by UL-VWF multimers, which have an increased biological activity.[4,17] The activity of VWF is also increased in VWD type 2B. In this disease a gain-of-function mutation induces spontaneous VWF-platelet interactions.[5] The role of VWF in HELLP syndrome and the antiphospholipid syndrome is less clear. VWF antigen levels are dramatically increased in HELLP syndrome, with a normal multimeric pattern.[2,3] The multimeric pattern of VWF has been reported to be abnormal in 37% of the patients suffering from the antiphospholipid syndrome. Interestingly, the presence of highly active multimers was found to correlate with the degree of thrombosis in these patients.[6]

In order to gain more insight in the role of VWF in the onset of thrombocytopenia in these four diseases, we addressed the following questions:

1) Can we develop an assay to distinguish between VWF in the resting state and VWF in a GpIbα-binding conformation? (Chapter 2)

2) Does VWF circulate in a GpIbα-binding conformation in VWD type 2B, TTP, HELLP syndrome and the antiphospholipid syndrome? (Chapter 2, 3 and 4)

3) What is the cause of the transition of VWF to a GpIb-binding conformation in the different diseases? (Chapter 2, 3 and 4)

4) Can we distinguish TTP from other, clinically related, diseases? (Chapter 5)

Reference List

(1) Bain BJ. Ethnic and sex differences in the total and differential white cell count and platelet count. J Clin Pathol. 1996;49:664-666.

(2) Friedman SA, Schiff E, Emeis JJ, Dekker GA, Sibai BM. Biochemical corroboration of endothelial involvement in severe preeclampsia. Am J Obstet Gynecol. 1995;172:202-203.

(3) Lattuada A, Rossi E, Calzarossa C, Candolfi R, Mannucci PM. Mild to moderate reduction of a von Willebrand factor cleaving protease (ADAMTS-13) in pregnant women with HELLP microangiopathic syndrome. Haematologica. 2003;88:1029-1034.

(4) Moake JL, Rudy CK, Troll JH et al. Unusually large plasma factor VIII:von Willebrand factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. N Engl J Med. 1982;307:1432-1435.

(5) Ruggeri ZM, Pareti FI, Mannucci PM, Ciavarella N, Zimmerman TS. Heightened interaction between platelets and factor VIII/von Willebrand factor in a new subtype of von Willebrand's disease. N Engl J Med. 1980;302:1047-1051.

(6) Schinco P, Borchiellini A, Tamponi G et al. Lupus anticoagulant and thrombosis: role of von Willebrand factor multimeric forms. Clin Exp Rheumatol. 1997;15:5-10.

(7) Savage B, Saldivar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. Cell. 1996;84:289-297.

(8) Weiss HJ, Sussman II, Hoyer LW. Stabilization of factor VIII in plasma by the von Willebrand factor. Studies on posttransfusion and dissociated factor VIII and in patients with von Willebrand's disease. J Clin Invest. 1977;60:390-404.

(9) Lenting PJ, Neels JG, van den Berg BM et al. The light chain of factor VIII comprises a binding site for low density lipoprotein receptor-related protein. J Biol Chem. 1999;274:23734-23739.

(10) Walker FJ, Chavin SI, Fay PJ. Inactivation of factor VIII by activated protein C and protein S. Arch Biochem Biophys. 1987;252:322-328.

(11) Koedam JA, Hamer RJ, Beeser-Visser NH, Bouma BN, Sixma JJ. The effect of von Willebrand factor on activation of factor VIII by factor Xa. Eur J Biochem. 1990;189:229-234.

(12) Nachman R, Levine R, Jaffe EA. Synthesis of factor VIII antigen by cultured guinea pig megakaryocytes. J Clin Invest. 1977;60:914-921.

(13) Jaffe EA, Hoyer LW, Nachman RL. Synthesis of antihemophilic factor antigen by cultured human endothelial cells. J Clin Invest. 1973;52:2757-2764.

(14) Wagner DD, Fay PJ, Sporn LA et al. Divergent fates of von Willebrand factor and its propolypeptide (von Willebrand antigen II) after secretion from endothelial cells. Proc Natl Acad Sci U S A. 1987;84:1955-1959.

(15) Sporn LA, Marder VJ, Wagner DD. Inducible secretion of large, biologically potent von Willebrand factor multimers. Cell. 1986;46:185-190.

(16) Dong JF, Moake JL, Nolasco L et al. ADAMTS-13 rapidly cleaves newly secreted ultralarge von Willebrand factor multimers on the endothelial surface under flowing conditions. Blood. 2002;100:4033-4039.

(17) Arya M, Anvari B, Romo GM et al. Ultralarge multimers of von Willebrand factor form spontaneous high-strength bonds with the platelet glycoprotein Ib-IX complex: studies using optical tweezers. Blood. 2002;99:3971-3977.

(18) Xie L, Chesterman CN, Hogg PJ. Control of von Willebrand factor multimer size by thrombospondin-1. J Exp Med. 2001;193:1341-1349.

(19) Zheng X, Chung D, Takayama TK et al. Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura. J Biol Chem. 2001;276:41059-41063.

(20) Dent JA, Galbusera M, Ruggeri ZM. Heterogeneity of plasma von Willebrand factor multimers resulting from proteolysis of the constituent subunit. J Clin Invest. 1991;88:774-782.

(21) Sadler JE. Biochemistry and genetics of von Willebrand factor. Annu Rev Biochem. 1998;67:395-424.

(22) Furlan M, Robles R, Lamie B. Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. Blood. 1996;87:4223-4234.

(23) Dong JF. Cleavage of ultra-large von Willebrand factor by ADAMTS-13 under flow conditions. J Thromb Haemost. 2005;3:1710-1716.

(24) De Cristofaro R, Peyvandi F, Palla R et al. Role of chloride ions in modulation of the interaction between von Willebrand factor and ADAMTS-13. J Biol Chem. 2005;280:23295-23302.

(25) Nishio K, Anderson PJ, Zheng XL, Sadler JE. Binding of platelet glycoprotein Ibalpha to von Willebrand factor domain A1 stimulates the cleavage of the adjacent domain A2 by ADAMTS13. Proc Natl Acad Sci U S A. 2004;101:10578-10583.

(26) Bowen DJ. An influence of ABO blood group on the rate of proteolysis of von Willebrand factor by ADAMTS13. J Thromb Haemost. 2003;1:33-40.

(27) Fukuda K, Doggett TA, Bankston LA et al. Structural basis of von Willebrand factor activation by the snake toxin botrocetin. Structure. 2002;10:943-950.

(28) Crawley JT, Lam JK, Rance JB et al. Proteolytic inactivation of ADAMTS13 by thrombin and plasmin. Blood. 2005;105:1085-1093.

(29) Pimanda JE, Ganderton T, Maekawa A et al. Role of thrombospondin-1 in control of von Willebrand factor multimer size in mice. J Biol Chem. 2004;279:21439-21448.

(30) Bernardo A, Ball C, Nolasco L, Moake JF, Dong JF. Effects of inflammatory cytokines on the release and cleavage of the endothelial cell-derived ultralarge von Willebrand factor multimers under flow. Blood. 2004;104:100-106.

(31) Wada H, Kaneko T, Ohiwa M et al. Plasma cytokine levels in thrombotic thrombocytopenic purpura. Am J Hematol. 1992;40:167-170.

(32) Huizinga EG, Tsuji S, Romijn RA et al. Structures of glycoprotein Ibalpha and its complex with von Willebrand factor A1 domain. Science. 2002;297:1176-1179.

(33) Ruggeri ZM. Von Willebrand factor, platelets and endothelial cell interactions. J Thromb Haemost. 2003;1:1335-1342.

(34) Berndt MC, Du XP, Booth WJ. Ristocetin-dependent reconstitution of binding of von Willebrand factor to purified human platelet membrane glycoprotein Ib-IX complex. Biochemistry. 1988;27:633-640.

(35) Scott JP, Montgomery RR, Retzinger GS. Dimeric ristocetin flocculates proteins, binds to platelets, and mediates von Willebrand factor-dependent agglutination of platelets. J Biol Chem. 1991;266:8149-8155.

(36) von Willebrand EA. Hereditär pseudohemofili. Finska Läkaresällskaptes Handlingar. 672, 7-112. 1926. Finska Läkaresällskaptes Handlingar.

(37) Zimmerman TS, Ratnoff OD, Powell AE. Immunologic differentiation of classic hemophilia (factor 8 deficiency) and von Willebrand's dissase, with observations on combined deficiencies of antihemophilic factor and proaccelerin (factor V) and on an acquired circulating anticoagulant against antihemophilic factor. J Clin Invest. 1971;50:244-254.

(38) Sadler JE. A revised classification of von Willebrand disease. For the Subcommittee on von Willebrand Factor of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. Thromb Haemost. 1994;71:520-525.

(39) Ngo KY, Glotz VT, Koziol JA et al. Homozygous and heterozygous deletions of the von Willebrand factor gene in patients and carriers of severe von Willebrand disease. Proc Natl Acad Sci U S A. 1988;85:2753-2757.

(40) Eikenboom JC, Ploos van Amstel HK, Reitsma PH, Briet E. Mutations in severe, type III von Willebrand's disease in the Dutch population: candidate missense and nonsense mutations associated with reduced levels of von Willebrand factor messenger RNA. Thromb Haemost. 1992;68:448-454.

(41) Nishino M, Girma JP, Rothschild C, Fressinaud E, Meyer D. New variant of von Willebrand disease with defective binding to factor VIII. Blood. 1989;74:1591-1599.

(42) Mazurier C, Dieval J, Jorieux S, Delobel J, Goudemand M. A new von Willebrand factor (vWF) defect in a patient with factor VIII (FVIII) deficiency but with normal levels and multimeric patterns of both plasma and platelet vWF. Characterization of abnormal vWF/FVIII interaction. Blood. 1990;75:20-26.

(43) Schneppenheim R, Budde U, Krey S et al. Results of a screening for von Willebrand disease type 2N in patients with suspected haemophilia A or von Willebrand disease type 1. Thromb Haemost. 1996;76:598-602.

(44) Meyer D, Fressinaud E, Gaucher C et al. Gene defects in 150 unrelated French cases with type 2 von Willebrand disease: from the patient to the gene. INSERM Network on Molecular Abnormalities in von Willebrand Disease. Thromb Haemost. 1997;78:451-456.

(45) Lyons SE, Bruck ME, Bowie EJ, Ginsburg D. Impaired intracellular transport produced by a subset of type IIA von Willebrand disease mutations. J Biol Chem. 1992;267:4424-4430.

(46) Dent JA, Berkowitz SD, Ware J, Kasper CK, Ruggeri ZM. Identification of a cleavage site directing the immunochemical detection of molecular abnormalities in type IIA von Willebrand factor. Proc Natl Acad Sci U S A. 1990;87:6306-6310.

(47) Ginsburg D, Konkle BA, Gill JC et al. Molecular basis of human von Willebrand disease: analysis of platelet von Willebrand factor mRNA. Proc Natl Acad Sci U S A. 1989;86:3723-3727.

(48) Gaucher C, Dieval J, Mazurier C. Characterization of von Willebrand factor gene defects in two unrelated patients with type IIC von Willebrand disease. Blood. 1994;84:1024-1030.

(49) Holmberg L, Karpman D, Isaksson C et al. Ins405AsnPro mutation in the von Willebrand factor propeptide in recessive type 2A (IIC) von Willebrand's disease. Thromb Haemost. 1998;79:718-722.

(50) Ribba AS, Lavergne JM, Bahnak BR et al. Duplication of a methionine within the glycoprotein Ib binding domain of von Willebrand factor detected by denaturing gradient gel electrophoresis in a patient with type IIB von Willebrand disease. Blood. 1991;78:1738-1743.

(51) Meyer D, Fressinaud E, Hilbert L et al. Type 2 von Willebrand disease causing defective von Willebrand factor-dependent platelet function. Best Pract Res Clin Haematol. 2001;14:349-364.

(52) Casonato A, Sartori MT, Bertomoro A et al. Pregnancy-induced worsening of thrombocytopenia in a patient with type IIB von Willebrand's disease. Blood Coagul Fibrinolysis. 1991;2:33-40.

(53) Casonato A, Sartori MT, de Marco L, Girolami A. 1-Desamino-8-D-arginine vasopressin (DDAVP) infusion in type IIB von Willebrand's disease: shortening of bleeding time and induction of a variable pseudothrombocytopenia. Thromb Haemost. 1990;64:117-120.

(54) Donner M, Kristoffersson AC, Lenk H et al. Type IIB von Willebrand's disease: gene mutations and clinical presentation in nine families from Denmark, Germany and Sweden. Br J Haematol. 1992;82:58-65.

(55) Levy GG, Nichols WC, Lian EC et al. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. Nature. 2001;413:488-494.

(56) Moake JL. Thrombotic microangiopathies. N Engl J Med. 2002;347:589-600.

(57) Levy GG, Motto DG, Ginsburg D. ADAMTS13 turns 3. Blood. 2005;106:11-17.

(58) Furlan M, Robles R, Solenthaler M et al. Deficient activity of von Willebrand factor-cleaving protease in chronic relapsing thrombotic thrombocytopenic purpura. Blood. 1997;89:3097-3103.

(59) Furlan M, Lammle B. Aetiology and pathogenesis of thrombotic thrombocytopenic purpura and haemolytic uraemic syndrome: the role of von Willebrand factor-cleaving protease. Best Pract Res Clin Haematol. 2001;14:437-454.

(60) Rock GA, Shumak KH, Buskard NA et al. Comparison of plasma exchange with plasma infusion in the treatment of thrombotic thrombocytopenic purpura. Canadian Apheresis Study Group. N Engl J Med. 1991;325:393-397.

(61) Kappers-Klunne MC, Wijermans P, Fijnheer R et al. Splenectomy for the treatment of thrombotic thrombocytopenic purpura. Br J Haematol. 2005;130:768-776.

(62) Yomtovian R, Niklinski W, Silver B, Sarode R, Tsai HM. Rituximab for chronic recurring thrombotic thrombocytopenic purpura: a case report and review of the literature. Br J Haematol. 2004;124:787-795.

(63) Fakhouri F, Vernant JP, Veyradier A et al. Efficiency of curative and prophylactic treatment with rituximab in ADAMTS13-deficient thrombotic thrombocytopenic purpura: a study of 11 cases. Blood. 2005;106:1932-1937.

(64) Zheng XL, Kaufman RM, Goodnough LT, Sadler JE. Effect of plasma exchange on plasma ADAMTS13 metalloprotease activity, inhibitor level, and clinical outcome in patients with idiopathic and nonidiopathic thrombotic thrombocytopenic purpura. Blood. 2004;103:4043-4049.

(65) Bohm M, Betz C, Miesbach W et al. The course of ADAMTS-13 activity and inhibitor titre in the treatment of thrombotic thrombocytopenic purpura with plasma exchange and vincristine. Br J Haematol. 2005;129:644-652.

(66) Furlan M, Robles R, Solenthaler M, Lammle B. Acquired deficiency of von Willebrand factor-cleaving protease in a patient with thrombotic thrombocytopenic purpura. Blood. 1998;91:2839-2846.

(67) Veyradier A, Girma JP. Assays of ADAMTS-13 activity. Semin Hematol. 2004;41:41-47.

(68) Hamasaki K, Mimura T, Kanda H et al. Systemic lupus erythematosus and thrombotic thrombocytopenic purpura: a case report and literature review. Clin Rheumatol. 2003;22:355-358.

(69) Roberts G, Gordon MM, Porter D, Jardine AG, Gibson IW. Acute renal failure complicating HELLP syndrome, SLE and anti-phospholipid syndrome: successful outcome using plasma exchange therapy. Lupus. 2003;12:251-257.

(70) Gordon LI, Kwaan HC. Thrombotic microangiopathy manifesting as thrombotic thrombocytopenic purpura/hemolytic uremic syndrome in the cancer patient. Semin Thromb Hemost. 1999;25:217-221.

(71) Sutor GC, Schmidt RE, Albrecht H. Thrombotic microangiopathies and HIV infection: report of two typical cases, features of HUS and TTP, and review of the literature. Infection. 1999;27:12-15.

(72) van der Plas RM, Schiphorst ME, Huizinga EG et al. von Willebrand factor proteolysis is deficient in classic, but not in bone marrow transplantation-associated, thrombotic thrombocytopenic purpura. Blood. 1999;93:3798-3802.

(73) Medina PJ, Sipols JM, George JN. Drug-associated thrombotic thrombocytopenic purpura-hemolytic uremic syndrome. Curr Opin Hematol. 2001;8:286-293.

(74) George JN. The association of pregnancy with thrombotic thrombocytopenic purpura-hemolytic uremic syndrome. Curr Opin Hematol. 2003;10:339-344.

(75) Shamseddine A, Chehal A, Usta I et al. Thrombotic thrombocytopenic purpura and pregnancy: report of four cases and literature review. J Clin Apher. 2004;19:5-10.

(76) McMinn JR, Jr., Thomas IA, Terrell DR et al. Complications of plasma exchange in thrombotic thrombocytopenic purpura-hemolytic uremic syndrome: a study of 78 additional patients. Transfusion. 2003;43:415-416.

(77) George JN. How I treat patients with thrombotic thrombocytopenic purpura-hemolytic uremic syndrome. Blood. 2000;96:1223-1229.

(78) Thompson CE, Damon LE, Ries CA, Linker CA. Thrombotic microangiopathies in the 1980s: clinical features, response to treatment, and the impact of the human immunodeficiency virus epidemic. Blood. 1992;80:1890-1895.

(79) Bianchi V, Robles R, Alberio L, Furlan M, Lammle B. Von Willebrand factor-cleaving protease (ADAMTS13) in thrombocytopenic disorders: a severely deficient activity is specific for thrombotic thrombocytopenic purpura. Blood. 2002;100:710-713.

(80) Moore JC, Hayward CP, Warkentin TE, Kelton JG. Decreased von Willebrand factor protease activity associated with thrombocytopenic disorders. Blood. 2001;98:1842-1846.

(81) Hulstein JJ, Rison CN, Kappers-Klunne MC et al. [Activity loss of Von Willebrand factor cleaving protein (ADAMTS-13) is diagnostic for primary and pregnancy-related thrombotic thrombocytopenic purpura]. Ned Tijdschr Geneeskd. 2004;148:1972-1976.

(82) Peyvandi F, Ferrari S, Lavoretano S, Canciani MT, Mannucci PM. von Willebrand factor cleaving protease (ADAMTS-13) and ADAMTS-13 neutralizing autoantibodies in 100 patients with thrombotic thrombocytopenic purpura. Br J Haematol. 2004;127:433-439.

(83) Sibai BM. The HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets): much ado about nothing? Am J Obstet Gynecol. 1990;162:311-316.

(84) Redman CW, Sacks GP, Sargent IL. Preeclampsia: an excessive maternal inflammatory response to pregnancy. Am J Obstet Gynecol. 1999;180:499-506.

(85) Vince GS, Starkey PM, Austgulen R, Kwiatkowski D, Redman CW. Interleukin-6, tumour necrosis factor and soluble tumour necrosis factor receptors in women with pre-eclampsia. Br J Obstet Gynaecol. 1995;102:20-25.

(86) Madazli R, Aydin S, Uludag S, Vildan O, Tolun N. Maternal plasma levels of cytokines in normal and preeclamptic pregnancies and their relationship with diastolic blood pressure and fibronectin levels. Acta Obstet Gynecol Scand. 2003;82:797-802.

(87) Haeger M, Unander M, Norder-Hansson B, Tylman M, Bengtsson A. Complement, neutrophil, and macrophage activation in women with severe preeclampsia and the syndrome of hemolysis, elevated liver enzymes, and low platelet count. Obstet Gynecol. 1992;79:19-26.

(88) Haeger M, Unander M, Andersson B et al. Increased release of tumor necrosis factor-alpha and interleukin-6 in women with the syndrome of hemolysis, elevated liver enzymes, and low platelet count. Acta Obstet Gynecol Scand. 1996;75:695-701.

(89) Visser W, Beckmann I, Bremer HA, Lim HL, Wallenburg HC. Bioactive tumour necrosis factor alpha in pre-eclamptic patients with and without the HELLP syndrome. Br J Obstet Gynaecol. 1994;101:1081-1082.

(90) Reubinoff BE, Schenker JG. HELLP syndrome--a syndrome of hemolysis, elevated liver enzymes and low platelet count--complicating preeclampsia-eclampsia. Int J Gynaecol Obstet. 1991;36:95-102.

(91) Magann EF, Martin JN, Jr. Critical care of HELLP syndrome with corticosteroids. Am J Perinatol. 2000;17:417-422.

(92) de Groot PG, Derksen RH. Pathophysiology of the antiphospholipid syndrome. J Thromb Haemost. 2005;3:1854-1860.

(93) Miyakis S, Lockshin MD, Atsumi T et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost. 2006;4:295-306.

(94) Wilson WA, Gharavi AE, Koike T et al. International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome: report of an international workshop. Arthritis Rheum. 1999;42:1309-1311.

(95) Arnout J, Vermylen J. Current status and implications of autoimmune antiphospholipid antibodies in relation to thrombotic disease. J Thromb Haemost. 2003;1:931-942.

(96) McNeil HP, Simpson RJ, Chesterman CN, Krilis SA. Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: beta 2-glycoprotein I (apolipoprotein H). Proc Natl Acad Sci U S A. 1990;87:4120-4124.

(97) de Laat HB, Derksen RH, Urbanus RT, Roest M, de Groot PG. beta2-glycoprotein I-dependent lupus anticoagulant highly correlates with thrombosis in the antiphospholipid syndrome. Blood. 2004;104:3598-3602.

(98) Cuadrado MJ, Mujic F, Munoz E, Khamashta MA, Hughes GR. Thrombocytopenia in the antiphospholipid syndrome. Ann Rheum Dis. 1997;56:194-196.

(99) Godeau B, Piette JC, Fromont P et al. Specific antiplatelet glycoprotein autoantibodies are associated with the thrombocytopenia of primary antiphospholipid syndrome. Br J Haematol. 1997;98:873-879.

(100) Macchi L, Rispal P, Clofent-Sanchez G et al. Anti-platelet antibodies in patients with systemic lupus erythematosus and the primary antiphospholipid antibody syndrome: their relationship with the observed thrombocytopenia. Br J Haematol. 1997;98:336-341.

(101) Biasiolo A, Pengo V. Antiphospholipid antibodies are not present in the membrane of gel-filtered platelets of patients with IgG anticardiolipin antibodies, lupus anticoagulant and thrombosis. Blood Coagul Fibrinolysis. 1993;4:425-428.

(102) Martinuzzo ME, Maclouf J, Carreras LO, Levy-Toledano S. Antiphospholipid antibodies enhance thrombin-induced platelet activation and thromboxane formation. Thromb Haemost. 1993;70:667-671.

(103) Le Thi TD, Tieulie N, Costedoat N et al. The HELLP syndrome in the antiphospholipid syndrome: retrospective study of 16 cases in 15 women. Ann Rheum Dis. 2005;64:273-278.

(104) Amoura Z, Costedoat-Chalumeau N, Veyradier A et al. Thrombotic thrombocytopenic purpura with severe ADAMTS-13 deficiency in two patients with primary antiphospholipid syndrome. Arthritis Rheum. 2004;50:3260-3264.

2

Chapter 2

A novel nanobody that detects the gain-of-function phenotype of Von Willebrand Factor in ADAMTS13 deficiency and Von Willebrand disease type 2B

Janine J.J. Hulstein
Philip G. de Groot
Karen Silence
Agnès Veyradier
Rob Fijnheer
Peter J. Lenting

Blood. 2005 Nov 1;106(9):3035-42

Abstract

Von Willebrand factor (VWF) is unable to interact spontaneously with platelets because this interaction requires a conversion of the VWF A1 domain into a Glycoprotein Ibα (GpIbα) binding conformation. Here, we discuss a llama-derived antibody fragment (AU/VWFa-11) that specifically recognizes the GpIbα-binding conformation. AU/VWFa-11 is unable to bind VWF in solution, but efficiently interacts with ristocetin- or botrocetin-activated VWF, VWF comprising type 2B mutation R1306Q, or immobilized VWF. These unique properties allowed us to use AU/VWFa-11 for the detection of activated VWF in plasma of patients characterized by spontaneous VWF-platelet interactions: von Willebrand disease (VWD) type 2B and thrombotic thrombocytopenic purpura (TTP). For VWD type 2B, levels of activated VWF were increased 12-fold ($p<0.001$) compared to levels in healthy volunteers. An inverse correlation between activated VWF levels and platelet count was observed ($R^2=0.74$, $p<0.003$). With regard to TTP, a 2-fold ($p<0.001$) increase in activated VWF levels was found in plasma of patients with acquired TTP, whereas an 8-fold increase ($p<0.003$) was found in congenital TTP. No overlap in levels of activated VWF could be detected between acquired and congenital TTP, suggesting that AU/VWFa-11 could be used to distinguish between both disorders. Furthermore, it could provide a tool to investigate the role of VWF in the development of thrombocytopenia in various diseases.

Introduction

Adhesion of platelets to the injured vessel wall is a multistep process, involving several components, including von Willebrand Factor (VWF). VWF is an adhesive glycoprotein that circulates in plasma as an array of multimeric subunits.[1] This multimeric structure allows VWF to function as a molecular bridge between the subendothelial matrix and the platelet-surface Glycoprotein (Gp) Ib/IX/V receptor complex. Complex formation between VWF and the GpIb/IX/V receptor is of particular importance for the tethering of platelets on vascular surfaces exposed to rapidly flowing blood.[2]

The interaction between VWF and the GpIb/IX/V complex is mediated by specific regions of both components: VWF residues 1238-1481 (the so-called A1 domain) comprises an interactive site for residues 1-290 of GpIbα.[3] Although the structure of this complex has been solved at the atomic level[4], some issues regarding this interaction have remained unclear. For instance, despite the notion that VWF and GpIbα coexist in the circulation, their interaction does not occur under normal conditions. In contrast, the isolated recombinant A1 domain does display spontaneous binding to GpIbα. Apparently, a shift from a nonbinding to a binding mode of the VWF A1 domain in its multimeric environment is required to induce complex formation.[4] However, the molecular basis of this activation-step is largely unknown.

Activation of the A1 domain can be induced by several means. Nonphysiological activation of the A1 domain occurs through direct immobilization of purified VWF onto artificial surfaces, such as glass or plastic. *In vitro* activation of VWF is also achieved by the addition of modulators, such as the snake-venom component botrocetin or the antibiotic ristocetin.[5,6] Furthermore, physiologic activation of VWF is induced on its binding to the subendothelial matrix component collagen or under conditions of very high shear stress.[7] Also, various pathologic conditions may lead to premature complex formation between VWF and platelet GpIbα. Some gain-of-function mutations in the VWF A1 domain may increase the affinity for GpIbα. Such mutations are associated with von Willebrand disease (VWD) type 2B; these patients are characterized by loss of high-molecular-weight multimers from plasma, increased ristocetin-induced platelet aggregation, a prolonged bleeding time, and thrombocytopenia.[8,9] Another condition that has been reported to allow spontaneous platelet adhesion relates to the size of multimeric VWF. Multimeric VWF is stored in Weibel-Palade bodies

in endothelial cells, and released upon stimulation.[10-12] The newly released VWF is enriched in ultra-large (UL)-VWF multimers, which have the potential to bind platelets in the absence of any modulators or high shear stress.[13] Direct release of the UL-VWF molecules in the circulation is prevented by proteolysis of these multimers at the endothelial surface.[14] This process is mediated by the recently identified protease ADAMTS13, which cleaves mature VWF between residues M1605 and Y1606.[15,16] Once cleaved by ADAMTS13, the residual multimers have lost the ability to bind platelets spontaneously. The importance of ADAMTS13 activity is illustrated by the life-threatening disease Thrombotic Thrombocytopenic Purpura (TTP), in which ADAMTS13 activity is low or absent. ADAMTS13 deficiency is caused by inhibiting antibodies (acquired TTP)[17], or by mutations in the gene encoding ADAMTS13 (congenital TTP)[18,19]. In the absence of ADAMTS13 activity, an excess of UL-VWF multimers is released into the circulation, which leads to spontaneous platelet binding and subsequent thrombus formation in the microvasculature.[20] This causes haemolytic anemia, renal failure, neurological deficits, fever and, if not treated well, coma and death.[21]

Although VWD type 2B and TTP are associated with different phenotypic appearances, they have in common that at least part of the circulating VWF multimers should exist in an active conformation. The presence of activated VWF can be determined indirectly by measuring ristocetin-dependent platelet aggregation. However, this method is rather insensitive and is difficult to use when VWF antigen levels are low. In the present paper we describe a recombinant llama-derived antibody fragment that recognizes immobilized VWF but not VWF in solution, suggesting that this antibody fragment recognizes an epitope within the VWF A1 domain that becomes exposed on activation of VWF. This antibody fragment was subsequently used to monitor the presence of activated VWF in plasma samples from patients with VWD type 2B and TTP. The analysis revealed that in the circulation of both patient groups the levels of activated VWF are elevated 2 to 12-fold compared to healthy individuals.

Materials and Methods

Proteins and antibodies

Recombinant GpIbα (residues 1-290) was expressed and purified as described.[4] Botrocetin was purchased from Kordia laboratory supplies (Leiden, The Netherlands). Plasma derived (pd)-VWF was purified from cryoprecipitate (Haemate P 250 IE, Behringwerke AG, Marburg, Germany) as described.[22] Bovine serum albumin (BSA) and human placental collagen type III were from Sigma (St. Louis, MO) and human albumin (Fraction V) was from MP Biochemicals (Irvine, CA USA). Polyclonal antibodies against VWF and Horseradish peroxidase (HRP)-conjugated antibodies against VWF and HRP-conjugated rabbit-anti-mouse antibody were obtained from Dakocytomation (Glostrup, Denmark).

Construction and expression of recombinant proteins

Construction of expression vector pNUT encoding wild type (wt) VWF and VWF with VWD type 2B mutation R1306Q was described previously.[23,24] VWF/A1(1261-1468) and VWF/A1(1261-1468)-R1306Q were cloned into expression vector pPIC9 and overexpressed in Pichia Pastoris.[4] pNUT-VWF/A1(1238-1481) was constructed by generating a polymerase chain reaction (PCR) product with forward primer 5'–GGATCCCAGGAGCCGGGAGGCCTGG TGG–3' and reverse primer 5'–GCGGCCGCCCCCGGGCCCACAGTGACTTG–3', for which pNUT-VWF served as template. After sequence analysis, the *Bam*HI-*Not*I fragment was ligated into a *Bam*HI-*Not*I digested pNUT vector containing a C-terminal 6-histidine tag. Wt VWF, VWF/R1306Q and VWF/A1(1238-1481) were stably expressed in baby hamster kidney-cells, which also overexpress furin for proper removal of the propeptide.[23] The full-length proteins were purified from conditioned serum-free medium as described.[25] VWF/A1(1238-1481) was purified from expression medium using Ni^{2+}/NTA chromatography.[4] VWF/A1(1261-1468) and VWF/A1(1261-1468)-R1306Q were purified on heparin-Sepharose, followed by gel filtration.[4] Analysis on Sodium dodecy sulfate (SDS)-polyacrylamide gel electrophoresis showed that all recombinant proteins were purified to homogeneity. The multimeric structure of wt VWF and VWF/R1306Q was analyzed using 0.1% SDS, 1% agarose gel electrophoresis as described previously.[26]

Production and selection of recombinant llama antibody fragments (nanobodies) via phage-display technology

Llama antibodies were raised by immunization with a wt-VWF preparation containing high molecular weight multimers using standard immunization protocols. Total RNA was isolated from peripheral blood lymphocytes and used as a template for the preparation of cDNA to amplify the repertoire of variable domains of the antibody heavy chains.[27] This repertoire was then subcloned to allow selection of nanobodies via phage-display.[27] For the selection of the nanobodies, the wells of a maxisorp-microtiter plate (NUNC, Roskilde, Denmark) were coated with 5 µg/ml VWF/A1(1238-1481) in 50 mM NaHCO3 (pH 9.6, overnight at 4°C). After washing and blocking (Phosphate buffered saline [PBS]/1 % casein), wells were incubated with phages for 2 h at room temperature. Wells were then washed extensively with PBS, and bound phages were eluted with 0.2 M glycin (pH 2.4, 20 min at room temperature). The eluted phages were added to exponentially growing *Escherichia coli* TG1-cells, which were then plated onto Luria broth (LB)-ampicilin. In the second round of selection, phages were resuspended in the presence of 10 µg/ml wt VWF before incubation in wells coated with VWF/A1(1238-1481). This selection strategy was chosen to isolate nanobodies that specifically recognize A1 domain in its GpIbα-binding conformation and not A1 domain in its resting conformation. After washing the wells 7 times for 30 min in the presence of 10 µg/ml wt VWF, bound phages were eluted and used for transduction of TG1 cells to isolate single clones after plating on LB-ampicilin. Subsequently, single colonies were picked for reinfection of TG1-cells and expression of phage gen3-nanobody fusion proteins was induced upon the addition of 1 mM isopropyl-1-thio-ß-D-galactopyranoside. Periplasmic proteins were extracted as described[28] and analyzed for binding to immobilized VWF/A1(1238-1481). DNA of positive clones was then analyzed via *Hinf*I digestion and those with appropriate inserts were used for transformation to the non-suppressor *E. coli* WK-6 strain[27], which allows the expression of nanobodies in the absence of the phage gen3-protein. Periplasmic samples were prepared as described[27] and nanobodies were purified to homogeneity using Ni^{2+}/NTA resin. The control nanobody used in the present study was obtained after immunization and selection using the isolated recombinant VWF A1 domain.

Competition assay

The specificity of the interaction between AU/VWFa-11 and activated VWF was assessed in an immunosorbent assay, in which purified pd VWF (3.7 nM, based on monomeric structure) was immobilized in microtiter wells (Costar, Cambridge, MA). Wells were blocked with PBS containing 3% BSA and 0.1% Tween-20 for 1 h at 37ºC and incubated with different concentrations of biotinylated control nanobody and AU/VWFa-11 (0-625 nM) in PBS for 1 h at 37ºC. Binding of the nanobodies was monitored with HRP-conjugated streptavidin and the nanobody concentration at half maximum binding was determined. These concentrations were used in the competition assay, in which pd VWF-coated wells were incubated with either of the nanobodies in the presence or absence of the indicated concentrations of wt-VWF or wt-VWF preincubated with 1 mg/ml ristocetin (5 min at room temperature; 0-115 nM soluble VWF for the control nanobody and 0-38 nM for AU/VWFa-11) or in the presence of a 10-fold molar excess of VWF/R1306Q or VWF/A1(1261-1468)-R1306Q. After washing, wells were incubated with HRP-conjugated streptavidin (DakoCytomation) and binding was detected by measuring HRP activity using *o*-phenylenediamine (OPD) as substrate.

Surface Plasmon Resonance Analysis

Surface Plasmon Resonance (SRP) binding studies were performed using a Biacore 2000 system (Biacore AB, Uppsala, Sweden). AU/VWFa-11 was immobilized on a CM5 sensor chip using the amine-coupling kit as instructed by the supplier (Biacore). V128H, a nanobody recognizing the A3 domain of VWF, was used as a control. Binding of VWF/A1(1238-1481), VWF/A1(1261-1468) and VWF/A1(1261-1468)-R1306Q to the AU/VWFa-11 coated channel was corrected for binding to the V128H coated channel. Binding of VWF constructs to the immobilized nanobodies was performed in 150 mM NaCl, 25 nM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 0.005% Tween-20 (pH 7.4) at 25°C with a flow rate of 20 µl/min. Regeneration of the surface was performed by subsequent application of 50 mM triethyl amine and formate buffer (10 mM $NaHCO_2$ and 150 mM NaCl pH 2.0).

Binding of AU/VWFa-11 and GpIbα to VWF/R1306Q in immunosorbant assay

Microtiter wells (Costar) were coated overnight at 4°C with 5 µg/ml AU/VWFa-11 and blocked with PBS/3% BSA/0.1% Tween-20 (30 min at 37°C). After washing with PBS/0.1% Tween-20, wells were incubated with 3.7 nM VWF/R1306Q (1 h at 37°C).

Wells were washed and incubated 1 h at 37°C with GpIbα (0.12 – 31.1 nM). After washing 3 times, wells were incubated with a monoclonal anti-GpIbα antibody and binding was detected with an HRP-conjugated rabbit-anti-mouse antibody.

Static adhesion of CHO-cells expressing the GpIb-IX-V complex to immobilized VWF

Wt-VWF (37 nM) was immobilized overnight at 4°C in microtiter wells (Nunclon, NUNC) in 50mM $NaHCO_3$ (pH 9.6). Wells were blocked with 0.5% poly-vinyl-pyrrolidone (PNP) in PBS for 1 h at room temperature and incubated with 1.25 μM AU/VWFa-11 or control nanobody (1 h at room temperature). After washing 3 times with PBS, CHO cells expressing the GpIb/IX/V complex (generous gift of Dr. J.A. Lopez[29], 1 x 10^5 cells in Dulbecco modified Eagle medium [DMEM] containing 0.1% BSA) were allowed to bind to immobilized VWF (90 min at 37°C) in the presence or absence of control nanobody or AU/VWFa-11 (1.25 μM). Wells were washed and binding of cells was detected by measuring the intrinsic alkaline phophatase activity of the CHO-cells, using p-nitrophenyl phosphate (PNP, Sigma) as a substrate diluted in lysisbuffer (3 mg/ml PNP in 1% Triton-X-100, 50mM acetic acid, pH 5.0).

Platelet adhesion to Collagen type III and VWF

Perfusions over collagen type III were carried out with whole blood, drawn from healthy volunteers who denied ingestion of aspirin or other nonsteroidal anti-inflammatory drugs for the preceding 10 days, into 0.1 volume of 50 μg/ml PPACK (H-D-Phe-Pro-Arg-Chloromethylketone, Bachem, Torrence, CA) and 20 U/ml Pentasaccharide. Thermanox coverslips (NUNC) were coated with collagen type III[30] and whole blood was perfused over the coverslips for 5 min at 1600 s^{-1}. Perfusions over VWF coated coverslips was performed with reconstituted blood. To obtain reconstituted blood, platelets were mixed with red cells to a platelet count of 200.000 platelets/μL and a hematocrit of 40%.[30] Perfusion was performed at a shear rate of 1600 s^{-1}. After perfusion, slides were washed, fixed and stained[30] and platelet adhesion was evaluated using computer-assisted analysis with OPTIMAS 6.0 software (Dutch Vision Systems, Breda, The Netherlands). All perfusions were performed 3 times.

Patient materials

Plasma samples of healthy donors (n=9), patients with VWD type 2B (n=10) and patients with acquired (n=12) or congenital TTP (n=5) were collected in 3.1% citrate using a Vacutainer system. VWD type 2B was diagnosed in families with the typical pattern of an autosomal inherited bleeding disorder with thrombocytopenia, low

ristocetin-induced platelet aggregation, and the absence of high multimeric VWF multimers on gel electrophoresis. The mutations in VWF of these patients have not been determined. Patients with acquired TTP were characterized by thrombocytopenia, Coombs negative hemolytic anemia and the presence of fragmented erythrocytes in peripheral blood. Other causes for hemolytic anemia and thrombocytopenia were excluded and all TTP patients were treated with plasma exchange. Response to plasma exchange was observed in all patients. Plasma samples were taken before treatment and ADAMTS13 activity was found to be absent in these samples. Five patients with a congenital form of TTP and a severe deficiency of ADAMTS13 activity were described previously.[31] Platelet poor plasma was aliquoted and frozen at -80°C. For normal pool plasma (NPP), platelet poor plasma of 40 healthy donors was pooled and was stored in aliquots at -80°C. All patients gave informed consent for the sampling of blood for scientific purposes, per the Declaration of Helsinki. Approval was obtained from the institutional review boards of the University Medical Center Utrecht (Utrecht, The Netherlands) and the Robert Debre hospital (Paris, France) for those studies.

Immunosorbent assay for activated VWF

VWF antigen levels were quantified as described before.[26] Microtiter wells (Maxisorb, NUNC) were coated overnight at 4°C with 5 µg/ml AU/VWFa-11 in 50 mM $NaHCO_3$ (pH 9.6) and blocked with PBS/3% BSA/0.1% Tween-20 for 30 min at 37°C. Wells were washed 3 times with PBS/0.1% Tween-20 and incubated with culture medium containing wt VWF or VWF-R1306Q in the absence or presence of ristocetin (0.08 – 1 mg/ml) or botrocetin (0.2 U/ml), or plasma samples (1 h 37°C). All samples were diluted in PBS to reach a VWF concentration between 0.23 and 1.85 nM. After washing 3 times with PBS/0.1% Tween-20, plates were incubated with HRP-conjugated polyclonal anti-VWF (1.3 µg/ml) in PBS for 1 h at 37°C. Plates were washed 3 times and binding was detected by measuring the HRP activity using OPD as a substrate (Merck, Darmstadt, Germany). NPP was used as standard in every assay. The ratio between the slope for the different plasma samples over the slope for NPP was designated activation factor. All patient plasmas have been measured in at least 2 individual experiments.

Variation of the AU/VWFa-11 immunosorbent assay

To determine the intraexperiment variation of the AU/VWFa-11 immunosorbent assay, 10 randomly chosen wells of an AU/VWFa-11 coated microtiter plate were incubated with one sample (healthy individual no. 7). Moreover, sample no. 7 was measured in 10 different experiments to determine the interexperiment variation. NPP was used as standard and the activation factor was calculated. The intraexperiment variation was 7.1% and the variation between different experiments was 13.7%.

Data analysis and statistics

Analysis of data obtained from SPR analysis and immunosorbent assays was performed using the GraphPad Prism program (GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego, CA). All data were expressed as mean with SD. An unpaired *t* test with Welch correction was performed to compare the mean levels of activated VWF between the different patient groups. For comparison of 3 groups, one-way ANOVA was performed followed by Bonferroni correction for the calculation of differences between each group separately. *P* below 0.05 was considered significant.

Results

Nanobody AU/VWFa-11 specifically recognizes the active conformation of VWF

To obtain antibodies that predominantly recognize activated but not native VWF, a llama was immunized with purified VWF preparations that contained UL-VWF (multimer size exceeding 20 subunits). Subsequently, the antibody repertoire of the animal was cloned, and nanobodies were selected for their ability to bind to immobilized VWF A1 domain in the presence of soluble full-length VWF. Of the nanobodies that were obtained, one was selected for further analysis, AU/VWFa-11. Throughout the study, a nanobody obtained after immunization with the isolated A1 domain was used as control nanobody. Nanobody AU/VWFa-11 was examined for its ability to distinguish between native VWF and activated VWF. To this end, purified recombinant wt-VWF was immobilized in microtiter wells. This procedure leads to conformational changes in the molecule, allowing the binding of nanobodies that are specific for activated VWF. As expected, both biotinylated nanobodies bound to immobilized VWF in a dose-dependent and saturable manner. Half maximum

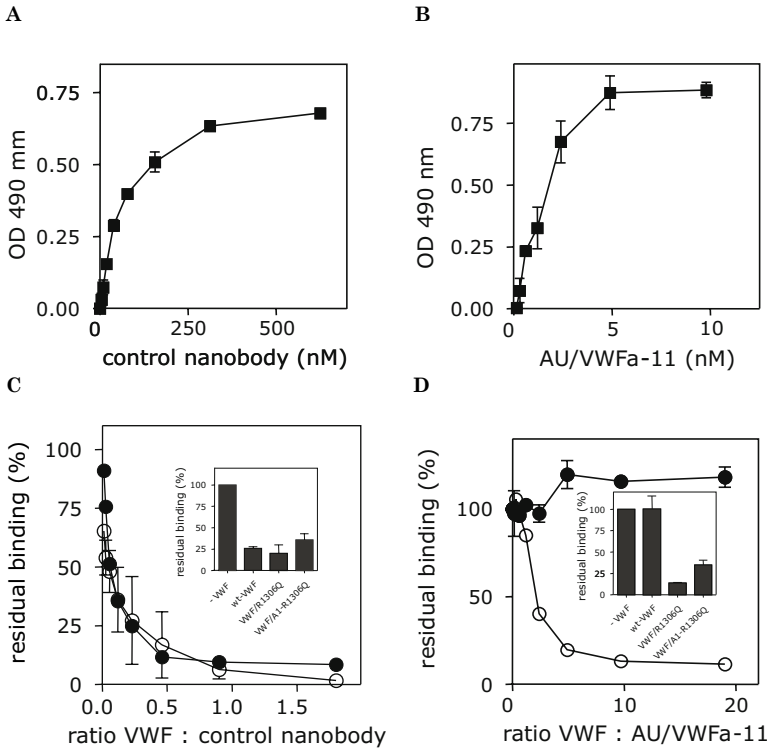

Figure 1: Differential binding of the control nanobody and AU/VWFa-11 to wt-VWF and ristocetin-activated VWF. A/B: Pd-VWF was immobilized in microtiter wells (1 µg/ml, overnight at 4°C) and incubated with different concentrations biotinylated control nanobody (**A**, 0-625 nM), or AU/VWFa-11 (**B**, 0-10 nM). Bound nanobody was detected with HRP-conjugated streptavidin. **C/D**: Pd-VWF-coated microtiter wells were incubated with biotinylated control nanobody (62.5 nM, **C**) or AU/VWFa-11 (1.9 nM, **D**) in the absence or presence of different concentrations of wt-VWF preincubated with 1 mg/ml ristocetin (5 minutes at room temperature; open circles) or wt-VWF (closed circles). Concentrations varied from 0-90 nM for control nanobody and 0-20nM for AU/VWFa-11. Inset shows incubation of control nanobody (62.5 nM) or AU/VWFa-11 (1.9 nM) in the presence of a 10-fold molar excess of VWF/R1306Q or VWF/A1(1261-1468)-R1306Q. Bound nanobody was detected using streptavidin-HRP. Binding in the absence of competitors was set to be 100%. Residual binding in the presence of VWF was plotted against the VWF / nanobody ratio. Data represent the mean ± SD of 3 experiments.

binding was obtained at 62.5 nM for the control nanobody or at 1.9 nM for AU/VWFa-11 (Figure 1A/B). These concentrations were used in a competition assay, in which binding of the nanobodies was studied in the presence of different concentrations of soluble wt-VWF, wt-VWF preincubated with ristocetin, VWF/R1306Q or VWF/A1(1261-1468). The presence of these competitors considerably reduced binding of biotinylated control nanobody (Figure 1C). Ristocetin-activated VWF, VWF/R1306Q, and VWF/A1(1261-1468)-R1306Q interfered with binding of biotinylated AU/VWFa-11 (Figure 1D). In contrast, binding of AU/VWFa-11 remained unaffected

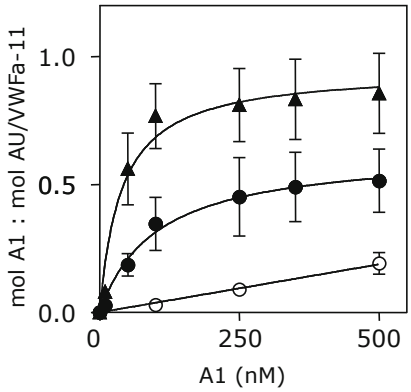

Figure 2: Interaction between AU/VWFa-11 and the VWF A1 domain. Different concentrations of VWF/A1(1238-1481) (open circles), VWF/A1(1261-1468) (closed circles) or VWF/A1(1261-1468)-R1306Q (triangles) (500 nM) were perfused over a CM5-sensor chip coated with 0.06 pmol/mm^2 AU/VWFa-11 at a flowrate of 20 µl/min. The response at equilibrium (mol A1 / mol AU/VWFa-11) was plotted against the concentration of VWF/A1 that was perfused over the chip.

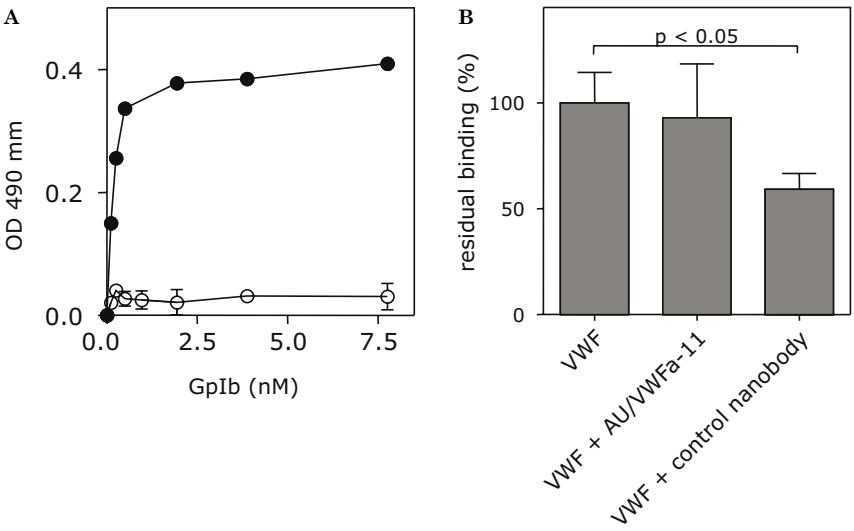

Figure 3: VWF-GpIbα interaction is unaffected by AU/VWFa-11 in static adhesion. A: Microtiter wells coated with 5 µg/ml AU/VWFa-11 (closed circles) or control nanobody (open circles) were blocked 30 min at 37°C with PBS/3% BSA/ 0.1% Tween-20 and incubated with 3.7 nM VWF/R1306Q. After washing, wells were incubated with GpIbα (0.12 – 31.1 nM) and subsequently with a monoclonal anti-GpIb antibody. Wells were washed and incubated with an HRP-conjugated rabbit-anti-mouse antibody. Binding was detected by measuring the peroxidase activity. **B**: Microtiter wells were coated with wt-VWF (37 nM in 50mM $NaHCO_3$ buffer), overnight at 4°C. After blocking wells 1 h at room temperature with 0.5% polyvinylpyrrolidone in PBS, wells were incubated with AU/VWFa-11 or control nanobody (1.25 µM in PBS, 1 h, room temperature). After washing with PBS, CHO-cells expressing the GpIb/IX/V complex (1 x 10^5 cells in DMEM containing 0.1% BSA) were allowed to bind in the presence or absence of the control nanobody or AU/VWFa-11 (1.25 µM). Binding of these cells was monitored by measuring the intrinsic alkaline phosphatase activity of the cells and set to be 100% in the absence of nanobodies. Data represent the mean ± SEM of 3 experiments.

in the presence of a 20-fold molar excess of wt-VWF. Apparently, AU/VWFa-11 has the potential to selectively recognize VWF that is, at least in part, in an activated conformation.

Interaction between AU/VWFa-11 and VWF A1 domain

The interaction between nanobody AU/VWFa-11 and the A1 domain of VWF was investigated in more detail. Binding of AU/VWFa-11 to 3 different variants of the A1 domain was compared in a quantitative manner using SPR analysis. VWF/A1(1261-1468), VWF/A1(1238-1481), which comprises longer flanking regions of the A1 domain than VWF/A1(1261-1468), and VWF/A1(1261-1468)-R1306Q (containing the type 2B mutation Arg1306Gln) were perfused over immobilized AU/VWFa-11 (0.06 pmol/mm^2) at a flow rate of 20 µl/min until equilibrium was reached. Binding isotherms using the response at equilibrium were used to calculate K_D (Figure 2). This analysis revealed an apparent K_D for VWF/A1(1238-1481) of 1704 nM. Removal of the flanking peptides of the A1 domain resulted in a higher affinity for AU/VWFa-11 (K_D = 81 nM). Moreover, VWF/A1(1261-1468)-R1306Q was bound even more efficiently with an apparent K_D of 44 nM. These results suggest that exposure of the epitope of nanobody AU/VWFa-11 indeed relies on conformational changes within the A1 domain.

GpIbα and AU/VWFa-11 bind different regions within the A1 domain

The conformation induced by ristocetin, immobilization of VWF, or a VWD type 2B mutation, promotes binding to GpIbα and this conformation is also specifically recognized by AU/VWFa-11. Therefore, the possibility was considered that AU/VWFa-11 and GpIbα bind to similar regions in the A1 domain. This was first tested in an immunosorbent assay in which binding of GpIbα to nanobody-bound VWF/R1306Q was studied. Immobilization of VWF/R1306Q via the control nanobody interfered with binding of GpIbα. In contrast, GpIbα was able to bind to AU/VWFa-11-bound VWF/R1306Q in a dose-dependent manner (Figure 3A), suggesting that the binding site for AU/VWFa-11 is distinct from the binding site for GpIbα. This was confirmed in a static adhesion assay. Binding of CHO cells expressing the GpIb/IX/V complex to immobilized VWF remained unaffected even in the presence of an excess of AU/VWFa-11 (Figure 3B).

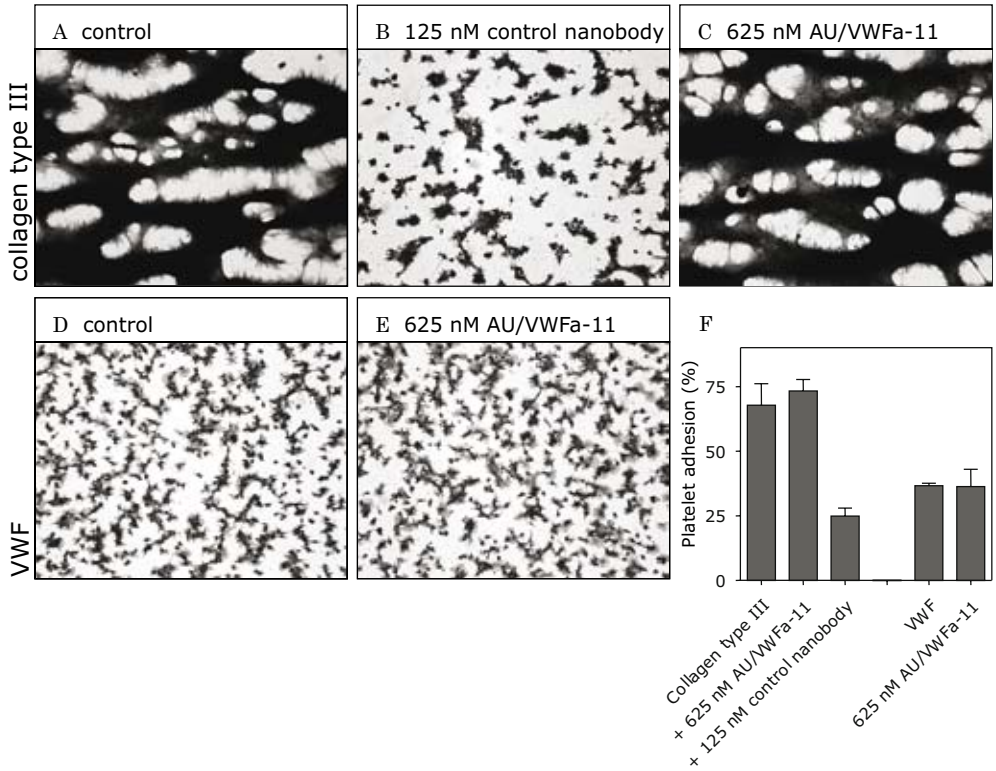

Figure 4: Different binding sites for GpIbα and AU/VWFa-11. A-C: Whole blood was perfused over coverslips coated with collagen type III (30 µg/cm^2 in 0.05 M/l acetic acid) in the absence (**A**) or presence of the control nanobody (125 nM, **B**) or AU/VWFa-11 (625 nM, **C**) at a shear rate of 1600 s^{-1}. **D/E:** Reconstituted blood was perfused over coverslips coated with pd-VWF (15 µg/ml) in the absence (**D**) or presence of 625 nM AU/VWFa-11 (**E**) at a shear rate of 1600 s^{-1}. After perfusion adhered platelets were fixed in 0.5% glutaraldehyde in PBS, dehydrated in methanol and stained with May-Grünwald and Giemsa. Platelet aggregates are represented by the dark regions. **F**: Platelet adhesion was evaluated using computer-assisted analysis and was expressed as the percentage of surface covered with platelets (n=3). Adhered platelets were visualized using light microscopy (Leitz Diaplan; Leicca, Rijswijk, The Netherlands) and computer-assisted analysis (AMS 40-10 Saffron, Walden, United Kingdom). Original magnification was 400 x (40 x/1.00 NA objective lens).

Because the effect of both nanobodies on the binding of VWF to GpIbα may be different under flow conditions, we further examined their effect in a perfusion assay. Human whole blood was perfused over a collagen type III surface at high shear (1600 s^{-1}). Under these conditions, platelet adhesion is fully dependent on the interaction between VWF and GpIbα.[32] In the absence of nanobodies, these conditions resulted in a platelet coverage of 67.8% ± 8.3% (n=3) (Figure 4A). The presence of the control nanobody was associated with a decreased platelet coverage (49.8% ± 4.5% and 24.9% ± 3.1% in the presence of 31.3 and 125 nM nanobody, respectively; Figure 4B). In

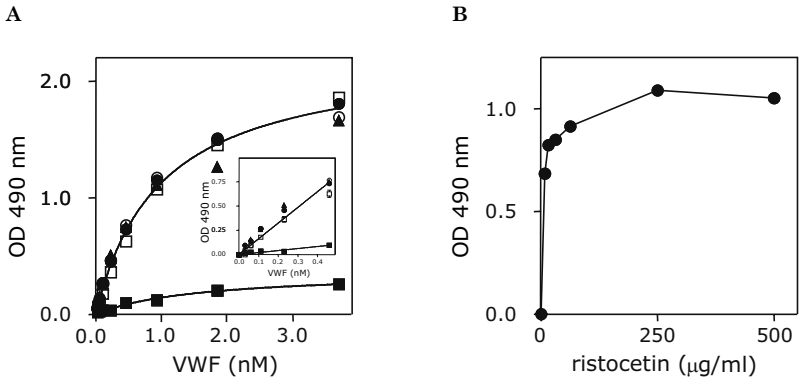

Figure 5: Effect of ristocetin, botrocetin and the R1306Q mutation on binding to AU/VWFa-11. A: Microtiter wells were coated overnight at 37°C with AU/VWFa-11 (5 µg/ml in 50 mM $NaHCO_3$, pH 9.6) and blocked 30 min at 37°C with 3% BSA, 0.1% Tween-20 in PBS. After washing, microtiter wells were incubated with medium containing different concentrations of wt-VWF (squares, triangles) or VWF/R1306Q (circles) (0-3.7 nM). Binding was allowed for 1 h at 37°C in the absence of modulators (closed squares and circles), or in the presence of 1 mg/ml ristocetin (open squares and circles) or 0.2 U/ml botrocetin (closed triangles). Microtiter wells were washed using 0.1% Tween-20 in PBS and incubated with HRP-conjugated polyclonal anti-VWF antibody. Bound VWF was detected by measuring peroxidase activity. An enlargement of the linear part of the binding curves was provided in the inset. Data represent the mean ± SD of 3 experiments. **B:** AU/VWFa-11 coated microtiter wells were incubated with wt-VWF in the presence or absence of various concentrations ristocetin (0.08 –1 mg/ml). After washing, wells were incubated with an HRP-conjugated polyclonal anti-VWF antibody. Bound VWF was detected by measuring the peroxidase activity.

contrast, even in the presence of 625 nM nanobody AU/VWFa-11, platelet coverage was still similar to that in its absence (73.3% ± 4.4%, Figure 4C). Moreover, platelet adhesion to a VWF-surface remained unaffected in the presence of AU/VWFa-11 (Figure 4D/E). These data are compatible with the view that GpIbα and nanobody AU/VWFa-11 bind to different regions of the A1 domain.

Immunosorbent assay for detection of activated VWF in solution

Although GpIbα and nanobody AU/VWFa-11 bind to distinct sites within VWF, both have in common that they only recognize VWF in its activated form, which is for instance induced by immobilization, ristocetin or VWD type 2B mutations. This unique feature allowed us to use this nanobody for the detection of activated VWF in solution. As a first example, the binding of recombinant wt-VWF and recombinant VWF/R1306Q to immobilized nanobody AU/VWFa-11 was compared. Therefore, this particular nanobody was immobilized in microtiter wells and incubated with various concentrations (0-3.7 nM) of wt-VWF and VWF/R1306Q in the absence and presence of ristocetin or botrocetin. The amount of bound VWF was subsequently monitored using HRP-conjugated polyclonal antibodies against VWF. With regard to wt-VWF,

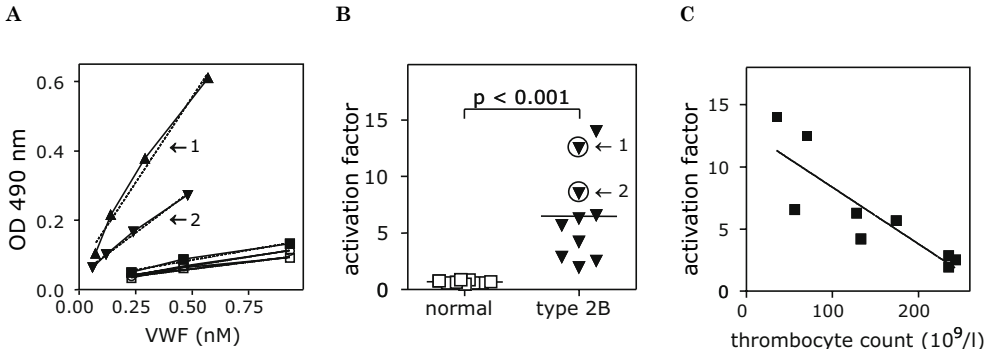

Figure 6: Activated VWF present in VWD type 2B plasma. A: Microtiter wells coated with AU/VWFa-11 (5 µg/ml) were blocked 30 min with 3% BSA, 0.1% Tween-20 in PBS. NPP (closed squares), plasma from normal individuals (n=9, open symbols) and VWD type 2B plasma (n=12, triangles) were diluted in PBS to obtain a concentration range (0.23-0.93 nM). After washing, wells were incubated 1 h at 37°C with the diluted plasmas. Bound VWF was detected using HRP-conjugated polyclonal anti-VWF antibody. The concentration of VWF in the diluted samples was plotted against the measured OD 490 nm. The slope found for NPP was set to be 1. Arrows indicate the slopes found for 2 different VWD type 2B patients. **B**: The activation factors were calculated and plotted in a scatter plot. Arrows indicate the activation factors calculated for patient 1 and 2 from figure A. The activation factor found for VWD type 2B patients was significantly higher than for the healthy individuals ($p<0.001$). Data represent the mean ± SD. **C**: The activation factor calculated for 9 VWD type 2B patients was plotted against the thrombocyte counts in these samples and a correlation was found to be significant ($p<0.003$, $R^2=0.7401$).

some binding could be observed, but absorbance values remained below 0.3 (Figure 5A). The addition of ristocetin or botrocetin resulted in a strong increase in binding, represented by a 6-fold increase in absorbance (up to 1.85). We quantified this difference by calculating the respective slopes of the initial, linear parts of the curve. This revealed that the slope for VWF/ristocetin was increased 7.2-fold compared to wt-VWF alone. A similar increase in slope compared to wt-VWF could be observed for VWF/R1306Q in the absence of ristocetin (Figure 5A inset). Moreover, this increase was not further enhanced in the presence of ristocetin. Because activation of VWF with 1 mg/ml ristocetin can induce nonspecific interactions, such as flocculation of VWF, wt-VWF was incubated with various concentrations of ristocetin. This revealed that maximal binding to AU/VWFa-11 was already reached at low ristocetin concentrations (250 µg/ml) (Figure 5B). Taken together, this assay provides a useful tool to detect circulating VWF containing an A1 domain in active conformation.

Detection of activated VWF in patient plasma

Because nanobody AU/VWFa-11 was particularly efficient in the detection of active VWF in solution, we tested whether this nanobody could be used for the detection of active VWF in plasma from patients. First, we analyzed the plasma from patients

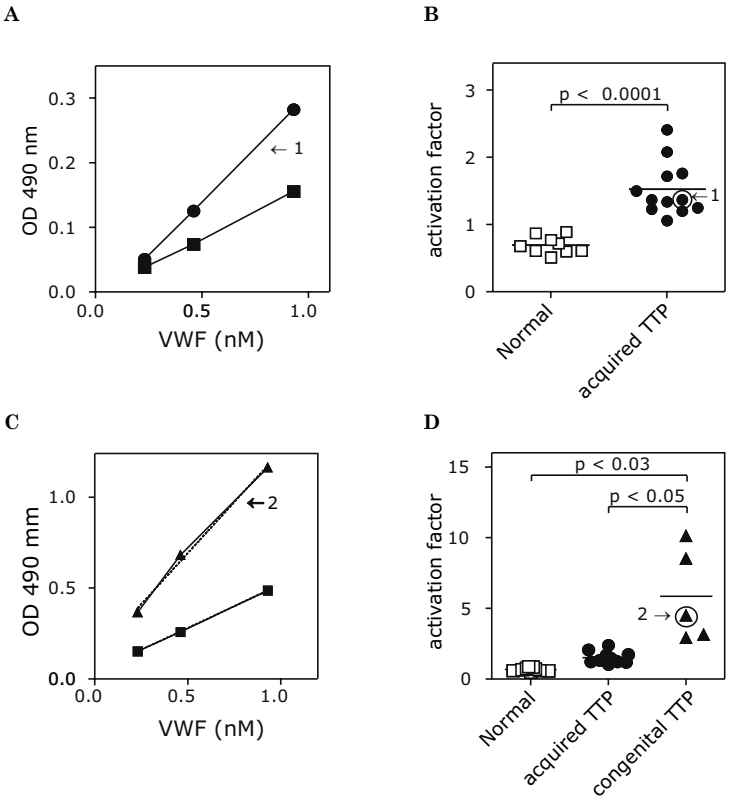

Figure 7: Detection of activated VWF in TTP plasma. A/C: AU/VWFa-11-coated microtiter wells were incubated with NPP (closed squares), plasma from healthy individuals (n=9, open squares) and plasma from patients suffering from acquired TTP (**A**, n=12, closed circles) or congenital TTP (**C**, n=5, triangles). Plasma was diluted before incubation to obtain a concentration range of VWF (0.23-0.93 nM). Bound VWF was monitored with HRP-conjugated anti-VWF antibody. The amount of VWF in the diluted sample was plotted against the HRP activity (OD 490 nm). Slopes were calculated and the slope found for NPP was set to be 1. Arrows indicate the slopes found for a patient suffering from acquired TTP (1) or congenital TTP (2) **B/D**: The activation factor was calculated and plotted in a scatter plot. Arrows indicate the values found for the patients plotted in figure A and C. Activation factors found for acquired and congenital TTP were significantly higher than for the healthy individuals (acquired TTP vs. healthy: $p<0.0001$ and congenital TTP vs. healthy $p<0.03$). The activation factor found for congenital TTP was also significantly elevated when compared to acquired TTP ($p<0.05$). Data represent the mean ± SD.

previously defined as type 2B, as well as a group of healthy individuals. As a reference, NPP was used (see "Patients, materials and methods"). The absorbance values obtained for NPP remained low (Figure 6A), and the slope was set to be 1. Also for each of the healthy individuals, only low absorbance values were detected, suggesting low amounts of active VWF in their plasma. A typical example of the slopes found for VWF type 2B patients is presented in Figure 6A. The mean slope compared to NPP was 0.70 ± 0.13 (n=9; Figure 6B). In contrast, high amounts of active VWF could

be determined in the plasmas of the VWD type 2B patients, as illustrated by the strongly increased absorbance values (Figure 6A). The mean slope of was calculated to be 8.4 ± 4.5 (n=10), a 12-fold increase compared to healthy individuals (p=0.001; Figure 6B). Thus, this assay indeed seems to be useful to analyze the presence of active VWF in plasma of patients.

Correlation between VWF activation and platelet count

Interestingly, the activation factor determined for VWF in plasma from patients with VWD type 2B varied considerably (1.95 – 14.0). This variation was not found in plasma from healthy individuals (0.57 –0.83). VWF containing type 2B mutations binds spontaneously to platelets, leading to enhanced clearance of both platelets and VWF. We therefore considered the possibility that the relative activation of VWF in circulation influenced the formation of platelet-VWF complexes. To address this question, we plotted the platelet counts obtained from the blood taken to prepare the plasma samples versus the corresponding activation factor of these plasmas. A strong inverse correlation was found between these parameters (Figure 6C, $p<0.003$, $R^2=0.7401$).

Detection of active VWF in plasma from TTP patients

A second group of patients analyzed for the presence of active VWF in their plasma were patients lacking ADAMTS13 activity, which was clinically manifested as TTP. Two groups could be distinguished: one group was defined as patients having a congenital deficiency of ADAMTS13 (n=5), whereas a second group had an acquired deficiency of ADAMTS13 (n=12). Compared to the group of healthy individuals (see "Detection of activated VWF in patient plasma"), patients having acquired ADAMTS13 deficiency appeared to have increased levels of active VWF in their plasma (Figure 7A). The mean slope was calculated to be 1.52±0.40, which is 2-fold higher compared to the group of healthy individuals ($p<0.001$, Figure 7B). Also the patients with congenital ADAMTS13 deficiency contained active VWF in their plasma (Figure 7C). Interestingly, the amount of active VWF (slope=5.85 ± 3.3) was increased 8-fold compared to the healthy individuals ($p<0.03$), but also 4-fold compared to the acquired ADAMTS13-deficient patients ($p<0.05$, Figure 7D). Moreover, no overlap in slope values was observed between each of the normal individuals and the two patient groups. This suggests that the assay may be useful for the diagnosis of ADAMTS13 deficiency in that it allows rapid distinction between acquired and congenital TTP.

Discussion

During the adhesion of platelets to the injured vessel wall, VWF functions as a molecular bridge between the exposed subendothelial matrix and the GpIb/IX/V complex. For the interaction between GpIbα and the A1 domain of VWF, the latter requires a conversion from its resting state into a GpIbα-binding conformation. Under several pathological conditions, such as VWD type 2B and TTP, VWF is forced into its GpIbα-binding conformation leading to undesired, spontaneous interactions between VWF and platelets, a process that is often manifested via thrombocytopenia. Here, we describe a recombinant llama-derived antibody fragment that is able to distinguish between the resting and GpIbα-binding state of VWF. In addition, we show that this nanobody can be applied in the detection of such activated VWF in the plasma of different patient groups.

Functional properties of nanobody AU/VWFa-11

Llamas produce a substantial part of their functional immunoglobulins as homodimers of heavy chains, so called nanobodies. By using a phage-display approach, we have been able to isolate a nanobody (ie. AU/VWFa-11) that recognizes VWF in its GpIbα-binding conformation but not VWF in its soluble, resting state (Figure 1). Because our selection strategy included the isolated A1 domain of VWF, it is obvious that this nanobody is directed against this region of the molecule. Interestingly, AU/VWFa-11 displayed distinct interactions with the various recombinant A1 domain variants (Figure 2). First, the A1 domain variant with short flanking regions (VWF/A1(1261-1468)) was more efficient in binding to this nanobody compared to its counterpart with longer flanking regions (VWF/A1(1238-1481)). This suggests that the N- and C-terminal regions of the A1 domain may play a role in the exposure of the binding site for AU/VWFa-11. It is of interest to mention that these flanking regions also modulate the interaction between VWF and GpIbα.[33] Binding of VWF/A1(1261-1468) to AU/VWFa-11 was even more increased upon the introduction of a type 2B mutation (R1306Q). This mutation also induces spontaneous binding between VWF and GpIbα.[24] Comparison of the various crystal structures revealed that the R1306Q replacement results in a number of conformation changes within the A1 domain, which may be responsible for improved GpIbα binding.[34] It seems conceivable that these changes also promote binding of nanobody AU/VWFa-11. In view of the similarities between this nanobody and GpIbα in their interaction with VWF, one

would expect that both proteins share a similar binding site. Surprisingly, GpIbα and AU/VWFa-11 can interact simultaneously with VWF (Figure 3A). Moreover, nanobody AU/VWFa-11 is unable to interfere with the interaction between VWF and GpIbα (Figs 3B and 4). Thus, this nanobody and GpIbα seem to recognize different regions within the A1 domain, suggesting that changes in conformation on activation of the A1 domain are not limited to the GpIbα binding site. At present, studies are in progress to define the epitope of AU/VWFa-11 in more detail.

Application of AU/VWFa-11 in the detection of active VWF in plasma from patients

Measurement of active VWF in plasma is currently assessed in an indirect manner by monitoring platelet aggregation in the presence of various concentrations of ristocetin. The unique properties of our nanobody allow the direct detection of active VWF in plasma, even under conditions of low antigen levels of VWF. This makes the immunosorbent assay suitable to analyze the presence of active VWF in the plasma of patients suffering pathological conditions associated with reduced platelet numbers. In an initial attempt, we applied our assay to compare the presence of active VWF in healthy individuals and patients diagnosed as having VWD type 2B or TTP. Plasma of healthy individuals showed only little binding of VWF to immobilized AU/VWFa-11 (Figure 6), confirming that the majority of the circulating VWF is in a nonbinding conformation. Some residual binding was found, indicating that even under physiological conditions a minor proportion of the VWF molecules could circulate in an activated conformation. Alternatively, some activation might have been introduced as a result of blood sampling and plasma preparation. An interesting issue in this regard is whether or not the introduction of the conformation changes is a reversible phenomenon. Unfortunately, our data do not distinguish between both possibilities, and further studies are therefore required.

Compared to the plasma from healthy individuals, increased amounts of active VWF were present in the plasma from patients with VWD type 2B or TTP (Figure 6 and 7). Although phenotypically distinct, both diseases are characterized by thrombocytopenia, caused by spontaneous platelet-VWF interaction. Indeed, an inverse correlation was found between the level of activated VWF in plasma from patients with VWD type 2B and their platelet count (Figure 6C). This may point to a direct role for activated VWF in the onset of thrombocytopenia.

Interestingly, when we incubated AU/VWFa-11 with plasma from congenital TTP

patients, we found that the amount of activated VWF was not only significantly elevated compared to healthy individuals, but also compared to patients with acquired TTP. Plasma samples of patients with acquired TTP were collected on admission to the hospital, before treatment with plasma exchange. At this time, a substantial part of the activated VWF is most likely present in the platelet-rich thrombi found in the microvasculature. This could explain the differences in levels of activated VWF between acquired and congenital TTP. On the other hand, the different molecular background of acquired and congenital TTP could also account for the difference in activation of VWF. Although further studies must be performed, the AU/VWFa-11 immunosorbent assay seems to have the potential to distinguish between acquired and congenital TTP.

In summary, we introduce a novel antibody fragment that is able to discriminate between the resting and activated state of VWF, and application of this nanobody enables us to detect activated VWF in plasma. As a consequence, this assay may be of particular interest for investigating the contribution of VWF to the pathogenesis of various vascular diseases that are characterized by thrombocytopenia.

Acknowledgements

We thank Dr J.A. Lopez for the GpIb-expressing CHO cells, Dr J.P. Girma for helpful comments and K. De Smet, B. Van der Jeugt, M.J. IJsseldijk and Dr S. Tsuji for excellent technical contributions.

Reference List

(1) Ruggeri ZM. Von Willebrand factor. Curr Opin Hematol. 2003;10:142-149.

(2) Savage B, Saldivar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. Cell. 1996;84:289-297.

(3) Vicente V, Houghten RA, Ruggeri ZM. Identification of a site in the alpha chain of platelet glycoprotein Ib that participates in von Willebrand factor binding. J Biol Chem. 1990;265:274-280.

(4) Huizinga EG, Tsuji S, Romijn RA et al. Structures of glycoprotein Ibalpha and its complex with von Willebrand factor A1 domain. Science. 2002;297:1176-1179.

(5) Berndt MC, Du XP, Booth WJ. Ristocetin-dependent reconstitution of binding of von Willebrand factor to purified human platelet membrane glycoprotein Ib-IX complex. Biochemistry. 1988;27:633-640.

(6) Scott JP, Montgomery RR, Retzinger GS. Dimeric ristocetin flocculates proteins, binds to platelets, and mediates von Willebrand factor-dependent agglutination of platelets. J Biol Chem. 1991;266:8149-8155.

(7) Ruggeri ZM. Von Willebrand factor, platelets and endothelial cell interactions. J Thromb Haemost. 2003;1:1335-1342.

(8) Ruggeri ZM, Pareti FI, Mannucci PM, Ciavarella N, Zimmerman TS. Heightened interaction between platelets and factor VIII/von Willebrand factor in a new subtype of von Willebrand's disease. N Engl J Med. 1980;302:1047-1051.

(9) Ruggeri ZM, Zimmerman TS. von Willebrand factor and von Willebrand disease. Blood. 1987;70:895-904.

(10) Ruggeri ZM, Ware J. The structure and function of von Willebrand factor. Thromb Haemost. 1992;67:594-599.

(11) Sadler JE. Biochemistry and genetics of von Willebrand factor. Annu Rev Biochem. 1998;67:395-424.

(12) Wagner DD, Marder VJ. Biosynthesis of von Willebrand protein by human endothelial cells. Identification of a large precursor polypeptide chain. J Biol Chem. 1983;258:2065-2067.

(13) Arya M, Anvari B, Romo GM et al. Ultralarge multimers of von Willebrand factor form spontaneous high-strength bonds with the platelet glycoprotein Ib-IX complex: studies using optical tweezers. Blood. 2002;99:3971-3977.

(14) Dong JF, Moake JL, Nolasco L et al. ADAMTS13 rapidly cleaves newly secreted ultralarge von Willebrand factor multimers on the endothelial surface under flowing conditions. Blood. 2002;100:4033-4039.

(15) Fujikawa K, Suzuki H, McMullen B, Chung D. Purification of human von Willebrand factor-cleaving protease and its identification as a new member of the metalloproteinase family. Blood. 2001;98:1662-1666.

(16) Gerritsen HE, Robles R, Lammle B, Furlan M. Partial amino acid sequence of purified von Willebrand factor-cleaving protease. Blood. 2001;98:1654-1661.

(17) Moake JL. Thrombotic microangiopathies. N Engl J Med. 2002;347:589-600.

(18) Levy GG, Nichols WC, Lian EC et al. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. Nature. 2001;413:488-494.

(19) Remuzzi G, Galbusera M, Noris M et al. von Willebrand factor cleaving protease (ADAMTS13) is deficient in recurrent and familial thrombotic thrombocytopenic purpura and hemolytic uremic syndrome. Blood. 2002;100:778-785.

(20) Moake JL, Turner NA, Stathopoulos NA, Nolasco LH, Hellums JD. Involvement of large plasma von Willebrand factor (vWF) multimers and unusually large vWF forms derived from endothelial cells in shear stress-induced platelet aggregation. J Clin Invest. 1986;78:1456-1461.

(21) George JN. How I treat patients with thrombotic thrombocytopenic purpura-hemolytic uremic syndrome. Blood. 2000;96:1223-1229.

(22) Sodetz JM, Pizzo SV, McKee PA. Relationship of sialic acid to function and in vivo survival of human factor VIII/von Willebrand factor protein. J Biol Chem. 1977;252:5538-5546.

(23) Lankhof H, van Hoeij M, Schiphorst ME et al. A3 domain is essential for interaction of von Willebrand factor with collagen type III. Thromb Haemost. 1996;75:950-958.

(24) Lankhof H, Damas C, Schiphorst ME et al. Functional studies on platelet adhesion with recombinant von Willebrand factor type 2B mutants R543Q and R543W under conditions of flow. Blood. 1997;89:2766-2772.

(25) Lenting PJ, Westein E, Terraube V et al. An experimental model to study the in vivo survival of von Willebrand factor. Basic aspects and application to the R1205H mutation. J Biol Chem. 2004;279:12102-12109.

(26) Romijn RA, Westein E, Bouma B et al. Mapping the collagen-binding site in the von Willebrand factor-A3 domain. J Biol Chem. 2003;278:15035-15039.

(27) Arbabi GM, Desmyter A, Wyns L, Hamers R, Muyldermans S. Selection and identification of single domain antibody fragments from camel heavy-chain antibodies. FEBS Lett. 1997;414:521-526.

(28) Skerra A, Pluckthun A. Assembly of a functional immunoglobulin Fv fragment in Escherichia coli. Science. 1988;240:1038-1041.

(29) Lopez JA, Leung B, Reynolds CC, Li CQ, Fox JE. Efficient plasma membrane expression of a functional platelet glycoprotein Ib-IX complex requires the presence of its three subunits. J Biol Chem. 1992;267:12851-12859.

(30) Lisman T, Moschatsis S, Adelmeijer J, Nieuwenhuis HK, de Groot PG. Recombinant factor VIIa enhances deposition of platelets with congenital or acquired alpha IIb beta 3 deficiency to endothelial cell matrix and collagen under conditions of flow via tissue factor-independent thrombin generation. Blood. 2003;101:1864-1870.

(31) Veyradier A, Lavergne JM, Ribba AS et al. Ten candidate ADAMTS13 mutations in six French families with congenital thrombotic thrombocytopenic purpura (Upshaw-Schulman syndrome). J Thromb Haemost. 2004;2:424-429.

(32) Wu YP, Vink T, Schiphorst M et al. Platelet thrombus formation on collagen at high shear rates is mediated by von Willebrand factor-glycoprotein Ib interaction and inhibited by von Willebrand factor-glycoprotein IIb/IIIa interaction. Arterioscler Thromb Vasc Biol. 2000;20:1661-1667.

(33) Nakayama T, Matsushita T, Dong Z et al. Identification of the regulatory elements of the human von Willebrand factor for binding to platelet GPIb. Importance of structural integrity of the regions flanked by the CYS1272-CYS1458 disulfide bond. J Biol Chem. 2002;277:22063-22072.

(34) Dumas JJ, Kumar R, McDonagh T et al. Crystal structure of the wild-type von Willebrand factor A1-glycoprotein Ibalpha complex reveals conformation differences with a complex bearing von Willebrand disease mutations. J Biol Chem. 2004;279:23327-23334.

Chapter 3

Acute activation of the endothelium results in increased levels of active Von Willebrand Factor in HELLP syndrome

Janine J. J. Hulstein
Pieter J. van Runnard Heimel
Arie Franx
Peter J. Lenting
Hein W. Bruinse
Karen Silence
Philip G. de Groot
Rob Fijnheer

Accepted for publication in J Thromb Haemost 2006

Summary

Background: HELLP(hemolysis, elevated liver enzymes and low platelets)-syndrome is a severe complication of preeclampsia in pregnancy, characterized by microvascular platelet-thrombi. Activation of the endothelium is thought to play a key role in preecclampsia and HELLP-syndrome. Activation of endothelial-cells may lead to release of Von Willebrand Factor(VWF)-multimers, that are highly reactive with platelets. Normally newly-released multimers are cleaved by ADAMTS13, resulting in less reactive derivatives. **Objective:** We hypothesized that HELLP-syndrome is characterized by increased amounts of active-VWF compared to healthy pregnancy and preecclampsia, due to acute activation of endothelial-cells. This might contribute to thrombocytopenia and thrombotic-microangiopathy.

Methods: Active-VWF and ADAMTS13 activity were measured in healthy pregnant volunteers(n=9), patients with preeclampsia(n=6) and patients with HELLP-syndrome(n=14) at similar gestational age. To study the role of endothelial-cell activation, the propeptide/mature-VWF ratio was determined and VWF released by cultured endothelial-cells was analyzed.

Results: Active-VWF levels were increased 2.1-fold in HELLP-syndrome compared to healthy pregnant volunteers ($p<0.001$) and 1.6-fold compared to patients with preeclampsia ($p=0.001$). ADAMTS13 activity was moderately decreased in patients with HELLP-syndrome compared to healthy pregnant volunteers ($p<0.004$), but not compared to preeclampsia. The propeptide/mature-VWF ratio was increased 1.7-fold compared to healthy pregnant volunteers ($p<0.001$) and 1.5-fold compared to patients with preeclampsia ($p<0.05$). A significant correlation was found between this ratio and the activation factor of VWF ($r=0.68$, $p<0.001$). The amount of active-VWF was increased 1.4-fold in medium of stimulated endothelial-cells as compared to non-stimulated cells ($p<0.05$).

Conclusion: Acute endothelial-cell activation in HELLP-syndrome and decreased ADAMTS13 activity result in increased amounts of active-VWF. This might explain the consumptive thrombocytopenia and thrombotic-microangiopathy associated with HELLP-syndrome. Inhibition of circulating active-VWF could be a potential new approach in the treatment of patients with the HELLP-syndrome.

Introduction

Preeclampsia is present in 2-4% of pregnant women, but it is one of the most important causes of morbidity and mortality of both the mother and the foetus.[1] Deficient placentation plays a central role in preeclampsia. However, poor placentation is unlikely to be the exclusive cause of the syndrome and is better considered as a predisposing factor. Debris shed from the syncitial surface of the placenta has been suggested to constitute the inflammatory stimulus in all pregnancies.[2] The generalized intravascular inflammatory response seems to be exaggerated in preeclampsia. This results in endothelial activation[3], which clinically manifests as hypertension and proteinuria.[1] HELLP (hemolysis, elevated liver enzymes and low platelets) syndrome is considered to be a severe form of preeclampsia with the features of a thrombotic microangiopathy: consumptive thrombocytopenia with the formation of microvascular platelet thrombi, resulting in microangiopathic hemolytic anemia and hepatic damage.[4] The presence of HELLP syndrome further increases the risk of maternal and neonatal morbidity and mortality.[5]

Several studies have shown that in patients with pregnancies complicated by preeclampsia and HELLP syndrome VWF antigen levels are markedly increased.[6,7] VWF is synthesized by megakaryocytes and endothelial cells as a pre-propeptide. During maturation of VWF, the propeptide is cleaved off and VWF is multimerized. Endothelial cells secrete part of the produced VWF via the constitutive pathway, but a substantial part of the multimerized VWF is stored in the Weibel Palade bodies in a 1:1 ratio with the VWF-propeptide. Upon stimulation of the endothelium, the stored VWF is secreted via the regulated pathway. Differences in half life between propeptide and VWF result in a molar ratio between propeptide and mature VWF of 0.13 in healthy individuals.[8] Acute activation of the endothelium leads to a rapid increase of this ratio, which makes the propeptide/mature-VWF ratio a very sensitive tool to measure acute endothelial activation.[9]

The main function of VWF is to mediate adhesion and aggregation of platelets, by forming a molecular bridge between exposed subendothelial matrix and the platelet-surface GpIb/IX/V receptor-complex.[10] Under physiological conditions VWF is unable to interact spontaneously with platelets. To induce an interaction, conversion of the VWF A1-domain into a GpIbα-binding conformation is required.[11] This conformation can be induced by binding of VWF to the exposed collagen. Moreover, newly secreted

VWF multimers have been reported to interact spontaneously with platelets.[12] Under normal conditions newly secreted VWF multimers are rapidly cleaved into smaller, less reactive multimers by the metalloproteinase ADAMTS13.

Recently, a llama-derived antibody fragment has been described, AU/VWFa-11.[13] This nanobody specifically recognizes VWF in the active, GpIbα-binding conformation. These unique properties allowed us to use AU/VWFa-11 for the detection of active VWF in plasma of patients characterized by spontaneous VWF-platelet interactions.

We hypothesized that plasma levels of active VWF are increased in patients with HELLP syndrome. Exposure of the GpIbα-binding site on VWF may result in a consumptive thrombocytopenia and thrombotic microangiopathy. Furthermore, we investigated the source of active VWF in HELLP syndrome by measuring ADAMTS13 activity and the propeptide/mature-VWF ratio in plasma, and by studying human endothelial cells *in vitro*.

Material and methods

Patient inclusion and blood collection

The study was conducted at the Departments of Hematology and Perinatal Medicine, the latter being a tertiary obstetric care facility, of the University Medical Center in Utrecht, The Netherlands. The Institutional Review Board approved the research protocol and all volunteers gave written informed consent before enrolment. The mother of the baby with Von Willebrand Disease (VWD) type 2B gave written informed consent to use the umbilical cord for endothelial cell culture.

Preeclampsia and HELLP syndrome were defined according to widely accepted criteria. Preeclampsia was defined as persistently high blood pressure of at least 140/90 mmHg with proteinuria of at least 0.3 g/day, in the second half of pregnancy in a previously normotensive and non-proteinuric woman.[14] HELLP syndrome was defined by hemolysis (serum lactate dehydrogenase (LDH) > 620 IU/L and/or haptoglobin ≤ 0.3 g/L), elevated liver enzymes (serum aspartate aminotransferase (AST) > 50 U/L and/or serum alanine aminotransferase (ALT) > 50 U/L), and a low platelet count (< 100 x10^9/L). Patients with HELLP syndrome were only included if LDH, liver enzymes and platelet count were concomitantly abnormal. As controls,

we recruited 10 healthy pregnant volunteers, matched for gestational age, with an uneventful pregnancy at our obstetric outpatient clinic. Maternal venous blood samples were collected at the time of diagnosis in a vacutainer containing citrate. Plasma was harvested after 10 minutes of refrigerated centrifugation at 3000 rpm and stored at -80˚C until analysis took place. For normal pooled plasma (NPP), platelet depleted plasma of 40 healthy volunteers was pooled and stored in aliquots at -80°C.

Laboratory assays

Primary human umbilical vein endothelial cells (HUVEC) obtained from healthy individuals and from a new born with VWD type 2B (carrying a 3916C>T mutation resulting in an 1306 ARG>TRP substitution) were isolated according to Jaffe et al[15] with minor modifications[16] and cultured in EBM-2 Bulletkit culture medium (EBM-2 supplemented with EGM-2 SingleQuots (hEGF, hydrocortisone, fetal bovine serum, VEGF, hFGF-B, R3-IGF-1, Ascorbic Acid, GA-1000, heparin) (Clonetecs). Cells of the first passage were used. Cells were seeded into a 6 wells plate (NUNC, Denmark) and grown until a confluent cell layer was formed. Medium was changed for medium without supplements 4 h before activation. Cells were activated with 160 nM phorbol 12-myristate 13-acetate (PMA, Sigma) overnight at 37°C. Medium was subsequently harvested.

Measurement of VWF antigen was performed by an immunoturbidimetric assay using latex particles (STA® Liatest® VWF: Diagnostica Stago, Asnière, France) with a Behring Coagulation System (BCS, Dade Behring, Marburg, Germany). Ristocetin cofactor activity (VWF:RCo) was measured using the aforementioned BCS. VWF propeptide concentrations were measured by an enzyme-linked immunosorbent assay (ELISA) as described previously.[8] The VWF propeptide concentration was expressed as a ratio with the VWF antigen concentration. Active VWF in plasma samples was determined using the AU/VWFa-11 based immunosorbent assay, as described.[13] The multimeric pattern of VWF was analyzed using 0.1% agarose gel electrophoresis, followed by immunoblotting according to Raines et al.[17] ADAMTS13 activity was determined using the fluorescence resonance energy transfer (FRET) assay for ADAMTS13 activity (Peptides International, Inc., USA).[18] The mean ± 2 SD of ADAMTS13 activity in 40 healthy controls was 61-142%.

Statistical analysis

Statistical analysis was performed with the SPSS software package (version 12.0.1, Chicago, Illinois). Differences between all groups were tested using the Kruskal-Wallis test, followed by the Mann-Whitney U test for the calculation of differences between each group separately. Bivariate correlation of variables was assessed using Spearman's rank correlation coefficient. $P < 0.05$ values were considered to indicate statistical significance.

Results

Patient characteristics are shown in Table I. All patients with preeclampsia or HELLP syndrome developed symptoms and signs prior to 34 weeks gestation. The mean maternal age was not significantly different between the three groups.

To study the role of VWF in preeclampsia and HELLP syndrome, we first measured VWF antigen levels in the three different groups. The plasma concentration of VWF in patients with preeclampsia was significantly increased as compared to healthy pregnant volunteers (median (range) 272 % (182-424) versus 168 % (127-221), $p < 0.005$, Figure 1A). Levels of VWF were also significantly increased in HELLP syndrome compared to healthy pregnant volunteers, but they were similar to the levels found in preeclampsia (339 % (177-500), Figure 1A).

The amount of VWF that can be activated by ristocetin is a measure of the functionality of VWF and can be determined using the ristocetin cofactor activity assay (VWF:RCo). VWF:RCo was not significantly increased in preecclampsia as compared to healthy pregnant volunteers (151 ± 41 and 196 ± 54, respectively; Figure 1B), whereas the VWF:RCo was significantly increased in HELLP syndrome compared to healthy pregnant volunteers (253 ± 71; $p < 0.001$). However, the amount of VWF that could be activated by ristocetin was similar in preeclampsia and HELLP syndrome.

With the AU/VWFa-11 based immunosorbent assay we next determined the amount of VWF that circulates in a GpIbα-binding conformation in vivo. In this assay, AU/VWFa-11 coated microtiter wells were incubated with plasma of healthy pregnant volunteers, patients with preecclampsia or patients with HELLP syndrome. All samples were diluted in PBS to reach a VWF concentration between 0.2 and 1.8

Table I: Patient characteristics

		Healthy pregnant controls n = 9	Patients with preeclampsia without HELLP syndrome n = 6	Patients with preeclampsia with HELLP syndrome n = 14
Maternal age (years)	median (range)	29 (23-34)	28 (20-39)	31 (26-37)
Obstetric history:	G1P0	4/9	4/6	7/14
	G2P0	-	2/6	1/14
	G2P1	5/9	-	4/14
	other	-	-	2/14
Gestational age (weeks)	median (range)	29.0 (27.9-30.4)	29.4 (27.1-32.6)	28.3 (26.0 30.1)
ABO bloodgroup:	O	33.3% (3/9)	33.3% (2/6)	35.7% (5/14)
Platelet count (10^9/L)	median (range)	-	251 (173-292)	70 (35-93)
AST (U/L),	median (range)	-	22 (10-25)	174 (51-392)
ALT (U/L),	median (range)	-	-	147 (54-324)
LDH (U/L) ,	median (range)	-	-	1554 (980-3015)
Drugs:	none	9/9	-	5/14
	methyldopa	-	6/6	6/14
	magnesiumsulfate	-	4/6	7/14
	nepresol	-	1/6	3/14
	ketanserin	-	-	2/14
Neonatal weight	growth restricted:	0/9	1/6	3/14

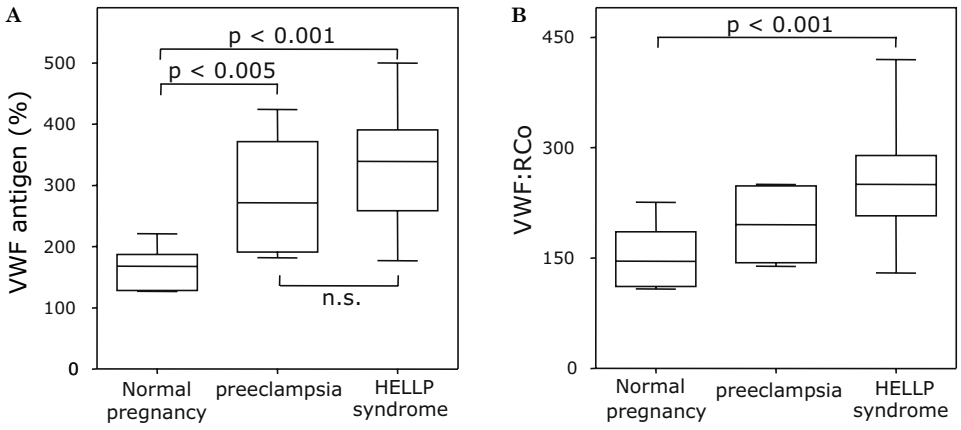

Figure 1: A: In healthy pregnant volunteers (n=9), patients with preeclampsia (n=6) and patients with HELLP syndrome (n=14) VWF antigen levels were determined and plotted in a box and whiskers plot. **B:** Ristocetin cofactor activity was measured and plotted in a box and whiskers plot.

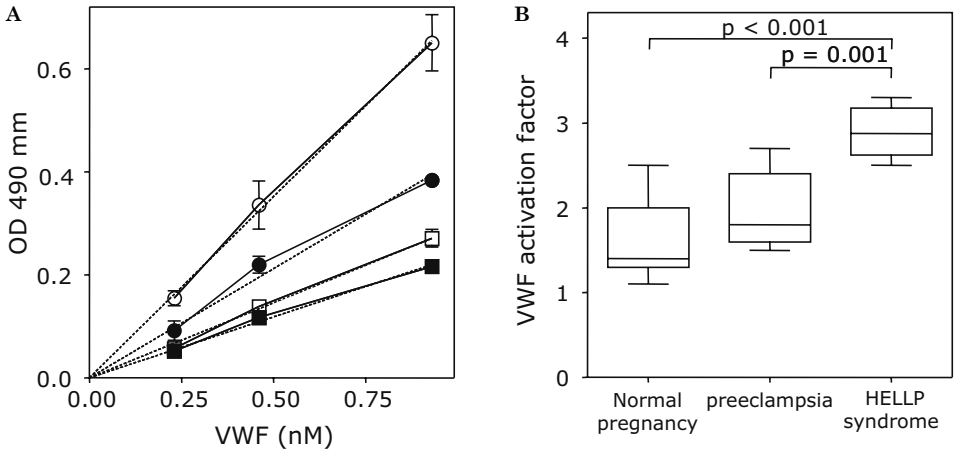

Figure 2: A: Microtiter wells coated with AU/VWFa-11 (5 µg/ml) were blocked 30 minutes with 3% BSA, 0.1% Tween-20 in PBS. NPP (closed squares), plasma from a healthy pregnant volunteers (open squares), plasma from a patient with preecclampsia (closed circles) or plasma from a patient with HELLP syndrome (open circles) were diluted in PBS to obtain a concentration range of VWF (0-1.8 nM). After washing, wells were incubated 1 hour at 37 °C with the diluted plasmas. Bound VWF was detected using HRP-conjugated polyclonal anti-VWF antibody. The concentration of VWF in the diluted samples was plotted against the measured OD 490 nm. The slope was determined from the initial linear part of the binding curve and the slope for NPP was set at 1. **B:** Activation factors were calculated for all plasma samples and plotted in a box and whiskers plot.

nM. The amount of bound VWF was monitored using an HRP-conjugated polyclonal antibody against VWF. The slope of the initial linear part of the binding curve was calculated for each sample and for NPP. The ratio between the slope for the different plasma samples over the slope for NPP was calculated. This ratio was designated VWF activation factor. A typical example of the slopes found for healthy pregnant volunteers, patients with preecclampsia and patients with HELLP syndrome is presented in Figure 2A. The activation factor was similar in patients with preecclampsia as compared to healthy pregnant controls (1.8 (1.5-2.7) and 1.4 (1.1-2.5) respectively, Figure 2B). However, in HELLP syndrome the amount of active VWF was increased 2.1-fold compared to healthy pregnant volunteers ($p < 0.001$). Moreover, the amount of active VWF was also significantly increased as compared to patients with preecclampsia (2.9 (2.5-3.3) versus 1.8 (1.5-2.7), $p = 0.001$, Figure 2B).

Subsequently, the activity of the VWF-cleaving protease was determined. ADAMTS13 activity was normal in healthy pregnant volunteers (105 ± 15%) and in patients with preeclampsia (90 ± 14%; Figure 3A). In patients with HELLP syndrome the

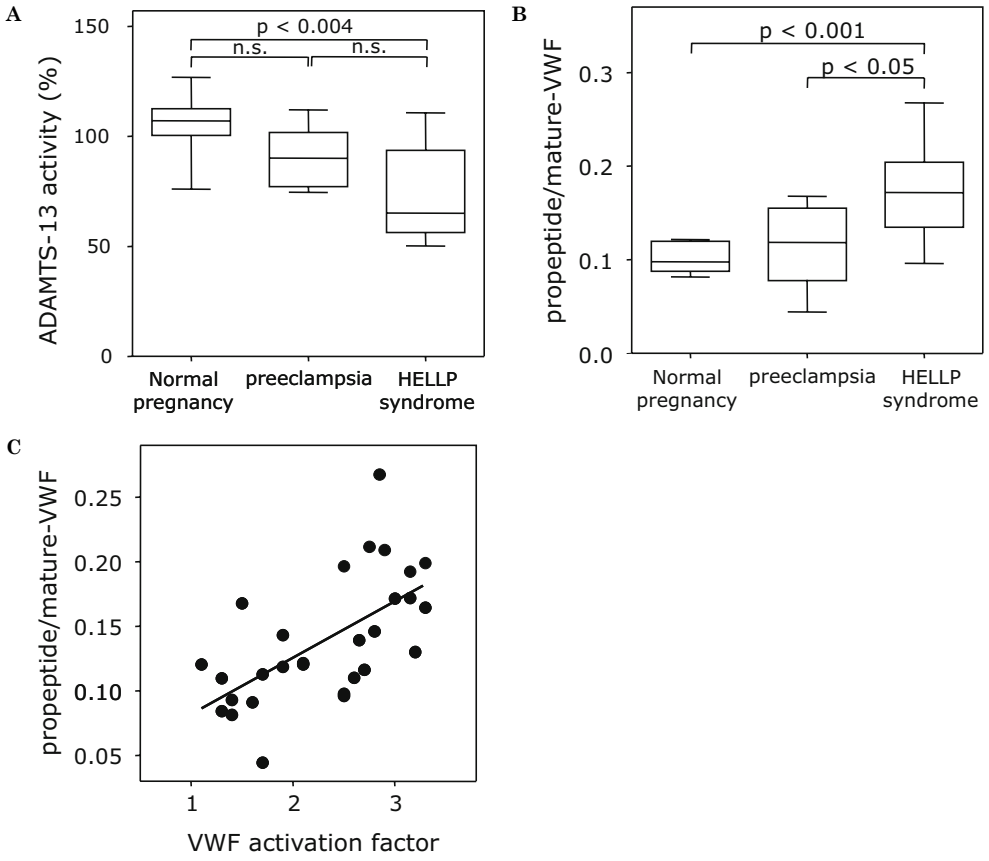

Figure 3: A: ADAMTS13 activity was measured using the FRETS-VWF73 assay and the activity was plotted for the three patient groups. **B:** Propeptide levels were measured and the propeptide/mature-VWF ratio was calculated for the different patient groups. **C:** The VWF activation factor was plotted against the propeptide/mature-VWF and correlation was found to be significant ($p<0.001$, $R^2=0.68$).

ADAMTS13 activity was significantly decreased compared to healthy pregnant volunteers (74 ± 21, $p < 0.004$; Figure 3A). However, ADAMTS13 activity in patients with HELLP syndrome was found to be within the normal range (61-142%).

In order to get more insight into the origin of the increased levels of active VWF, propeptide levels were determined. Propeptide levels and VWF antigen levels were concomitantly increased in healthy pregnant volunteers and in preeclampsia. Therefore, propeptide/mature-VWF ratio remained within normal range: 0.10 (0.08-0.12) in healthy pregnant volunteers and 0.12 (0.04-0.17) in preeclampsia (Figure 3B). In contrast, propeptide levels were increased up to 3-fold in HELLP syndrome compared to healthy pregnant volunteers, resulting in a significantly increased

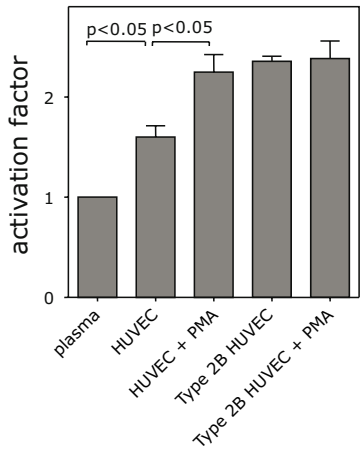

Figure 4: Normal HUVEC and HUVEC carrying a VWD type 2B mutation were stimulated with PMA (160nM). Medium from the non-stimulated and stimulated HUVEC was harvested and VWF antigen levels and the level of active VWF (activation factor) were determined.

propeptide/mature-VWF ratio as compared to healthy pregnant controls and patients with preeclampsia (0.17 (0.10-0.27) $p < 0.05$; Figure 3B). A significant correlation between propeptide/mature VWF and VWF activation factor was found ($r= 0.684$, $p < 0.001$, Figure 3C).

To investigate if VWF is secreted in a GpIbα-binding conformation by activated endothelial cells, the amount of active VWF secreted by stimulated normal HUVEC was compared with the amount secreted by non-stimulated HUVEC. First, the amount of active VWF in medium of non-stimulated normal HUVEC was measured. A 1.6-fold increase in active VWF levels was found in medium containing constitutively secreted VWF compared to NPP ($p < 0.05$, Figure 4). The activation factor calculated for VWF from PMA-stimulated normal HUVEC was significantly increased compared to the constitutively secreted VWF (2.23 (2.08-2.46), $n = 4$ versus 1.59 (1.52-1.73), $n = 4$, $p < 0.05$, Figure 4). As a positive control we used HUVEC from a newborn with VWD type 2B. VWF circulates in a GpIbα-binding conformation in VWD type 2B, due to a gain-of-function mutation in the A1-domain of VWF. The activity of VWF obtained from HUVEC carrying VWD type 2B mutation Arg1306Trp was increased, regardless of the secretion pathway (Figure 4). These data suggest that activated endothelial cells could be the source of active VWF in HELLP syndrome.

Discussion

HELLP syndrome is a severe complication of preeclampsia that has all the characteristics of a thrombotic microangiopathy, such as consumptive thrombocytopenia and platelet-rich thrombi. The underlying mechanism of this disease is largely unknown. Recently, we have shown that VWF circulates in a GpIbα-binding conformation in another thrombotic microangiopathy, TTP.[13] In the present paper we show that increased amounts of active VWF also circulate in HELLP syndrome and that these amounts strongly correlate with the propeptide/mature-VWF ratio, which is indicative for acute endothelial cell activation. This suggests that acutely activated endothelium is the main source for active VWF in HELLP syndrome. The sudden increase in active VWF might provide an explanation for the observed consumptive thrombocytopenia in HELLP syndrome.

The activity of VWF was measured in two distinct assays. The first assay, the ristocetin cofactor activity assay, measures the amount of VWF that can be activated in vitro by the addition of ristocetin. Using this assay, we found that the platelet-binding function of VWF was the same in patients with preecclampsia and patients with HELLP syndrome (Figure 1B). However, when we measured the amount of VWF that actually circulated in a GpIbα-binding conformation, we found that these amounts were significantly increased in HELLP syndrome as compared to preecclampsia (Figure 2). In contrast to the ristocetin cofactor activity assay, we correct for the total amount of VWF in plasma before incubation of the samples in the AU/VWFa-11 based immunosorbent assay. Therefore, the VWF activation factor represents the percentage of VWF in an active conformation. Since VWF antigen levels are also increased in HELLP syndrome, the absolute amount of active VWF is 2-fold increased in HELLP syndrome as compared to preecclampsia and up to 4-fold increased as compared to healthy pregnant controls.

The presence of increased amounts of active VWF in TTP can probably be ascribed to the absence of ADAMTS13 activity. Due to the absence of ADAMTS13 activity ultralarge VWF multimers circulate in TTP, which are biologically more active. The activity of ADAMTS13 was found to be decreased in HELLP syndrome as compared to healthy pregnancy, but it was still within the normal range (61-142%). Moreover, ADAMTS13 activity was not significantly different between the group of patients with preecclampsia and the group with HELLP syndrome. These data indicate that

consumption of ADAMTS13 may play a role in the onset of preecclampsia and HELLP syndrome, but cannot account for the increased amounts of active VWF in HELLP syndrome as compared to preecclampsia.

It has been suggested that preeclampsia is caused by extensive endothelial cell activation, which is part of an increased maternal inflammatory response as compared to normal pregnancy.[3] Endothelial cell activation has previously been reported to result in increased levels of soluble-thrombomodulin, E-selectin and VWF.[19] We also found increased VWF antigen levels in preecclampsia and HELLP syndrome, confirming the endothelial cell activation reported by other groups. Since VWF antigen levels are influenced by ABO blood[20], the percentage of patients with blood group O, and with non-O was kept similar in all our patient groups (Table I). The ratio propeptide/mature VWF provides additional information about the status of the endothelial cell activation (acute or chronic). Acute activation of the endothelial cell layer, or vascular damage, leads to an increase in this ratio. When a condition of endothelial cell activation persists and becomes chronic, the molar ratio normalizes.[9] Propeptide and VWF antigen levels were concomitantly increased in preeclampsia, indicating chronic endothelial activation. In contrast, we found an increased propeptide/mature-VWF ratio in HELLP syndrome, suggesting the presence of an extra acute activation. Since VWF stored in the Weibel Palade bodies is enriched in VWF multimers, which are more reactive with platelets, the extra acute activation of endothelial cells in HELLP could indeed be the source of active VWF.[12] The significant correlation between the ratio propeptide/mature VWF and the amounts of active VWF supports this suggestion.

In an *in vitro* study, we compared the activation factor of constitutively secreted VWF to the activation factor of VWF secreted from the Weibel Palade bodies upon stimulation. Constitutively secreted VWF and VWF secreted from Weibel Palade bodies of endothelial cells carrying the VWD type 2B mutation was equally active. However, medium from normal HUVEC after PMA-stimulation contained increased levels of active VWF compared to medium from non-stimulated normal HUVEC. These data further supported our hypothesis that acutely activated endothelial cells are the source for active VWF in HELLP syndrome.

In conclusion, acute activation of the endothelial cell layer in HELLP syndrome results in increased VWF antigen and VWF-propeptide levels. Moreover, the VWF secreted from the Weibel Palade Bodies contains increased amounts of VWF in a GpIbα-binding conformation. In patients developing HELLP syndrome, the transition from chronic activation of the endothelium to acute activation could result in increased levels of newly released, active VWF. Since ADAMTS13 activity is moderately decreased in HELLP syndrome, the excessive release of newly secreted VWF multimers may override the capacity of the available ADAMTS13, resulting in the presence of increased amounts of active VWF in circulation. This could explain the consumptive thrombocytopenia and the formation of platelet-rich thrombi in HELLP syndrome. Moreover, inhibition of the VWF-GpIbα interaction in HELLP syndrome could provide a new therapeutic intervention.

Acknowledgements

We thank Drs. B.B. van Rijn for providing us with plasma of healthy pregnant volunteers. The diagnostic division of Sanquin blood supply foundation (Amsterdam, The Netherlands) is gratefully acknowledged for excellent technical assistance.

Reference List

(1) Sibai B, Dekker G, Kupferminc M. Pre-eclampsia. Lancet. 2005;365:785-799.

(2) Redman CW, Sargent IL. Pre-eclampsia, the placenta and the maternal systemic inflammatory response--a review. Placenta. 2003;24 Suppl A:S21-S27.

(3) Redman CW, Sacks GP, Sargent IL. Preeclampsia: an excessive maternal inflammatory response to pregnancy. Am J Obstet Gynecol. 1999;180:499-506.

(4) Ruggenenti P, Remuzzi G. Pathophysiology and management of thrombotic microangiopathies. J Nephrol. 1998;11:300-310.

(5) Sibai BM, Ramadan MK, Usta I et al. Maternal morbidity and mortality in 442 pregnancies with hemolysis, elevated liver enzymes, and low platelets (HELLP syndrome). Am J Obstet Gynecol. 1993;169:1000-1006.

(6) Friedman SA, Schiff E, Emeis JJ, Dekker GA, Sibai BM. Biochemical corroboration of endothelial involvement in severe preeclampsia. Am J Obstet Gynecol. 1995;172:202-203.

(7) Lattuada A, Rossi E, Calzarossa C, Candolfi R, Mannucci PM. Mild to moderate reduction of a von Willebrand factor cleaving protease (ADAMTS-13) in pregnant women with HELLP microangiopathic syndrome. Haematologica. 2003;88:1029-1034.

(8) Borchiellini A, Fijnvandraat K, ten Cate JW et al. Quantitative analysis of von Willebrand factor propeptide release in vivo: effect of experimental endotoxemia and administration of 1-deamino-8-D-arginine vasopressin in humans. Blood. 1996;88:2951-2958.

(9) van Mourik JA, Boertjes R, Huisveld IA et al. von Willebrand factor propeptide in vascular disorders: A tool to distinguish between acute and chronic endothelial cell perturbation. Blood. 1999;94:179-185.

(10) Ruggeri ZM. Von Willebrand factor, platelets and endothelial cell interactions. J Thromb Haemost. 2003;1:1335-1342.

(11) Huizinga EG, Tsuji S, Romijn RA et al. Structures of glycoprotein Ibalpha and its complex with von Willebrand factor A1 domain. Science. 2002;297:1176-1179.

(12) Arya M, Anvari B, Romo GM et al. Ultralarge multimers of von Willebrand factor form spontaneous high-strength bonds with the platelet glycoprotein Ib-IX complex: studies using optical tweezers. Blood. 2002;99:3971-3977.

(13) Hulstein JJ, de Groot PG, Silence K et al. A novel nanobody that detects the gain-of-function phenotype of von Willebrand factor in ADAMTS13 deficiency and von Willebrand disease type 2B. Blood. 2005;106:3035-3042.

(14) von Dadelszen P, Magee LA, Roberts JM. Subclassification of preeclampsia. Hypertens Pregnancy. 2003;22:143-148.

(15) Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest. 1973;52:2745-2756.

(16) Willems C, Astaldi GC, de Groot PG et al. Media conditioned by cultured human vascular endothelial cells inhibit the growth of vascular smooth muscle cells. Exp Cell Res. 1982;139:191-197.

(17) Raines G, Aumann H, Sykes S, Street A. Multimeric analysis of von Willebrand factor by molecular sieving electrophoresis in sodium dodecyl sulphate agarose gel. Thromb Res. 1990;60:201-212.

(18) Kokame K, Nobe Y, Kokubo Y, Okayama A, Miyata T. FRETS-VWF73, a first fluorogenic substrate for ADAMTS13 assay. Br J Haematol. 2005;129:93-100.

(19) Nadar SK, Al YE, Blann AD, Lip GY. Thrombomodulin, von Willebrand factor and E-selectin as plasma markers of endothelial damage/dysfunction and activation in pregnancy induced hypertension. Thromb Res. 2004;113:123-128.

(20) Gill JC, Endres-Brooks J, Bauer PJ, Marks WJ, Jr., Montgomery RR. The effect of ABO blood group on the diagnosis of von Willebrand disease. Blood. 1987;69:1691-1695.

4

Chapter 4

Beta2-Glycoprotein I inhibits von Willebrand factor-dependent platelet adhesion and aggregation

Janine J.J. Hulstein
Peter J. Lenting
Bas de Laat
Ron H.W.M. Derksen
Rob Fijnheer
Philip G. de Groot

Submitted

Abstract

Rationale: Patients with the antiphospholipid syndrome are characterized by the association of thrombosis or pregnancy morbidity and the presence of anti-phospholipid antibodies. Particularly, antibodies against $ß_2$-glycoprotein ($ß_2$-GPI) correlate with thrombosis. This suggests a physiological function for $ß_2$-GPI.

Objectives: This study was performed in order to find a physiological function of plasma protein $ß_2$-GPI.

Findings: We found that $ß_2$-GPI inhibits von Willebrand factor (VWF)-induced platelet aggregation. Platelet adhesion to a VWF-coated surface was decreased up to 50% in the presence of $ß_2$-GPI ($p<0.03$). $ß_2$-GPI binds to the A1-domain of VWF when it is in its active Glycoprotein Ibα-binding conformation. Anti-$ß_2$-GPI antibodies isolated from patients with the antiphospholipid syndrome neutralized the interaction between $ß_2$-GPI and VWF and thus the inhibitory activity of $ß_2$-GPI. The amounts of active VWF in circulation were increased 1,7-fold in patients tested positive for lupus anticoagulant (LAC) due to anti-$ß_2$-GPI antibodies compared to healthy individuals ($p<0.0001$).

Conclusions: $ß_2$-GPI is a biological relevant inhibitor of VWF function and as such inhibits platelet adhesion. Anti-$ß_2$-GPI antibodies neutralize this inhibitory function of $ß_2$-GPI, resulting in increased levels of active VWF. This might explain thrombosis and consumptive thrombocytopenia observed in patients with anti-$ß_2$-GPI antibodies.

Introduction

The antiphospholipid syndrome is defined by the presence of antiphospholipid antibodies in plasma of patients with thrombotic complications or pregnancy morbidity.[1,2] In 1990 it was found that these antiphospholipid antibodies actually recognize phospholipid-binding plasma proteins, instead of phospholipids themselves.[3] In time, a large number of different plasma proteins has been described that are recognized by certain subpopulations of the so-called antiphospholipids antibodies. However, there is now a wealth of evidence that particularly autoantibodies directed against ß$_2$-glycoprotein I (GPI) expressing lupus anticoagulant (LAC) activity are the antibodies that are related with the clinical manifestations observed in the antiphospholipid syndrome.[4-6]

ß$_2$-GPI circulates in plasma as a 48 kDa glycoprotein at a relatively high concentration (approximately 3.5 μM). The sequence of ß$_2$-GPI is highly conserved among species, suggesting that ß$_2$-GPI is of biological relevance.[7] However, the few known individuals with ß$_2$-GPI deficiency[8] as well as ß$_2$-GPI deficient mice are not characterized by obvious hemostatic abnormalities[9]. This is surprising in view of the various *in vitro* studies that have suggested that ß$_2$-GPI can act as inhibitor of the intrinsic pathway of coagulation and fibrinolysis.[10] Furthermore, ß$_2$-GPI has previously been reported to cause partial inhibition of ADP-induced platelet aggregation.[11] In search for a physiological role of ß$_2$-GPI, we have re-evaluated the effect of ß$_2$-GPI on platelet adhesion and aggregation. These events both involve a complex process in which several components play a role, including von Willebrand factor (VWF).

VWF is a plasma protein that circulates with a concentration of about 50 nM. VWF is a multimeric protein involved in platelet adherence to the injured vessel wall at high shear rates, platelet-platelet interactions and it is a carrier protein for factor VIII. Adhesion of platelets at high shear rates is a multistep process in which VWF functions as a molecular bridge between the exposed subendothelial collagen and GpIb-IX-V receptor complex on platelets.[12] VWF and platelets are unable to interact spontaneously. To induce an interaction, a conversion of the VWF A1-domain into a Glycoprotein Ibα (GpIbα)-binding conformation is required.[13] Activation of the A1-domain can be induced by binding of VWF to a surface, or by incubation with

modulators such as botrocetin or ristocetin.[14,15] In von Willebrand Disease (VWD) type 2B, VWF circulates in a GpIbα-binding conformation, due to a gain-of-function mutation in the A1-domain of VWF.

In the present paper, we show that $ß_2$-GPI preferentially binds to the GpIbα-binding conformation of the A1-domain of VWF. This property of $ß_2$-GPI makes it a biological relevant inhibitor of platelet-VWF interaction. Autoantibodies against $ß_2$-GPI prevent the inhibitory effect of $ß_2$-GPI, which could contribute to the increased thrombotic risk observed in the antiphospholipid syndrome.

Materials and Methods

Purification of plasma proteins

For purification of plasma-derived $ß_2$-GPI, non-frozen human plasma was dialyzed against a solution containing 0.04 M Tris, 0.01 M succinate, 0.005% polybrene, 1 mM EDTA, 1 mM benzamidin, 43 mM NaCl and 0.02% NaN_3. The plasma was added to DEAE-Sephadex column, and the flow-through was collected, pooled and added to a protein-G-Sepharose column. The effluent pool was added to a mono S-Sepharose column. Bound $ß_2$-GPI was eluted by a linear salt-gradient (138 mM NaCl – 550 mM NaCl), and checked for purity by SDS-PAGE.[16] Purified $ß_2$-GPI was analyzed with gel filtration and showed a single peak at 48 kDa.

Plasma derived-VWF was purified from cryoprecipitate (Haemate P 250 IE, Behringwerke AG, Marburg, Germany) as described.[17]

Recombinant proteins

Recombinant full-length $ß_2$-GPI (DI-V) was obtained by a generous gift from Dr. Iverson of La Jolla Pharmaceutical Company.[18] SDS-PAGE revealed a slightly lower molecular weight for recombinant $ß_2$-GPI compared to plasma-purified $ß_2$-GPI, due to a lower level of glycosylation.

VWF/A1(1261-1468) and VWF/A1(1261-1468)-R1306Q were cloned into expression vector pPIC9 and overexpressed in Pichia Pastoris.[13] VWF/A1(1261-1468) and VWF/A1(1261-1468)-R1306Q were purified on heparin-Sepharose, followed by gel filtration.[13] VWF-wt, VWF-delta A1, -delta A2 and -delta A3 were stably expressed

in baby hamster kidney-cells, that also overexpress furin for proper removal of the propeptide.[19,20] Proteins were purified from conditioned serum-free medium as described.[21] SDS-PAGE showed that all recombinant proteins were purified to homogeneity. The multimeric structure of wt-VWF and VWF-deltaA1, -delta A2 and -delta A3 was confirmed using 0.1% SDS, 1% agarose gel electrophoresis as described previously.[22]

Antibodies

Anti-GpIbα antibody 6D1 was obtained from Dr. B.S. Collar (Mount Sinai Hospital, New York, NY). Anti-VWF antibody MoAb 9 was kindly provided by Dr. C.V. Denis (Le Kremlin-Bicêtre cedex, France.)[23] Llama anti-VWF antibody ALX0081 was described previously as control nanobody.[24] This antibody inhibits binding of VWF to GpIb. Anti-β_2-GPI antibodies 27G7 and 1F12 were kindly provided by Dr. J. Arnout (Leuven, Belgium) and and monoclonal 2B2 by Dr. A. Tincani (Brescia, Italy). Antibody 4F3 was described previously by Lutters et al.[25] Monoclonal anti-β_2-GPI antibody 3B7 was raised in our own laboratory by immunizing mice with plasma-purified β_2-GPI. The immunization was performed according to standard procedures. Monoclonal antibody 3B7 binds to domain I of β_2-GPI. HRP-conjugated antibodies against VWF and HRP-conjugated Streptavidin were obtained from Dakocytomation (Glostrup, Denmark).

Patients

30 patients with a diagnosis of SLE (systemic lupus erythematosus) (n=25) Lupus like disease (LLD, n=3), or primary antiphospholipid syndrome (n=2) were selected from a group of 198 patients that was described previously[4]; 10 patients without lupus anticoagulant (LAC)-activity, 10 patients with LAC-activity due to anti-β_2-GPI antibodies and 10 patients with LAC-activity not due to anti-β_2-GPI antibodies. Patients with SLE meet at least 4 criteria of the American College of Rheumatology for the classification of SLE and patients with LLD 1 to 3 of these criteria. Patients with primary antiphospholipid syndrome have antiphospholipid antibodies and a history of thrombosis in the absence of other signs for a systemic autoimmune disease. Patient characteristics are described in table I. The Institutional Review Board of the University Medical Center Utrecht approved this study and informed consent was obtained from all patients.

Table I: Patient characteristics

	LAC positive due to anti-ß$_2$-GPI antibodies[A] (n=10)	LAC positive not due to anti-ß$_2$-antibodies (n=10)	LAC negative SLE patients (n=10)
SLE	5	10	10
LLD	3	0	0
PAPS[B]	2	0	0
ACL IgG	9	7	3
ACL IgM	4	3	2
Anti-beta2GPI IgG	10	0	0
Anti-beta2GPI IgM	3	1	0
Anti-prothrombin IgG	8	5	4
Anti-prothrombin IgM	5	4	0
Thrombosis	10	1	3

[A] Determined as described in reference 4
[B] PAPS = primary antiphospholipid syndrome

Blood samples were taken at an arbitrary visit of the patients to the outpatient department and were collected by venapuncture using plastic tubes containing 3.8% trisodium citrate (0.129 M) as the anticoagulant. To obtain platelet-poor plasma samples were centrifuged twice at 2000 g for 10 minutes and subsequently stored at −80°C. All patient samples were measured with two LAC-assays, notably an activated partial thromboplastin time (PTT-LA, Diagnostica Stago) and a dilute Russell viper venom time (dRVVT, Dade-Behring, Marburg, Germany). Patients tested positive for LAC when they had a positive PTT-LA or/and a positive dilute RVVT test, as described previously[4]. Patients with a positive LAC were tested with the ß$_2$-GPI-dependent LAC assay as described by De Laat et al.[4] Anti-ß$_2$-GPI antibodies and anti-prothrombin antibodies were determined as described.[26]

Plasma of 16 healthy individuals was kindly provided by Dr. J.C. Meijers (Amsterdam, The Netherlands). For normal pooled plasma, platelet depleted plasma of 40 healthy volunteers was pooled and stored in aliquots at -80°C.

Purified IgGs and affinity-purified antibodies from patient plasma

To obtain affinity-purified patient antibodies, platelet poor plasma from patients (positive for either anti-ß$_2$-GPI IgG or anti-prothrombin IgG antibodies) were centrifuged twice at 2000 g for 10 minutes, and subsequently stored at -50°C until further use. The IgG fraction was purified by the use of a protein G-Sepharose column. The IgG fraction was added to a column coated with ß$_2$-GPI or prothrombin and anti-ß$_2$-GPI IgG antibodies or anti-prothrombin IgG antibodies were purified. The purity of the (affinity-) purified IgG fractions was checked using SDS-PAGE.

Laboratory assays

VWF antigen levels were quantified as described previously.[22] Active VWF in plasma samples was determined using the AU/VWFa-11 based immunosorbent assay, as described[24]. VWF propeptide concentrations were measured by an enzyme-linked immunosorbent assay (ELISA).[27] The ratio of VWF-propeptide / mature VWF-antigen concentration was calculated using molar concentrations of both proteins. ADAMTS13 activity was determined using the fluorescence resonance energy transfer-assay for ADAMTS13 activity.[28] The mean ADAMTS13 activity of 40 healthy donors was 101 ± 40% (mean ± 2SD).

Platelet aggregation with ADP, collagen, and ristocetin-activated VWF

Freshly drawn blood from healthy volunteers was collected into 3.8% trisodium citrate (0.129 M). Donors did not take any medication during 10 days prior to blood collection. Platelet-rich plasma (PRP) was prepared by centrifugation at 1100 RPM for 15 min at room temperature. Washed platelets were isolated as described.[29] Aggregation studies were performed with an optical aggregometer (Chrono-log Corporation, Havertown PA, USA). PRP was stimulated with various concentrations ADP (0-5.0 µM), collagen (0-2.0 µg/ml), or ristocetin (0.8-1.3 mg/ml) in the absence or presence of ß$_2$-GPI (2.2 µM), or an antibody mix. The antibody mix was composed of anti-ß$_2$-GPI antibodies 1F12, 2B2, 3B7, 4F3 and 27G7 and added to a final concentration of each of the antibodies of 20 µg/ml).

Washed pallets were stimulated with ADP (50 µM) and epinephrine (50 µM), collagen (2 µg/ml), or ristocetin-activated VWF (10 µg/ml VWF and 0.3 mg/ml ristocetin) in the presence or absence of 2.2 µM ß$_2$-GPI. Anti-GpIbα antibody 6D1, anti-VWF llama-antibody ALX0081 and anti-VWF-RGD MoAb 9 were used at a final concentration of

20 µg/ml. 27G7 and 1F12 were added to washed platelets at a final concentration of 2.2 µM. Patient antibodies (100 µg/ml) were preincubated 5 min at 37°C with washed platelets and 2.2 µM β_2-GPI before stimulation with 2 µg/ml collagen.

Platelet adhesion to VWF

Perfusions over VWF were carried out with reconstituted blood. To obtain reconstituted blood, washed platelets were mixed with red cells to a platelet count of 200.000 platelets/µl and a hematocrit of 40%.[30] For perfusions over VWF, glass coverslips coated with VWF (37 nM) were incubated with plasma derived β_2-GPI (1.1 or 2.2 µM) for 1 h at 22°C. Coverslips were subsequently blocked with 4% human albumin/PBS. Perfusion was performed at a shear rate of 300 s^{-1}. After perfusion, slides were washed, fixed and stained[30] and platelet adhesion was evaluated using computer-assisted analysis with OPTIMAS 6.0 software (Dutch vision systems, Breda, The Netherlands). All perfusions were performed 3 times.

Binding of β_2-GPI and VWF

Recombinant β_2-GPI (10 µg/ml, diluted in a Tris based solution (TBS): 50 mM Tris and 100 mM NaCl)) was immobilized onto a hydrophilic ELISA plate (Maxisorb, NUNC, Denmark) for 1 hour at 37°C. Plates were blocked with 2% non-fat dried milk dissolved in TBS/0.1% Tween-20 for 1 hour at 37°C and incubated with a concentration range of biotinylated VWF-wt (0-5 µg/ml), VWF-wt preincubated with ristocetin (1 mg/ml, 5 min at room temperature), VWF/A1, or VWF/A1-R1306Q (0-10 µg/ml). Plates were washed 3 times and bound VWF was detected using HRP-conjugated streptavidin. HRP-activity was measured with o-phenylenediamine as a substrate (Merck, Germany) and absorbance was measured at 490 nm.

Binding of deletion mutants of VWF to β_2-GPI

Wells coated with recombinant β_2-GPI (10 µg/ml, diluted in a TBS, 1 h 37°C) were blocked with 2% non-fat dried milk dissolved in TBS/0.1% Tween-20 for 1 hour at 37°C. After washing, 1 µM of VWF-wt, VWF-delta A1, delta A2 or delta A3 was allowed to bind to immobilized β_2-GPI (1 h at 37 °C). Bound VWF was detected using an HRP-conjugated polyclonal anti-VWF antibody.

Competition with VWF and ristocetin activated VWF

The specificity of the interaction between ß$_2$-GPI and VWF/A1-R1306Q was assessed in an immunosorbent assay, in which ß$_2$-GPI (10 µg/ml) was immobilized in microtiter wells (Costar, Cambridge MA, USA). Wells were blocked with 2% non-fat dried milk dissolved in TBS/0.1% Tween-20 for 1 h at 37°C and incubated with biotinylated VWF/A1-R1306Q (1.1 µg/ml) in the presence or absence of VWF-wt or VWF-wt preincubated with 1 mg/ml ristocetin (5 min at room temperature). After washing, wells were incubated with HRP-conjugated streptavidin (Dakocytomation, Denmark) and binding was detected by measuring HRP-activity using o-phenylenediamine as substrate.

Competition with anti-β_2-GPI antibodies

The influence of affinity purified anti-prothrombin or anti-ß$_2$-GPI antibodies from patient plasmas were studied in a competition assay. In this assay, wells coated with recombinant ß$_2$-GPI were first incubated 1 h at 37°C with the antibodies. Subsequently, VWF/A1-R1306Q was allowed to bind ß$_2$-GPI in the absence of presence of the antibodies. After washing, wells were incubated with HRP-conjugated streptavidin (Dakocytomation, Denmark) and binding was detected by measuring HRP-activity using o-phenylenediamine as substrate.

Binding of VWF to immobilized β_2-GPI in the presence of patient plasma

ß$_2$-GPI (10 µg/ml) was immobilized in microtiter wells (Costar, Cambridge MA, USA) and wells were blocked with 2% non-fat dried milk dissolved in TBS/0.1% Tween-20 for 1 h at 37°C. After washing, wells were incubated 2 h at 37°C with plasma of healthy individuals (n=16), plasma of patients tested negative for LAC-activity (n=10), patients tested positive for LAC-activity not due to anti-ß$_2$-GPI antibodies (n=10), or patients tested positive for both LAC-activity due to anti-ß$_2$-GPI antibodies (n=10). After this incubation, wells were washed and VWF/A1-R1306Q was allowed to bind to immobilized ß$_2$-GPI. Bound VWF was detected using HRP-conjugated streptavidin.

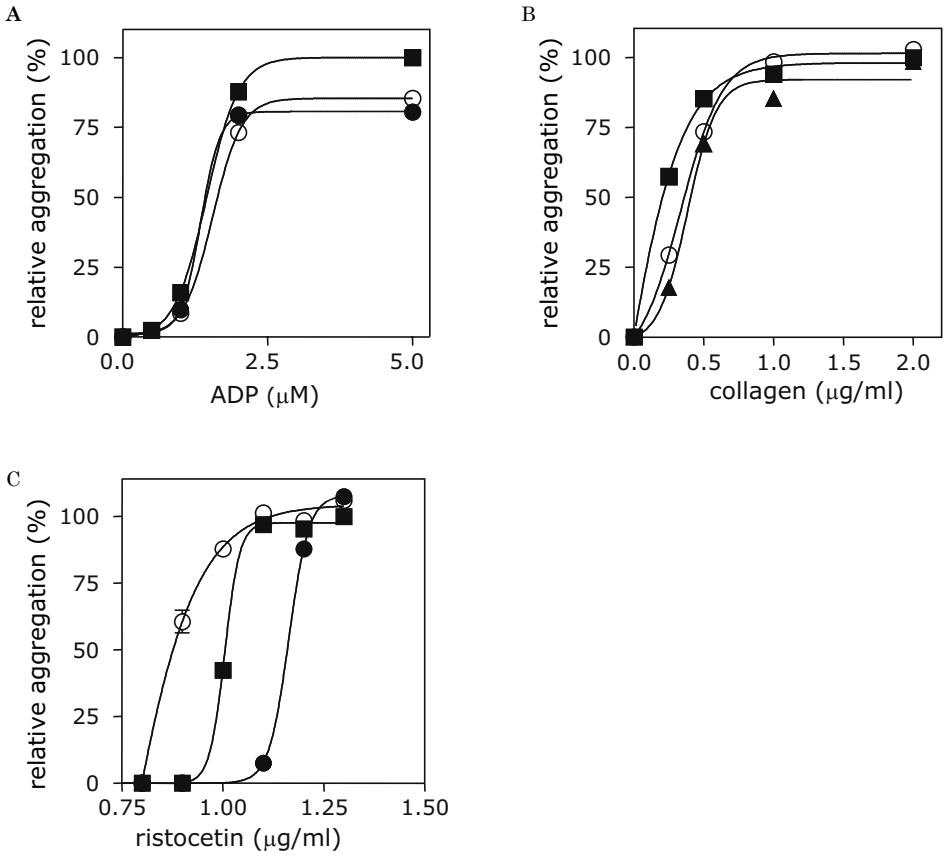

Figure 1: β_2-GPI selectively inhibits ristocetin-induced aggregation in PRP Aggregation of PRP was induced with various concentrations ADP (0-5.0 μM) **(A)**, collagen (0-2.0 μg/ml) **(B)**, or ristocetin (0.8-1.3 mg/ml) **(C)** in the absence (closed squares), or presence (closed circles) of β_2-GPI (2.2 μM), or an anti-β_2-GPI antibody mix (open circles). The antibody mix was composed of 1F12, 2B2, 3B7, 4F3 and 27G7, and was added at a final concentration of 20 μg/ml of each antibody. A typical example of each aggregation was shown. All aggregation experiments were performed using blood of 3 different donors.

Statistical analysis

Analysis of data obtained from aggregation studies, perfusion experiments and immunosorbent assays was performed using the GraphPad Prism program (GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego, CA). All data were expressed as mean with standard deviation. The t-test for paired observations was used to compare data obtained in the aggregation and perfusion experiments.

Statistical analysis of the patient-related data was performed with the SPSS software package (version 12.0.1, Chicago, Illinois). Differences between patient groups were tested using the Kruskal-Wallis test, followed by the Mann-Whitney U test for the calculation of differences between the separate groups. $P < 0.05$ values were considered to indicate statistical significance.

Results

β_2-GPI selectively inhibits ristocetin-induced platelet aggregation in PRP

The effect of ß$_2$-GPI on platelet aggregation was first studied in PRP. Aggregation was induced with various concentrations ADP (0-5.0 µM), collagen (0-2.0 µg/ml), or ristocetin (0.8-1.3 mg/ml). In each experiment total aggregation measured after stimulation with the highest concentration agonist was set at 100% and relative aggregation at other concentrations of agonist was calculated. Platelet aggregation induced by ADP, collagen and ristocetin were dose dependent (Figure 1A, B and C). ADP- and collagen-induced aggregations remained unaffected after addition of extra ß$_2$-GPI (2.2 µM) to PRP (Figure 1A and B). In contrast, after addition of ß$_2$-GPI, platelet agglutination induced by ristocetin could only be achieved at higher ristocetin concentrations (Figure 1C). Plasma contains 1.0-5.0 µM ß$_2$-GPI, To neutralize this plasma ß$_2$-GPI, we studied the influence of anti-ß$_2$-GPI antibodies on platelet aggregation. Addition of a mixture of monoclonal anti-ß$_2$-GPI antibodies at a final concentration of 20 µg/ml of each antibody did not affect platelet aggregation induced by ADP or collagen. However, ristocetin-induced agglutination was observed at lower ristocetin-induced concentrations. This indicates that ristocetin agglutination is regulated by plasma levels of ß$_2$-GPI.

Aggregation of washed platelets induced by various stimuli is inhibited by β_2-GPI

Subsequently, we studied the influence of ß$_2$-GPI on aggregation of washed platelets. Since ADP is a weak platelet activator in the absence of plasma fibrinogen, platelets were stimulated with ADP (50 µM) in combination with epinephrine (50 µM) to induce sufficient platelet aggregation. Furthermore, collagen (2 µg/ml) or plasma-derived VWF (10 µg/ml) preincubated with ristocetin (0.3 mg/ml) were used as platelet activator. Addition of 2.2 µM ß$_2$-GPI resulted in reduced platelet aggregation, regardless of the agonist (Figure 2A, B and C). In ADP- or collagen-induced platelet

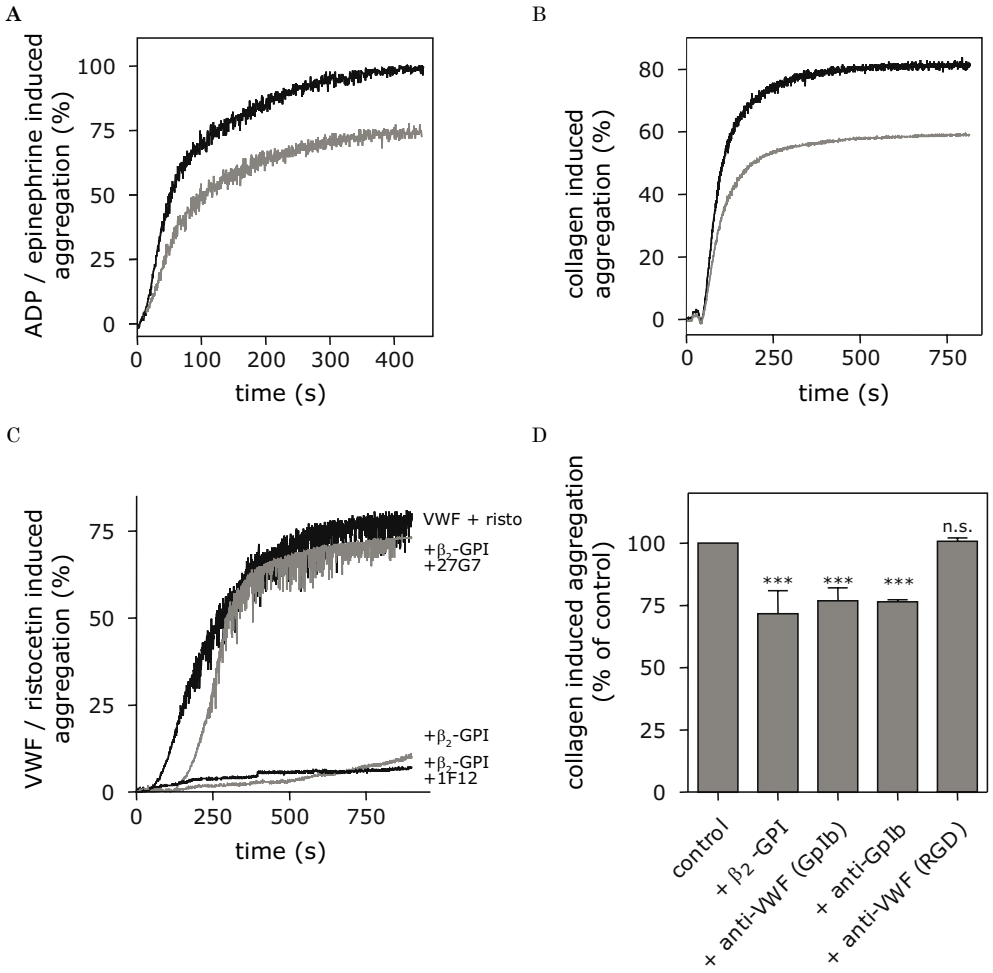

Figure 2: Aggregation of washed platelets induced by various stimuli is inhibited by β_2-GPI. Aggregation of washed platelets was induced with ADP (50 µM) and epinephrine (50 µM) **(A)**, or collagen (2 µg/ml) **(B)** in the absence (black line) or presence (gray line) of β_2-GPI (2.2 µM). **C:** Aggregation of washed platelets was induced with ristocetin-activated VWF (10 µg/ml VWF and 0.3 mg/ml ristocetin) in the absence (black line) or presence (gray line) of β_2-GPI (2.2 µM). To study the effect of anti-β_2-GPI antibodies, β_2-GPI (2.2 µM) and LAC negative anti-β_2-GPI antibody 1F12 or LAC positive anti-β_2-GPI antibody 27G7 were added at a final concentration of 2.2 µM to washed platelets. A typical example of the aggregation-curves is shown. All aggregation experiments were performed 3 times, using blood of different donors. **D:** Total aggregation of washed platelets induced with 2 µg/ml collagen was set at 100%. Relative aggregation (%) in the presence of β_2-GPI (2.2 µM), anti-VWF antibody ALX0081 recognizing the GpIbα-binding site (20 µg/ml), anti-GpIbα antibody 6D1 (20 µg/ml), or anti-VWF antibody MoAb 9 recognizing the $\alpha_{IIb}\beta_3$ binding site (20 µg/ml) was calculated. Data represent the mean ± SD of 5 experiments. ***$p < 0.0005$

aggregation, addition of β_2-GPI only influenced total extent of platelet aggregation, whereas the slope of the aggregation-curve remained unaffected. This effect could be

mimicked by the addition of an antibody against GpIbα (6D1) or VWF (ALX0081), both inhibiting the interaction between GpIbα and VWF (Figure 2D). Platelet aggregation remained unaffected by the addition of MoAb 9, an antibody that blocks the $\alpha_{IIb}\beta_3$-binding site on VWF (Figure 2D). Taken together, these results suggest that β_2-GPI inhibits ADP- and collagen-stimulated aggregation of washed platelets in a VWF-GPIb dependent manner.

Anti-β_2-GPI antibodies abrogate inhibitory effect of β_2-GPI on platelet aggregation

The influence of anti-β_2-GPI antibodies on the inhibitory effect of β_2-GPI in platelet aggregation was studied. For this, 27G7 was used, a monoclonal antibody directed against β_2-GPI with lupus anticoagulant (LAC)-activity. The effect of this antibody was compared with the effect of 1F12, an anti-β_2-GPI monoclonal antibody without LAC-activity. Stimulation of washed platelets with ristocetin-activated VWF induced a total platelet aggregation of 78% (Figure 2C). In the presence of 2.2 μM β_2-GPI a total aggregation of 10% was found, corresponding with 87% inhibition. This inhibition remained unaltered in the presence of 2.2 μM 1F12. In contrast, addition of 2.2 μM 27G7 completely abolished the inhibitory effect of β_2-GPI on platelet aggregation (Figure 2C). This suggests that anti-β_2-GPI antibodies, which comprise LAC-activity, influence the inhibitory function of β_2-GPI on ristocetin-induced platelet agglutination.

β_2-GPI interacts directly with VWF

We further investigated whether β_2-GPI interacts directly with VWF. The possible interaction between β_2-GPI and VWF was studied in an immunosorbent assay, in which β_2-GPI was immobilized onto microtiter wells. Wells were incubated with various concentrations of biotinylated plasma-derived VWF in the presence or absence of ristocetin. The amount of bound VWF was subsequently monitored with HRP-conjugated streptavidin. Some binding could be observed at higher concentrations of VWF (Figure 3A). However, addition of ristocetin resulted in significant binding at low concentrations of VWF (Figure 3A). The interaction between VWF and β_2-GPI was studied in more detail using various deletion-mutants of VWF. To induce sufficient binding of the different VWF deletion mutants, a high concentration VWF (1 μM) was used (Figure 3B). Binding of VWF variants with deletions of the A2- or A3-domain was similar compared to binding of VWF-wt (Figure 3B). However, deletion of the A1-domain of VWF resulted in significant decreased binding to immobilized β_2-GPI

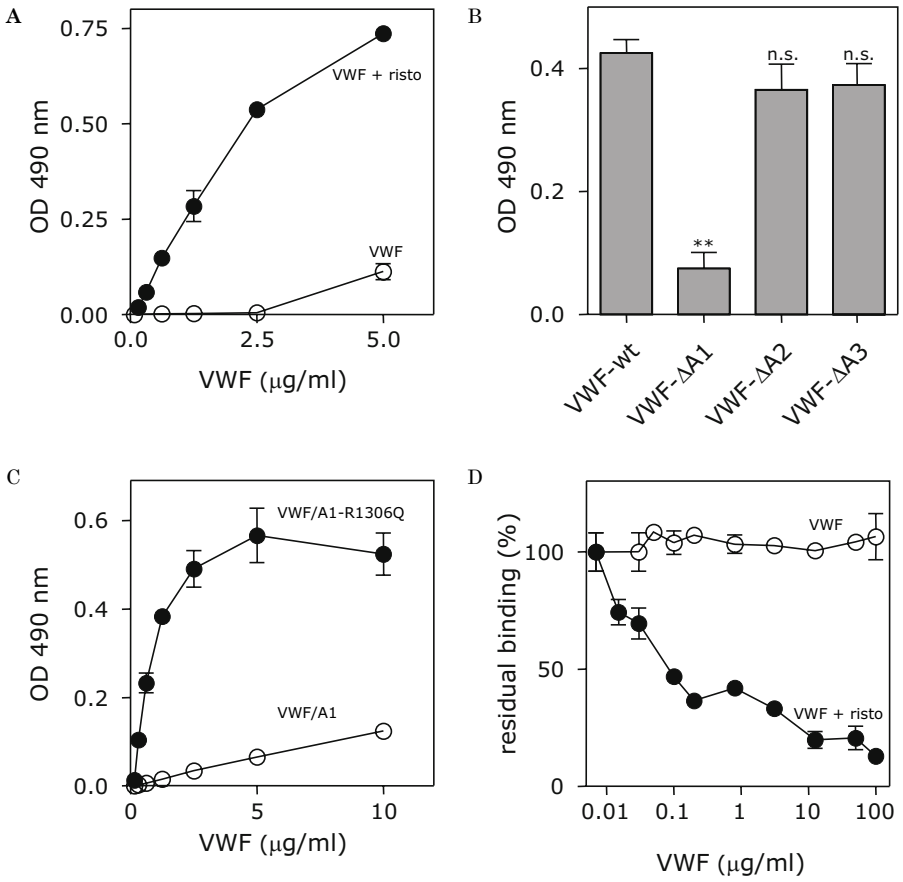

Figure 3: β_2-GPI interacts with the GpIb-binding conformation of the A1-domain of VWF. Full length β_2-GPI (10 μg/ml) was immobolized onto microtiter wells (1 h at 37°C) and incubated with different concentrations biotinylated VWF-wt (0-5 μg/ml) in the absence (open circles) or presence (closed circles) of ristocetin (1 mg/ml). Bound VWF was detected with HRP-conjugated streptavidin. **B:** Wells coated with full length β_2-GPI were incubated with 1 μM VWF-wt, VWF-delta A1, -delta A2, or -delta A3. Bound VWF was detected using HRP-conjugated anti-VWF. **p = 0.003 **C:** Immobilized β_2-GPI was incubated with different concentrations biotinylated VWF/A1 (open circles), VWF/A1-R1306Q (closed circles) (0-10 μg/ml). Bound VWF was monitored with streptavidin-HRP. **D:** β_2-GPI coated wells were incubated with biotinylated VWF/A1-R1306Q (1.1 μg/ml) in the presence of different concentrations plasma-derived VWF (0-100 μg/ml) without (open circles) or with (closed circles) ristocetin (1 mg/ml). Bound VWF/A1-R1306Q was monitored using HRP-conjugated streptavidin. All data represent the mean ± SD of 3 experiments.

(p = 0.003, Figure 3B), indicating that the A1-domain of VWF plays an important role in the interaction between β_2-GPI and VWF. Since the A1-domain seems to be of importance in the interaction with β_2-GPI and to avoid potential complications due to the presence of ristocetin, we tested direct binding to immobilized β_2-GPI of recombinant A1-domain and a variant of the A1-domain containing VWD type 2B

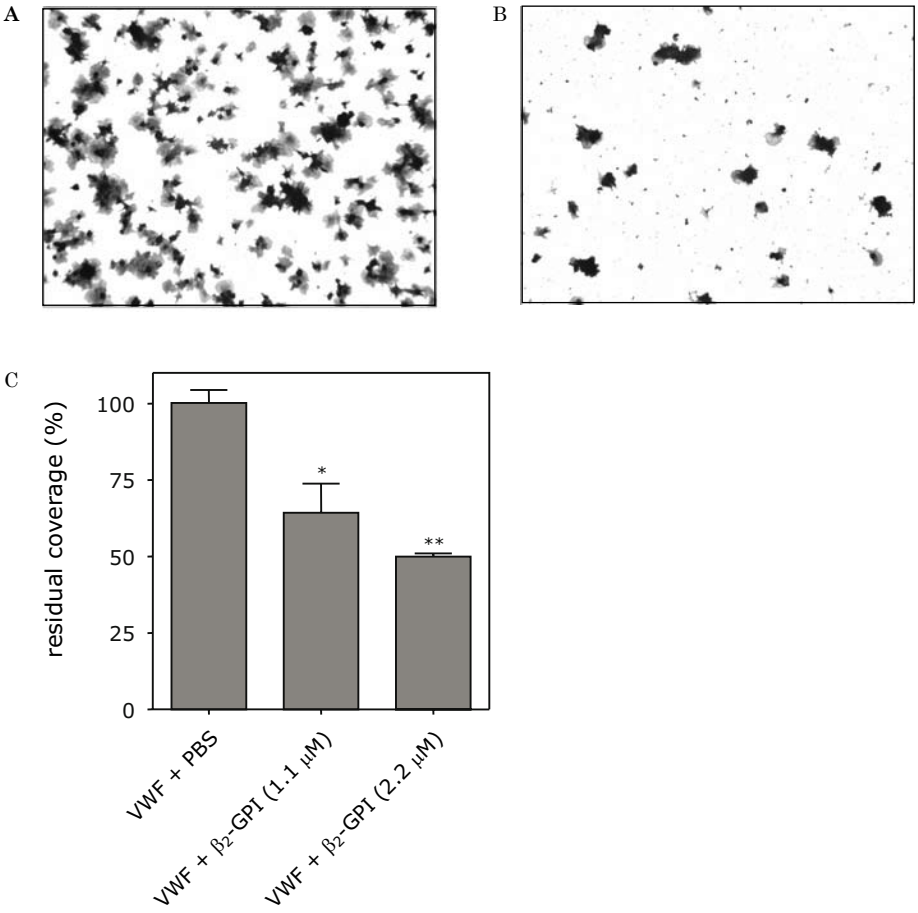

Figure 4: Platelet adhesion to VWF is inhibited by β_2-GPI. Reconstituted blood was perfused over glass coverslips coated with plasma derived-VWF (37 nM) at a shear rate of 300 s^{-1}. Before perfusion, coated coverslips were incubated with PBS **(A)** or β_2-GPI (1.1 or 2.2 μM) **(B)**. After perfusion, adhered platelets were fixed in 0.5% glutaraldehyde in PBS, dehydrated in methanol and stained with May-Grünwald and Giemsa. Adhered platelets were visualized using light microscopy (Leitz Diaplan; Leicca, Rijswijk, The Netherlands) and computer-assisted analysis (AMS 40-10 Saffron, Walden, United Kingdom). Original magnification was 400 x (40 x/1.00 NA objective lens). (dark regions represent platelet aggregates) **C:** Platelet adhesion was evaluated using computer-assisted analysis and was expressed as the percentage of surface covered with platelets. To compare the experiments platelet coverage on VWF was set to be 100% and relative coverage was calculated for the other conditions. Data represent the mean ± SD of 3 experiments. *p < 0.03, **p < 0.003

mutation R1306Q. Little binding of wt-A1-domain to β_2-GPI at concentrations up to 10 μg/ml was observed. In contrast, VWF/A1-R1306Q displayed efficient dose-dependent and saturable binding to β_2-GPI (half maximum binding at 1.1 μg/ml; Figure 3C). The specificity of this interaction was challenged in competition-assays using VWF or ristocetin-activated VWF. Binding of biotinylated VWF/A1-R1306Q (1.1 μg/ml) was

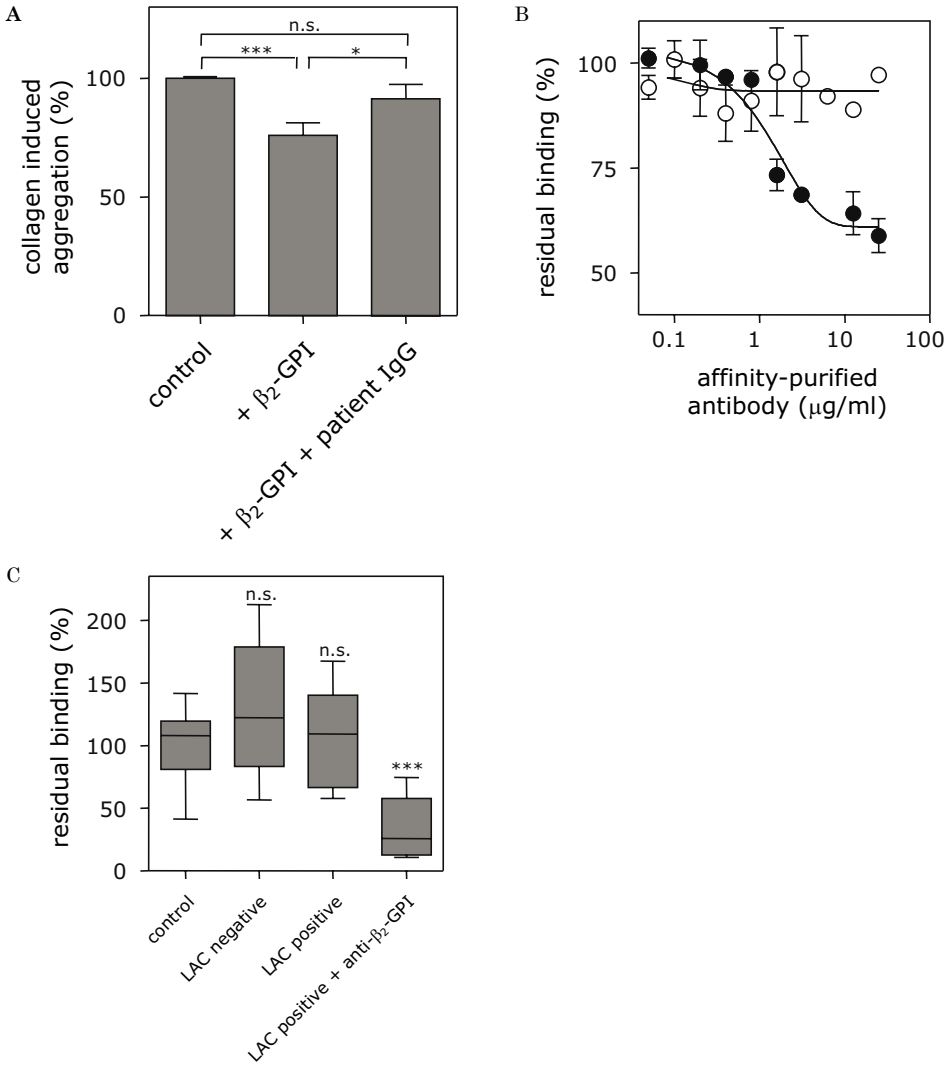

Figure 5A: Patient anti-β_2-GPI antibodies interfere with the interaction between VWF and β_2-GPI. Total aggregation of washed platelets induced with 2 μg/ml collagen was set at 100%. Relative aggregation in the presence of β_2-GPI (2.2 μM) and in the presence of β_2-GPI and patient IgG of 3 different patients (100 μg/ml) was calculated. All aggregation experiments were performed with platelets of 5 different blood donors. Data present the mean ± SD of the effect of IgG isolated from 1 patient and are representative for the data found with the IgGs from the other 2 patients. *p = 0.02, ***p = 0.0004 **B:** Wells coated with full length β_2-GPI were incubated with biotinylated VWF/A1-R1306Q (1.1 μg/ml) in the presence of different concentrations of affinity purified anti-thrombin antibodies (open circles) or anti-β_2-GPI antibodies (closed circles). Bound VWF/A1-R1306Q was detected using streptavidin-HRP. Experiments were performed with antibodies purified from plasma of 3 different patients, a typical example of the experiments was presented. Data show the mean ± SD of 3 experiments. **C:** Wells coated with full length β_2-GPI were blocked and incubated 2 h at 37°C with plasma of normal individuals (n=16), plasma of patients negative for LAC activity (n=10), positive for LAC activity not due to anti-β_2-GPI antibodies (n=10), or positive for LAC activity due to anti-β_2-GPI antibodies (n=10). After washing, wells were incubated with biotinylated VWF/A1-R1306Q (1.1 μg/ml) (30 min, 37°C). Bound VWF was detected with streptavidin-HRP. All data represent the median and range of 3 experiments. ***p = 0.0002

studied in the presence of different concentrations of VWF with or without 1 mg/ml ristocetin. Binding of VWF/A1-R1306Q to immobilized ß$_2$-GPI remained unaffected in the presence of up to 100 µg/ml plasma-derived VWF. However, ristocetin-activated VWF increasingly inhibited binding to ß$_2$-GPI, up to 87% at a concentration of 100 µg/ml VWF. Half maximal inhibition was observed at 0.1 µg/ml (Figure 3D).

β_2-GPI inhibits platelet adhesion to VWF under flow conditions

The effect of ß$_2$-GPI on platelet-VWF interaction could be different under flow, therefore the effect was studied in a perfusion system. Reconstituted blood was perfused over VWF-coated glass coverslips at a shear of 300 s^{-1}. Platelet coverage on VWF in the absence of ß$_2$-GPI was set at 100% (Figure 4A/C). Preincubation of the VWF-coated surface with different concentrations ß$_2$-GPI (1.1 µM or 2.2 µM) was associated with a significant and dose-dependent decrease of platelet coverage compared to the control (36 ± 9.5% and 50 ± 1.0% respectively; $p < 0.03$, n=3) (Figure 4B/C). A glass coverslip with directly coated ß$_2$-GPI did not support platelet adhesion (data not shown).

Anti-β_2-GPI antibodies abrogate inhibitory effect of β_2-GPI on platelet aggregation

The effect of anti-ß$_2$-GPI antibodies on platelet aggregation was studied. For this, IgGs were purified from plasma of 3 different patients with a ß$_2$-GPI–dependent LAC. Washed platelets were stimulated with collagen (2 µg/ml) in the presence or absence of ß$_2$-GPI. A 24 ± 5% reduction of platelet aggregation was found in the presence of ß$_2$-GPI (Figure 5A). The addition of patients IgGs neutralized the effect of ß$_2$-GPI, resulting in similar total platelet aggregation as compared with the control situation, in which only collagen was added to the platelets. The effect of each patient IgG sample was studied using isolated platelets of 5 different blood donors. A typical example of the effect of purified patient IgGs was shown in Figure 5A. Similar results were found using purified IgGs of the other 2 patients.

Patient anti-β_2-GPI antibodies inhibit the β_2-GPI - VWF interaction

Subsequently, we studied the influence of patient anti-ß$_2$-GPI antibodies on the interaction between VWF and ß$_2$-GPI. To this end, we performed a competition assay, in which biotinylated VWF/A1-R1306Q was allowed to bind to immobilized ß$_2$-GPI in the presence of affinity purified anti-ß$_2$-GPI antibodies from 3 different patients with the antiphospholipid syndrome. Affinity purified anti-prothrombin antibodies purified from plasma of 3 different patients with LAC positive SLE were used as

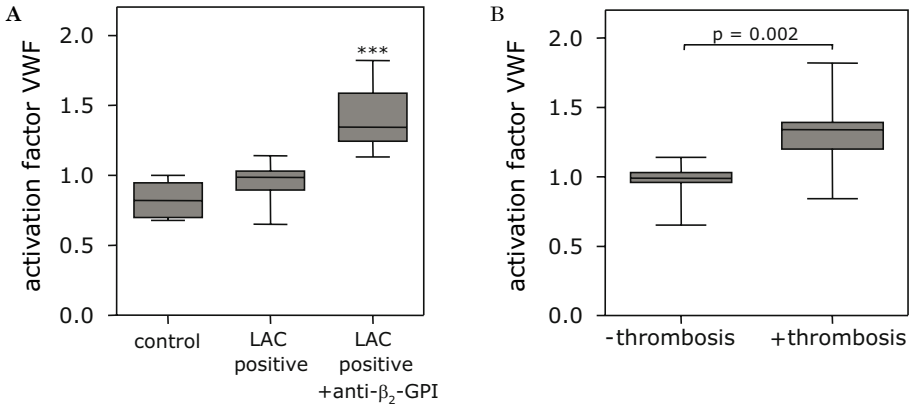

Figure 6A: Increased amounts of active VWF in patients with anti-β_2-GPI antibodies. In healthy individuals (n=10), in patients positive for lupus anticoagulant not due to anti-β_2-GPI antibodies (n=10) and in patients positive for lupus anticoagulant due to anti-β_2-GPI antibodies (n=10) the activation factor of VWF was determined and plotted in a box and whiskers plot. ***$p < 0.0001$ **B:** The activation factor of VWF was plotted for patients with (n=11), or without (n=9) thrombosis.

control. Binding of VWF/A1-R1306Q remained unaffected in the presence of anti-prothrombin antibodies. Addition of anti-β_2-GPI antibodies inhibited the interaction up to 41% (41 ± 4%) in the presence of 20 µg/ml of antibody. Due to a restricted amounts of these affinity-purified antibodies we were unable to study the effect of higher concentrations of the antibodies. A typical example of the effect of the patient antibodies on the interaction between VWF/A1-R1306Q and β_2-GPI is shown in figure 5B.

To test if antibodies present in plasmas of patients with the antiphospholipid syndrome interfere with the interaction between VWF and β_2-GPI, a binding assay was performed with different plasma samples. In this assay, immobilized β_2-GPI was incubated with plasmas of normal individuals (n=16), plasmas tested positive for anti-β_2-GPI antibodies and LAC-activity (n=10), plasmas with LAC-activity not due to anti-β_2-GPI antibodies (n=10), and plasmas from patients with SLE that were tested negative for LAC-activity (n=10). After incubation, wells were washed and incubated with biotinylated VWF/A1-R1306Q. Binding of VWF/A1-R1306Q after incubation with plasma of normal individuals was set at 100% (Figure 5C) and relative binding of VWF/A1-R1306Q after incubation with patient plasma was calculated. After incubation with plasma tested negative for lupus anticoagulant or plasma positive for LAC-activity not due to anti-β_2-GPI antibodies, binding of VWF/A1-R1306Q remained unaffected (127 ± 51% and 106 ± 39%, respectively) compared

Table II: propeptide and VWF-antigen levels and activity of ADAMTS13

	LAC positive due to anti-β_2-GPI antibodies (n=10)	LAC positive due to anti-prothrombin antibodies (n=10)	LAC negative SLE patients (n=10)
VWF-antigen (µg/ml)	23.4 ± 9.4	18.3 ± 4.5	22.1 ± 9.1
VWF-propeptide (µg/ml)	0.17 ± 0.10	0.13 ± 0.05	0.16 ± 0.14
Propeptide/mature VWF	0.09 ± 0.03	0.09 ± 0.03	0.08 ± 0.02
ADAMTS13 activity (%)	81.7 ± 28.5	71.7 ± 31.1	66.5 ± 20.8

All data represent mean ± SD

to normal plasma. However, a strong decrease of binding was found after incubation of immobilized β_2-GPI with plasma of patients positive for LAC-activity due to anti-β_2-GPI antibodies (34 ± 24% of normal plasma, p=0.0002). This indicates that also in plasma the interaction between β_2-GPI and VWF is influenced by the presence of anti-β_2-GPI antibodies in the circulation.

Increased amounts of active VWF in patients with LAC-activity caused by anti-β_2-GPI antibodies

The results of the previous experiments suggest that β_2-GPI interacts with the GpIbα-binding conformation of VWF and that patient antibodies neutralize this interaction. To support these observations we analyzed plasma of patients with the antiphospholipid syndrome for the presence of VWF in its active conformation in their circulation. To analyze this, nanobody AU/VWFa-11 was used, which specifically recognizes the GpIbα-binding conformation of VWF. In plasma of patients tested positive for LAC-activity not due to anti-β_2-GPI antibodies (n=10) the amounts of VWF in a GpIbα-binding conformation were similar to the amounts found in normal plasma (1.0 ± 0.2, Figure 6A). In contrast, in patients that tested positive for lupus anticoagulant due to anti-β_2-GPI antibodies (n=10), an increased amount of circulating active VWF was measured (1.4 ± 0.2; $p < 0.0001$, Figure 6A). Rearrangement of the patient group into a group with (n=11) and a group without (n=9) thrombosis showed that the amount of active VWF was significantly increased in the group with thrombosis (p=0.002), indicating that circulation of active VWF increases the risk for thrombosis (Figure 6B).

ADAMTS-13 activity and endothelial cell activation

In order to find a possible explanation for the increased amounts of active VWF in the circulation, a number of other parameters were measured. The ratio between VWF-propeptide and mature VWF-antigen is indicative for acute endothelial cell activation. The levels of VWF-antigen were found to be equally increased in all patient groups and propeptide levels were concomitantly increased. The ratio VWF-propeptide / mature VWF-antigen therefore remained within the normal range in all groups (Table II). Furthermore, we measured the ADAMTS-13 activity, since this enzyme determines the length and reactivity of the VWF multimers. The mean ADAMTS-13 activity was within the normal range in all patient groups (Table II).

Discussion

It is almost an axiom in haemostasis that every active (co)factor or enzyme has its own inhibitor(s) to prevent exaggerated haemostasis and unwanted thrombus formation. The first step in thrombus formation at higher shear stresses is the binding of VWF to an injured vessel wall. Despite this crucial function of VWF, no direct inhibitor of VWF-GpIb interaction has been identified up till now. In the present paper, we show that $ß_2$-GPI can interact directly with VWF. This interaction becomes far more efficient when VWF is in its GpIbα-binding conformation. Binding of $ß_2$-GPI to VWF inhibits ristocetin-induced platelet agglutination and platelet adhesion at physiological shear stresses. These observations show that $ß_2$-GPI can act as an inhibitor of active VWF *in vitro*. The presence of anti-$ß_2$-GPI antibodies in patients with the antiphospholipid syndrome strongly correlates with thrombosis. These antibodies interfere with the inhibitory role of $ß_2$-GPI, suggesting a role for $ß_2$-GPI as a VWF antagonist *in vivo* as well.

VWF circulates in plasma in a resting conformation, which does not interact with GpIbα. The GpIbα-binding conformation is induced by binding of VWF to subendothelial collagen, or by incubation with ristocetin.[12,14,15] Until now, this conformational change and proteolysis of very reactive ultralarge VWF multimers by ADAMTS13 are the only known regulators of VWF activity. In an ELISA set-up we found a direct interaction between $ß_2$-GPI and the A1-domain of VWF. However, a significant interaction with plasma derived VWF was only observed at non-

physiological concentrations of VWF. Addition of ristocetin strongly increased this interaction. To exclude the possibility that the observed effects were due to binding of β_2-GPI to ristocetin, we confirmed this high affinity of β_2-GPI for VWF in the GpIbα-binding conformation using recombinant VWF with a VWD type 2B mutation (VWF/A1-R1306Q). Moreover, a competition assay showed that the interaction of β_2-GPI with VWF/A1-R1306Q could only be inhibited by VWF, which was activated with ristocetin. These findings show that β_2-GPI preferentially binds the active, GpIbα-binding conformation of VWF. Since β_2-GPI will only act as an inhibitor when VWF is activated and bound to collagen, no complexes of β_2-GPI and VWF will be present in the circulation. We postulate that the major function of β_2-GPI is to prevent the presence of active VWF in the circulation. Only when massive amounts of activated VWF are present, as for instance in patients with thrombotic thrombocytopenic purpura, the levels of β_2-GPI are insufficient to prevent platelet clumping.

Based on *in vitro* experiments a number of possible functions for β_2-GPI have been postulated, among others a role as modulator of platelet aggregation.[11] In our re-evaluation of the influence of β_2-GPI on platelet function, we found that β_2-GPI is a circulating inhibitor of VWF function. More importantly, we show that a subpopulation of antibodies, found in patients with the antiphospholipid syndrome, interfere with the inhibitory function of β_2-GPI. This subset of antibodies are anti-β_2-GPI antibodies with LAC-activity, which correlate strongly with thrombosis.[4] These observations suggest that β_2-GPI is a physiological inhibitor of active VWF. Inhibition of this functional inhibitor by antibodies in patients with antiphospholipid syndrome results in the presence of circulating active VWF and is a possible explanation for the increased thrombotic tendency.

To confirm that active VWF is circulating in patients with the antiphospholipid syndrome, we have measured the level of active VWF with a nanobody that specifically recognizes VWF in its GpIbα-binding conformation.[24] We found significantly increased levels of active VWF in plasma of patients with an anti-β_2-GPI antibody dependent LAC-activity. Moreover, we found a significant higher level of active VWF in patients with thrombosis. The increased levels of active VWF could not be ascribed to a decrease of ADAMTS13 activity or an increased release of VWF from the endothelium. We therefore hypothesize that the increased amounts of VWF are caused by a loss of inhibition, due to the presence of circulating anti-β_2-GPI antibodies.

The absence of β_2-GPI in plasma not a known risk factor for thrombosis[31], although the presence of anti-β_2-GPI antibodies with LAC-activity in patients with the antiphospholipid syndrome correlate strongly with thrombo-embolic complications.[4-6] Not many families with β_2-GPI deficiency have been described. β_2-GPI deficiency is extremely rare in the Caucasian population while a few Japanese families have been described with a frame shift mutation resulting in absence of detectable levels of β_2-GPI.[8] Although the described siblings did not suffer from thrombotic manifestations, a significant number of their ancestors died of stroke. We have not enough information to draw firm conclusions, but it is possible that absence of β_2-GPI is a risk factor for thrombotic complications only in the presence of other challenges such as accelerated atherosclerosis at older age. In this respect, it is understandable that also mice lacking β_2-GPI do not develop thrombosis without a challenge.

The increased risk for thrombosis in patients with antiphospholipid syndrome has been proposed to coincide with the induction of a new function of β_2-GPI after binding of anti-β_2-GPI antibodies. β_2-GPI-anti-β_2-GPI complexes have an increased affinity for different cell types[2] and binding of these complexes to platelets, endothelial cells and monocytes results in activation of these cells. In the present paper, we show that β_2-GPI does have a physiological function and that this function is neutralized in the presence of anti-β_2-GPI antibodies with LAC activity. The risk for thrombosis in patients with the antiphospholipid syndrome might be explained by one of these two mechanisms, but more likely it is due to a combination of different effects of anti-β_2-GPI antibodies on the function of β_2-GPI. The presence of antibodies changes β_2-GPI from a anti-thrombotic protein (inhibitor of platelet adhesion) into a prothrombotic protein (cell activation)

We have previously shown that the amount of active VWF correlates strongly with a decreased platelet number in VWD type 2B.[24] Thrombocytopenia is also often observed in patients with the antiphospholipid syndrome. It is noteworthy that in older studies on additional risk factors for thrombosis in antiphospholipid syndrome, besides anti-phospholipid antibodies only thrombocytopenia was found to be an independent risk factor for thrombo-embolic complications.[32]

In summary, we show for the first time a direct interaction between VWF and β_2-GPI. This interaction seems to regulate binding of VWF to platelets, thereby inhibiting thrombus formation. Antibodies recognizing β_2-GPI compete for the interaction

between VWF and β_2-GPI, resulting in loss of the inhibitory, protective function of β_2-GPI. The increased amount of circulating, unprotected active VWF is able to interact spontaneously with platelets. This could explain the onset of thrombosis and consumptive thrombocytopenia in patients with a β_2-GPI dependent LAC.

Acknowledgements

We thank Drs. M.T. Pennings and. R.T. Urbanus for providing us with purified plasma β_2GPI. M. Compier, L. Rens and M. IJsseldijk are gratefully acknowledged for excellent technical assistance.

This study was supported by grants from the Netherlands Organization for Health Research and Development (ZON-MW 902-26-290) and from the Landsteiner Foundation of Blood Transfusion Research (LSBR grant nr. 0114). The financial support of the Noaber foundation, and the Hoge Dennen foundation is gratefully acknowledged.

Reference List

(1) Miyakis S, Lockshin MD, Atsumi T et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost. 2006;4:295-306.

(2) de Groot PG, Derksen RH. Pathophysiology of the antiphospholipid syndrome. J Thromb Haemost. 2005;3:1854-1860.

(3) Arnout J, Vermylen J. Current status and implications of autoimmune antiphospholipid antibodies in relation to thrombotic disease. J Thromb Haemost. 2003;1:931-942.

(4) de Laat HB, Derksen RH, Urbanus RT, Roest M, de Groot PG. beta2-glycoprotein I-dependent lupus anticoagulant highly correlates with thrombosis in the antiphospholipid syndrome. Blood. 2004;104:3598-3602.

(5) Zoghlami-Rintelen C, Vormittag R, Sailer T et al. The presence of IgG antibodies against beta2-glycoprotein I predicts the risk of thrombosis in patients with the lupus anticoagulant. J Thromb Haemost. 2005;3:1160-1165.

(6) Pengo V, Biasiolo A, Pegoraro C et al. Antibody profiles for the diagnosis of antiphospholipid syndrome. Thromb Haemost. 2005;93:1147-1152.

(7) Gao B, Virmani M, Romm E et al. Sequence of a cDNA encoding bovine apolipoprotein H. Gene. 1993;126:287-288.

(8) Yasuda S, Tsutsumi A, Chiba H et al. beta(2)-glycoprotein I deficiency: prevalence, genetic background and effects on plasma lipoprotein metabolism and hemostasis. Atherosclerosis. 2000;152:337-346.

(9) Sheng Y, Reddel SW, Herzog H et al. Impaired thrombin generation in beta 2-glycoprotein I null mice. J Biol Chem. 2001;276:13817-13821.

(10) Schousboe I. beta 2-Glycoprotein I: a plasma inhibitor of the contact activation of the intrinsic blood coagulation pathway. Blood. 1985;66:1086-1091.

(11) Nimpf J, Wurm H, Kostner GM. Beta 2-glycoprotein-I (apo-H) inhibits the release reaction of human platelets during ADP-induced aggregation. Atherosclerosis. 1987;63:109-114.

(12) Ruggeri ZM. Von Willebrand factor, platelets and endothelial cell interactions. J Thromb Haemost. 2003;1:1335-1342.

(13) Huizinga EG, Tsuji S, Romijn RA et al. Structures of glycoprotein Ibalpha and its complex with von Willebrand factor A1 domain. Science. 2002;297:1176-1179.

(14) Berndt MC, Du XP, Booth WJ. Ristocetin-dependent reconstitution of binding of von Willebrand factor to purified human platelet membrane glycoprotein Ib-IX complex. Biochemistry. 1988;27:633-640.

(15) Scott JP, Montgomery RR, Retzinger GS. Dimeric ristocetin flocculates proteins, binds to platelets, and mediates von Willebrand factor-dependent agglutination of platelets. J Biol Chem. 1991;266:8149-8155.

(16) Oosting JD, Derksen RH, Hackeng TM et al. In vitro studies of antiphospholipid antibodies and its cofactor, beta 2-glycoprotein I, show negligible effects on endothelial cell mediated protein C activation. Thromb Haemost. 1991;66:666-671.

(17) Sodetz JM, Pizzo SV, McKee PA. Relationship of sialic acid to function and in vivo survival of human factor VIII/von Willebrand factor protein. J Biol Chem. 1977;252:5538-5546.

(18) McNeeley PA, Dlott JS, Furie RA et al. Beta2-glycoprotein I-dependent anticardiolipin antibodies preferentially bind the amino terminal domain of beta2-glycoprotein I. Thromb Haemost. 2001;86:590-595.

(19) Lankhof H, Damas C, Schiphorst ME et al. von Willebrand factor without the A2 domain is resistant to proteolysis. Thromb Haemost. 1997;77:1008-1013.

(20) Lankhof H, van Hoeij M, Schiphorst ME et al. A3 domain is essential for interaction of von Willebrand factor with collagen type III. Thromb Haemost. 1996;75:950-958.

(21) Lenting PJ, Westein E, Terraube V et al. An experimental model to study the in vivo survival of von Willebrand factor. Basic aspects and application to the R1205H mutation. J Biol Chem. 2004;279:12102-12109.

(22) Romijn RA, Westein E, Bouma B et al. Mapping the collagen-binding site in the von Willebrand factor-A3 domain. J Biol Chem. 2003;278:15035-15039.

(23) Pietu G, Ribba AS, Cherel G et al. Epitope mapping of inhibitory monoclonal antibodies to human von Willebrand factor by using recombinant cDNA libraries. Thromb Haemost. 1994;71:788-792.

(24) Hulstein JJ, de Groot PG, Silence K et al. A novel nanobody that detects the gain-of-function phenotype of von Willebrand factor in ADAMTS13 deficiency and von Willebrand disease type 2B. Blood. 2005;106:3035-3042.

(25) Lutters BC, Derksen RH, Tekelenburg WL et al. Dimers of beta 2-glycoprotein I increase platelet deposition to collagen via interaction with phospholipids and the apolipoprotein E receptor 2'. J Biol Chem. 2003;278:33831-33838.

(26) Horbach DA, van Oort E, Donders RC, Derksen RH, de Groot PG. Lupus anticoagulant is the strongest risk factor for both venous and arterial thrombosis in patients with systemic lupus erythematosus. Comparison between different assays for the detection of antiphospholipid antibodies. Thromb Haemost. 1996;76:916-924.

(27) Borchiellini A, Fijnvandraat K, ten Cate JW et al. Quantitative analysis of von Willebrand factor propeptide release in vivo: effect of experimental endotoxemia and administration of 1-deamino-8-D-arginine vasopressin in humans. Blood. 1996;88:2951-2958.

(28) Kokame K, Nobe Y, Kokubo Y, Okayama A, Miyata T. FRETS-VWF73, a first fluorogenic substrate for ADAMTS13 assay. Br J Haematol. 2005;129:93-100.

(29) van Lier M, Lee F, Farndale RW et al. Adhesive surface determines raft composition in platelets adhered under flow. J Thromb Haemost. 2005;3:2514-2525.

(30) Lisman T, Moschatsis S, Adelmeijer J, Nieuwenhuis HK, de Groot PG. Recombinant factor VIIa enhances deposition of platelets with congenital or acquired alpha IIb beta 3 deficiency to endothelial cell matrix and collagen under conditions of flow via tissue factor-independent thrombin generation. Blood. 2003;101:1864-1870.

(31) Bancsi LF, van dL, I, Bertina RM. Beta 2-glycoprotein I deficiency and the risk of thrombosis. Thromb Haemost. 1992;67:649-653.

(32) Hasselaar P, Derksen RH, Blokzijl L et al. Risk factors for thrombosis in lupus patients. Ann Rheum Dis. 1989;48:933-940.

5

Chapter 5

FRETS-VWF73: a rapid and predictive tool for thrombotic thrombocytopenic purpura

Evelyn Groot
Janine J.J. Hulstein
Chantal N. Rison
Philip G. de Groot
Rob Fijnheer

J Thromb Haemost. 2006;4(3):698-9

Thrombotic thrombocytopenic purpura (TTP) is a rare disease characterized by thrombocytopenia and microangiopathic hemolytic anemia. Acute TTP manifests itself by fever, neurological dysfunction and renal insufficiency. It can present either as primary TTP, TTP without any demonstrable causes, or as a secondary effect of underlying diseases like sepsis, malignant hypertension, cancer, eclampsia, bone marrow transplantation and the hemolytic-uremic syndrome (HUS).[1-3] Plasma exchange reduced the mortality rate of primary TTP from 90% to approximately 20%[4], but survival is occasionally associated with severe complications.[5] Furthermore, plasma exchange is not always effective in other diseases bearing TTP symptoms.[2,3] As the diagnosis 'thrombocytopenia and hemolytic anemia' does not distinguish TTP from other diseases, additional criteria are necessary to be able to discriminate between both in order to start a tailor-made treatment rapidly.

The efficacy of plasma exchange was elucidated by the discovery that TTP patients are deficient in the Von Willebrand factor (VWF) cleaving protease[6] that was later found to be a member of the metalloprotease family named ADAMTS13[7]. Plasma exchange is thought to supply patients with ADAMTS13 and/or remove neutralizing autoantibodies against ADAMTS13. ADAMTS13 regulates the size of Von Willebrand factor (VWF) and its deficiency results in unusually large multimers of VWF. These ultra-large (UL-)VWF multimers can induce thrombosis by platelet agglutination. It was suggested that severe ADAMTS13 deficiency (<5-10%) is a good additional criterion for TTP, although the specificity and sensitivity of ADAMTS13 deficiency for TTP remains controversial.[8-10]

Several assays have been developed for detection of ADAMTS13 in plasma, among which the collagen-binding assay[11], the ristocetin-cofactor assay[12] and the proteolytic multimer assay[2]. In the latter, patient plasma is incubated with VWF and VWF multimers are separated by SDS-agarose followed by immunoblotting with anti-VWF antibodies. ADAMTS13 activity is taken to be absent in case UL-VWF multimers are still visible after proteolysis and present if the UL-VWF multimers are not visible anymore after proteolysis. With the introduction of the FRETS-VWF73 assay, a more rapid method became available that facilitates quantitative measurement of ADAMTS13 activity within 1 h.[13] A fluorescent signal is detected when the substrate comprising 73 amino acids of the VWF A2 domain is cleaved by ADAMTS13 in the patient plasma. ADAMTS13 activity is now expressed in terms of percentage

Table I Results of the proteolytic multimer assay and the FRETS-VWF73 assay regarding 79 patients who were considered TTP in the differential diagnosis stage. The final diagnosis was made by the treating physician without prior knowledge of the results of these assays. ADAMTS13 activity in terms of percentage was based on the results of the FRETS-VWF73 assay.

Final diagnosis upon hospital dismissal or death (number of patients)	Number of patients deficient in ADAMTS13 activity in proteolytic multimer assay (corresponding FRETS-VWF73 activity)	Number of patients demonstrating ADAMTS13 activity in proteolytic multimer assay (corresponding FRETS-VWF73 activity)
TTP (n = 22)	19 (0%)	3 (53-75%)
Pregnancy-related TTP (n = 5)	5 (0%)	0
HUS (n=8)	0	8 (30-95%)
TTP bone marrow transplantation (n = 11)	0	11 (32-83%)
Sepsis (n = 4)	0	4 (3-61%)
Malign hypertension (n = 4)	0	4 (31-74%)
HELLP (n = 8)	0	8 (35-87%)
Cancer (n = 7)	0	7 (28-103%)
Renal insufficiency (n = 4)	0	4 (25-48%)
Other diseases (n = 6)	0	6 (41-95%)
Total number of patients	24	55

compared to the activity found in normal pooled plasma. Such a rapid method, when proven valuable, can become of great importance for the adjustment of proper treatment of patients.

We performed a prospective study in 79 patients with Coomb's negative hemolytic anemia and thrombocytopenia, where TTP was considered. Plasma samples were collected before the onset of plasma exchange and tested for the presence of ADAMTS13 activity with both the proteolytic multimer assay and the FRETS-VWF73 assay. Plasma exchange was started independently of this result and the final diagnosis, TTP or an underlying disease causing thrombocytopenia and hemolytic anemia, was made by the treating physician upon hospital dismissal or death. Table 1 presents the results of the two ADAMTS13 activity assays in the 79 patients that were considered TTP in the differential diagnosis stage. Upon hospital dismissal or death, 22 patients were diagnosed as primary TTP, 5 as pregnancy related TTP, 8 as HUS, 11 as TTP after bone marrow transplantation, 4 as sepsis, 4 as malignant hypertension, 8 as hemolysis, elevated liver enzymes and low platelets (HELLP), 7 as cancer, 4 as renal insufficiency and another 6 as other diseases. The diagnosis was set without prior knowledge of the ADAMTS13 results.

Twenty-four of the 27 patients (89%) that were diagnosed as primary TTP or pregnancy-related TTP did not show VWF proteolysis in the proteolytic multimer assay. In the FRETS-VWF73 assay, activity in these 24 patients was found to be 0%. Three primary TTP patients (11%), who had normal proteolysis in the proteolytic multimer assay, appeared to have activities varying from 53%-75% in the FRETS-VWF73 assay. In the other 55 patients, where thrombocytopenia and hemolytic anemia was caused by underlying diseases, normal VWF proteolysis was found in the proteolytic multimer assay. Here, the activity in the FRETS-VWF73 assay was found to be between 25 and 103%. One patient with sepsis and normal proteolysis in the proteolytic multimer assay had an activity of 3% in the FRETS-VWF73 assay.

To summarize, we found that absence of ADAMTS13 activity was 89% sensitive and 100% specific for TTP. This was concluded from both the labour-intensive proteolytic multimeric assay and the rapid FRETS-VWF73 assay, pointing at a 100% consistency between these two tests. In our opinion, the easy use and rapidity of FRETS-VWF73 have made it to a valuable and predictive tool in TTP diagnostics.

Reference List

(1) Schwartz ML, Brenner WE. The obfuscation of eclampsia by thrombotic thrombocytopenic purpura. Am J Obstet Gynecol. 1978;131:18-24.

(2) van der Plas RM, Schiphorst ME, Huizinga EG et al. von Willebrand factor proteolysis is deficient in classic, but not in bone marrow transplantation-associated, thrombotic thrombocytopenic purpura. Blood. 1999;93:3798-3802.

(3) Ruggenenti P, Remuzzi G. Pathophysiology and management of thrombotic microangiopathies. J Nephrol. 1998;11:300-310.

(4) Rock GA, Shumak KH, Buskard NA et al. Comparison of plasma exchange with plasma infusion in the treatment of thrombotic thrombocytopenic purpura. Canadian Apheresis Study Group. N Engl J Med. 1991;325:393-397.

(5) Rizvi MA, Vesely SK, George JN et al. Complications of plasma exchange in 71 consecutive patients treated for clinically suspected thrombotic thrombocytopenic purpura-hemolytic-uremic syndrome. Transfusion. 2000;40:896-901.

(6) Furlan M, Robles R, Solenthaler M et al. Deficient activity of von Willebrand factor-cleaving protease in chronic relapsing thrombotic thrombocytopenic purpura. Blood. 1997;89:3097-3103.

(7) Fujikawa K, Suzuki H, McMullen B, Chung D. Purification of human von Willebrand factor-cleaving protease and its identification as a new member of the metalloproteinase family. Blood. 2001;98:1662-1666.

(8) Loof AH, van Vliet HH, Kappers-Klunne MC. Low activity of von Willebrand factor-cleaving protease is not restricted to patients suffering from thrombotic thrombocytopenic purpura. Br J Haematol. 2001;112:1087-1088.

(9) Bianchi V, Robles R, Alberio L, Furlan M, Lammle B. Von Willebrand factor-cleaving protease (ADAMTS13) in thrombocytopenic disorders: a severely deficient activity is specific for thrombotic thrombocytopenic purpura. Blood. 2002;100:710-713.

(10) Lammle B, Kremer Hovinga JA, Alberio L. Thrombotic thrombocytopenic purpura. J Thromb Haemost. 2005;3:1663-1675.

(11) Gerritsen HE, Turecek PL, Schwarz HP, Lammle B, Furlan M. Assay of von Willebrand factor (vWF)-cleaving protease based on decreased collagen binding affinity of degraded vWF: a tool for the diagnosis of thrombotic thrombocytopenic purpura (TTP). Thromb Haemost. 1999;82:1386-1389.

(12) Bohm M, Vigh T, Scharrer I. Evaluation and clinical application of a new method for measuring activity of von Willebrand factor-cleaving metalloprotease (ADAMTS13). Ann Hematol. 2002;81:430-435.

(13) Kokame K, Nobe Y, Kokubo Y, Okayama A, Miyata T. FRETS-VWF73, a first fluorogenic substrate for ADAMTS13 assay. Br J Haematol. 2005;129:93-100.

6

Chapter 6

General discussion

Science never solves a problem without creating ten more.

George Bernard Shaw

Binding of von Willebrand factor (VWF) to the subendothelium at a site of injury is the first step in thrombus formation at higher shear stresses. To prevent spontaneous binding of VWF to platelets, VWF circulates in a globular, inactive conformation. If VWF would circulate in an active conformation, spontaneous binding of VWF to platelets could occur, resulting in thrombosis and consumptive thrombocytopenia. Therefore, conversion of VWF into an active conformation must be subjected to regulatory mechanisms. In this thesis we have studied the regulation of VWF activation in four diseases associated with thrombocytopenia; von Willebrand disease (VWD) type 2B, thrombotic Thrombocytopenic purpura (TTP), the syndrome of hemolysis, elevated liver enzymes and low platelets (HELLP) and the antiphospholipid syndrome.

Active VWF

VWF circulates in a resting conformation, unable to bind to the Glycoprotein (Gp)Ib-IX-V receptor complex on platelets.[1] GpIbα and/or the A1-domain of VWF have to be "activated" in order to induce an interaction. Crystallographic analysis revealed that the interaction between GpIbα and the A1-domain of VWF involves 2 contact sites: (1) a large area involving leucine-rich repeats 5-8, the ß-switch and C-terminal flank of GpIbα and the α3-helix, α3ß4-loop and ß4-strand of the A1-domain and (2) a smaller contact site composed of the N-terminal ß-hairpin and the first leucine-rich repeat of GpIbα and loops α1ß2, ß3α2 and α3ß4 at the bottom-face of the A1-domain (Figure 1).[1]

GpIb and the A1-domain are present in an active conformation in platelet-type VWD and VWD type 2B, due to mutations in either GpIb or A1.[2-4] Studies regarding these mutations have provided more insight into the conformational changes necessary to induce a stable interaction between VWF and GpIbα. Gain-of-function mutations related to platelet-type VWD are all found in the ß-switch region of GpIbα and induce stabilization of ß-hairpin structures, resulting in an increased association of GpIbα to VWF.[1,5] The gain-of-function mutations in VWD type 2B are clustered at the bottom-face of A1[1], adjacent to the site that interacts with the ß-finger of GpIbα. Dumas and coworkers have suggested that VWD type 2B mutations R1306Q and I1546V induce a number of conformational changes mainly by changing the orientation of the α1ß2-loop in the A1-domain (Figure 2).[5]

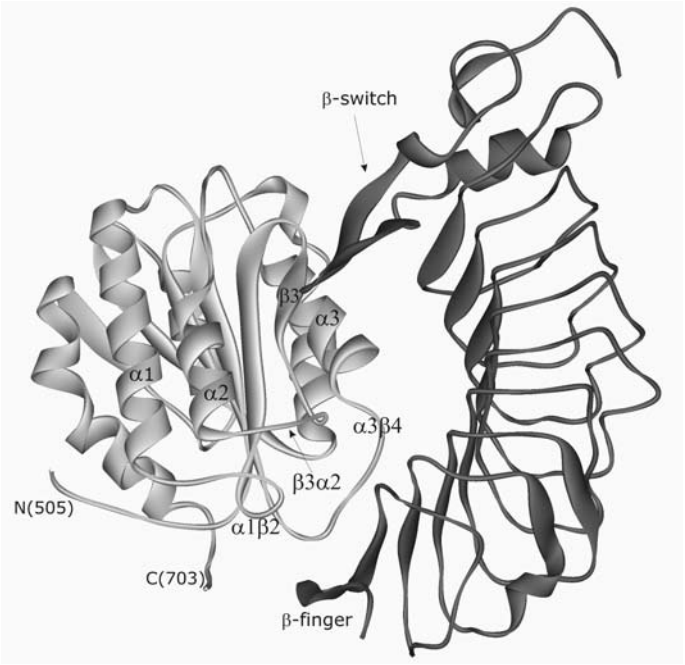

Figure 1: Structures of the complex of GpIbα-M 239V with A1-R1306Q. Ribbon presentation of the GpIbα-A1 complex. GpIbα is shown in dark gray and the A1-domain of VWF in light gray. The N-terminal ß-hairpin called ß-finger of GpIbα and the C-terminal flanking region, called ß-switch, are important in the contact with the A1-domain. The ß-switch of GpIbα adopts a ß-hairpin structure that aligns with the central ß-sheet of A1. The different regions of the A1-domain that are involved in complex formation are also indicated. (Figure adapted from Huizinga et al. Science 2002)

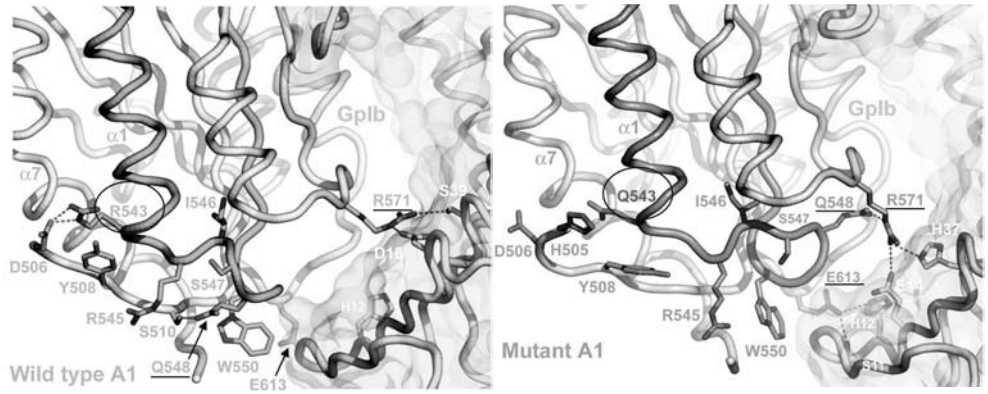

Figure 2: Comparison of GpIbα-A1 and GpIbα-M239V/A1-R1306Q, highlighting rearrangement of the α1β2-loop region of the A1-domain. Mutated amino acid 1306 is encircled. The conformational change is mainly visible in the α1ß2-loop region of the A1-domain. This significantly changes the position of the underlined amino acids A548, R571 and E613. Hydrogen bonds are indicated by dashed lines. (Figure adapted from Dumas et al. J Biol Chem. 2004)

Measurement of active VWF

The amount of VWF in circulation that can be activated in vitro, is normally measured by determining the ristocetin cofactor activity. In chapter 2, a novel llama-derived antibody fragment is described, developed to recognize the active conformation of VWF directly. The affinity of this novel nanobody, AU/VWFa-11, for the A1-domain of VWF increases 2-fold upon introduction of the R1306Q mutation in the A1-domain (Chapter 2). Since this mutation also promotes strong binding of the A1-domain to GpIbα, one might expect that GpIbα and AU/VWFa-11 share the same binding site on VWF. However, GpIbα and AU/VWFa-11 can interact simultaneously with VWF and nanobody AU/VWFa-11 is unable to interfere with the interaction between VWF and GpIbα. This indicates that more than one new epitope is revealed upon activation of the A1-domain.

Using AU/VWFa-11, active VWF was detected in plasma of patients with VWD type 2B. The amount of active VWF, expressed as the "Activation Factor", was found to vary considerably between patients. Interestingly, the activation factor correlated with platelet counts in VWD type 2B patients, a finding that has recently been confirmed by Federici and colleagues (personal communication). Increased amounts of active VWF were also found in TTP, HELLP syndrome and the antiphospholipid syndrome, disorders characterized among others by thrombocytopenia. These data suggest that the presence of active VWF is directly involved in the onset of thrombocytopenia.

Regulation of the amount of active VWF in circulation

An intriguing issue is whether activation of VWF is a reversible or an irreversible process. We showed that the activation factor of VWF released from the Weibel-palade bodies is 1.4-fold increased compared to the activation factor of VWF derived from the constitutive pathway, and 2.3-fold as compared to VWF in circulation (Chapter 3). Partially activated VWF secreted from the Weibel-palade bodies apparently becomes inactivated and circulates in a resting state until it is activated again at a site of injury. This would suggest that activation of VWF is a reversible process.

VWF activation is induced by binding of VWF to exposed subendothelial collagen. This indicates that the interaction of the A3-domain of VWF with collagen could influence the conformation of the A1-domain. Another region that seems to influence the activity of the A1-domain is the D'D3-region. It has been suggested that this region folds over the A1-domain of VWF, thereby shielding the GpIbα-binding site of the domain.[6] Apparently, shielding becomes disrupted by VWF immobilization onto collagen or non-physiological surfaces such as glass or plastic. We found that the affinity of AU/VWFa-11 for different size variants of the A1-domain was notably different (chapter 2). The longest A1-fragment, containing a part of the neighboring D'D3-region was bound with a K_D of 1.7 μM. The affinity was improved 20-fold, to a K_D of 81 nM upon removal of the flanking peptides, suggesting that the domains flanking the A1-domain may indeed play a role in exposure of the GpIbα-binding site of VWF. This is consistent with data showing that recombinant A1-domains lacking the N-terminal extension bind GpIbα with 5- to 10-fold increased affinity.[7] Moreover, several type 2B mutations have been described in the proximity of the A1-termini. It has been proposed that these mutations induce efficient binding of the A1-domain to GpIbα by altering the conformation of the N-and C-terminus. This would again suggests that the termini of the A1-domain regulate exposure of the GpIbα-binding site.[1,8]

If activation itself would be reversible without intervention by other factors, one would expect a balance between the inactive and the active state. Binding of the nanobody to the active conformation might then retain VWF in the GpIbα-binding conformation. The activated VWF would be able to support platelet agglutination without the addition of ristocetin. To investigate this, purified plasma derived (pd-) VWF was incubated with different concentrations AU/VWFa-11 at 37°C for 30 minutes, after which the preincubated VWF was added to washed platelets and platelet aggregation was measured using an optical aggregometer. In a pilot study we found that even a 340-fold excess of nanobody could not retain VWF in its active conformation (data not shown). Moreover, the activity of VWF in medium of stimulated endothelial cells did not decrease in time. These data suggest that the conformational change of VWF is not reversible without intervention by other factors. However, the exact mechanism of VWF activation and if this is a reversible or irreversible process remains an important issue to study in more detail.

ADAMTS13

Proteolysis of VWF into smaller multimers is thought to be one of the mechanisms by which the activity of VWF is down-regulated.[9] Non-proteolysed ultralarge (UL-)VWF multimers are biologically more active and spontaneously form high strength bonds with GpIbα.[10] The mechanism behind this increased biological activity remains unknown, although it has been suggested that the conformational state of UL-VWF multimers is more critical than their size.[10] This suggestion is based on the observation that UL-VWF multimers indeed form high strength bonds with GpIbα, but the strength of the individual bonds is similar for the isolated A1-domain. The question remains if UL-VWF multimers unfold more easily due to their size, or if another mechanisms determine the increased activity of the UL-VWF multimers.

ADAMTS13 (A Disintegrin And Metalloprotease with ThromboSpondin motif) cleaves a single peptide bond between tyrosine 1605 and methionine 1606 in the A2 domain of VWF. The susceptibility of the A2-domain for proteolysis depends on unfolding of the VWF molecule. Several reports have suggested that the conformation of the A1-domain strongly influences this susceptibility. Incubation of VWF with heparin or ristocetin might promote proteolysis and Nishio and coworkers have shown that binding of GpIbα to the A1-domain enhances proteolysis significantly.[11,12] This phenomenon might explain the differences in clinical manifestations observed in VWD type 2B and TTP. Although in both diseases VWF interacts spontaneously with platelets, TTP is associated with thrombosis in the microvasculature, whereas VWD type 2B is a bleeding diathesis that is rarely associated with thrombosis. The combination of A1 in its active conformation and the interaction with GpIb in VWD type 2B, may enhance susceptibility of the A2-domain for proteolysis by ADAMTS13, resulting in digestion of VWF bound to platelets. Moreover, platelet-VWF complexes might be removed more rapidly from the circulation. Proteolysis and clearance possibly prevent thrombosis in VWD type 2B. In TTP, the more reactive UL-VWF multimers also bind spontaneously to platelets. However, due to a deficiency of ADAMTS13 the VWF-platelet complexes cannot be removed from the circulation, platelet-platelet interaction will take place and VWF-rich thrombi are formed in the microvasculature.

The discovery that ADAMTS13 plays an important role in the onset of TTP was a major step forwards in the understanding of the disease. This discovery also initiated a search for rapid in vitro assays to determine the activity of ADAMTS13 in patient

plasma. Most assays start with incubation of a recombinant VWF with patient plasma in the presence of barium chloride and denaturants, such as urea. After digestion, the amount of multimers is analyzed using different methods.[13-17] Two multicenter comparison studies have been performed, in which the different methods were evaluated.[18,19] Reproducibility of most assays was found to be low and inter-laboratory agreement was poor. Only samples with severely reduced ADAMTS13 activity showed good inter-assay and inter-laboratory reproducibility. More recently, a fluorogenic substrate for ADAMTS13 has been developed, comprising 73 amino acids of the A2-domain of VWF.[20] With the introduction of this assay a more rapid method became available that facilitated quantitative measurement. This method has been compared to various other assays[21-23], amongst others to the multimer-analysis using SDS-agarose gels (Chapter 5). All studies showed that the new assay exhibited very reliable results to detect low, moderately reduced and normal levels of ADAMTS13. The accuracy and reproducibility of the new FRETS-VWF73 assay in combination with the fact that it can be performed within 1 hour, makes it an appropriate method for screening of patients with thrombotic microangiopathy. Moreover, the quantitative measurements also allowed to determine normal ranges of ADAMTS13 activity (61-141% activity), which makes it possible to get more insight in the mechanism underlying TTP. Absence of activity in the FRETS-assay was found to be 89% sensitive and 100% specific for TTP (Chapter 5). However, inter-laboratory reproducibility is an important issue that has to be addressed in the coming years. In the mean time, other tests for ADAMTS13 activity will be developed and assays for ADAMTS13 antigen levels and inhibitor levels might also be implicated in diagnostics of TTP.

A marginal comment at all the assays that measure ADAMTS13 activity, is the fact that they are all performed under static conditions. The influence of factors on for instance docking of ADAMTS13 onto VWF under flow conditions can therefore not be found. Not only the presence of certain inhibitory antibodies could be missed, but also the influence of a factor like cytokine IL-6. This cytokine has been suggested to influence ADAMTS13 activity only under flow conditions[24], possibly by interfering with the docking of ADAMTS13 to VWF under flow. Although an effect of IL-6 on VWF proteolysis has been suggested based on clinical observations[25], this effect has

not been confirmed in *in vitro* assays. Since IL-6 is increased in for instance HELLP syndrome, the actual activity of ADAMTS13 in these patients might be overestimated using the static assays.

Endothelial cell activation

VWF is secreted from endothelial cells via two separate pathways: the constitutive pathway and the regulated pathway. VWF from the regulated pathway is released upon stimulation or damage of the endothelial cell and is enriched in UL-VWF multimers. VWF from the constitutive pathway mainly contains lower VWF multimers that are less biologically active.[26,27] Release of the contents of Weibel-palade bodies is induced by numerous stimuli, including cytokines.[28] Endothelial cells are stimulated during infections and pregnancy, when the immune system is activated. In normal pregnancy, a slight activation of the endothelial cells is believed to add to a general procoagulant status. This is thought to be necessary to protect from massive blood loss during delivery.[29] An extra acute activation of the endothelium is present in HELLP syndrome (chapter 3).[30] During chronic activation, activity of released UL-VWF multimers can be re-balanced by adaptation of the velocity of proteolysis and/or clearance. Extra acute activation in HELLP syndrome might tip over the balance in favor of a highly procoagulant status. A similar trigger might also play a role in the onset of TTP. Infections and pregnancies have been mentioned many times as trigger for TTP episodes.[31] Moreover, a TTP-phenotype can be induced in ADAMTS13 knock-out mice by challenging the mice with Shigatoxin.[32] This suggests that endothelial cell activation is a very important step in the onset of VWF-mediated platelet consumption.

β_2-glycoprotein I

Next to conformational changes in VWF, proteolysis by ADAMTS13 and release of UL-VWF from the Weibel-palade bodies, we have demonstrated that β_2-glycoprotein (GP) I can also influence the function and amount of active VWF in circulation. We found that β_2-GPI binds specifically to the active GpIbα-binding conformation of VWF (Chapter 4). This property enables β_2-GPI to inhibit VWF induced platelet adhesion and aggregation. Antibodies recognizing β_2-GPI, found in patients with the antiphospholipid syndrome, neutralize this inhibitory function of β_2-GPI by preventing the interaction between β_2-GPI and VWF. Increased amounts of active VWF were found in plasma of patients with antiphospholipid syndrome caused by

anti-ß$_2$-GPI antibodies. These increased levels of active VWF could contribute to the onset of thrombosis and consumptive thrombocytopenia in the antiphospholipid syndrome. In this regard, it is interesting that the presence of a subpopulation of anti-ß$_2$-GPI antibodies, was also described to correlate best with thrombosis.[33]

ß$_2$-GPI levels range from 150-300 µg/ml in healthy individuals. Little is known about the levels of ß$_2$-GPI during inflammation or pregnancy. Recently, Lin and coworkers reported that ß$_2$-GPI levels negatively correlate with C-reactive protein, the largest reduction in ß$_2$-GPI occurring in patients with the highest levels of C-reactive protein.[34] Moreover, they found that levels of ß$_2$-GPI decrease significantly after the eighth week of pregnancy. This might suggest that inflammation or pregnancy not only triggers the release of active UL-VWF multimers form the endothelium, but also induces reduction of the protein that inhibits active VWF, ß$_2$-GPI. One might speculate that low levels of ß$_2$-GPI as compared to the levels of (active) VWF are an additional risk factor for consumptive thrombocytopenia and even for thrombosis in HELLP syndrome.

The triangle of endothelial cell activation, ADAMTS13 and β$_2$-GPI

Deficiency of ß$_2$-GPI alone does not induce the phenotype of antiphospholipid syndrome.[35,36] Also, deficiency of ADAMTS13 does not always result in clinical manifestations of TTP.[31] In some people ADAMTS13 activity remains strongly reduced or absent after an episode of TTP, without the presence of any symptoms. The same phenomenon is found in ADAMTS13 knock out mice.[32,37] Apparently, a second hit is necessary to induce the syndromes. This second hit might occur in inflammation or pregnancy. Under these circumstances, ß$_2$-GPI levels decrease and endothelial cells are activated and release the contents of the Weibel-palade bodies, including the active UL-VWF multimers. Moreover, activity of ADAMTS13 under flow conditions is inhibited by IL-6, which circulates during inflammation and is found to be increased in TTP, preeclampsia and HELLP syndrome.[38-40]

HELLP syndrome is often preceded by preecclampsia, but it remains unknown why some people develop this severe syndrome, whereas others do not. It might be possible to get more insight into this issue, when ß$_2$-GPI levels and amounts of active VWF

would be monitored in these patients. Individuals with low β_2-GPI levels might be more susceptible for HELLP syndrome or TTP. Challenges like pregnancy or inflammation might cause a disturbance of the balance between amounts of active VWF and levels of β_2-GPI, resulting in spontaneous platelet binding and thrombocytopenia.

The activation factor found for different patients with VWD type 2B varied considerably between patients (Chapter 2), although all patients were found to share the same mutation in the A1-domain (Arg1306Trp). Moreover, platelet counts vary in patients with VWD type 2B, even between patients with the same molecular defect. The amount of active VWF was found to correlate with platelet numbers. This indicates that the mutation is not the only determinant of the severity of the disease. The amount of active VWF and its effect on platelet count might depend on regulation of active VWF by for instance β_2-GPI. The phenotype of VWD type 2B induced by the same genotype might be more severe when β_2-GPI levels are more decreased. Severe thrombocytopenia during pregnancy might therefore also be ascribed to a fall in β_2-GPI levels starting in the eighth week, as described by Lin and coworkers.[34]

In patients with the antiphospholipid syndrome, the function of β_2-GPI is neutralized by the presence of antibodies, but inhibition of the function of β_2-GPI alone is not sufficient to explain the symptoms found in this syndrome. Under static conditions ADAMTS13 activity was found to be within the normal range (chapter 4). However, the activity might be inhibited when measured under flow conditions. Schinco and coworkers reported that UL-VWF multimers are present in patients with the antiphospholipid syndrome, which would suggest that proteolysis is impaired in patients with the antiphospholipid syndrome.[41] Interestingly, the presence of these UL-VWF multimers was found to correlate with the degree of thrombosis.[41] Moreover, binding of anti-β_2-GPI antibodies not only neutralizes the inhibitory function of β_2-GPI, but also induces new functions of the newly formed complex. The anti-β_2-GPI - β_2-GPI complexes have an increased affinity for platelets, endothelial cells and monocytes and binding of the complexes can induce activation of these celltypes.[42]

Implications for cardiovascular disease

Regulation of the amount of active VWF in circulation depends on a triangle: the amount of VWF released from the activated endothelium and regulation by ADAMTS13 activity and β_2-GPI (Figure 3). This triangle seems to be well regulated and loss of one of the inhibitory factors, ADAMTS13 or β_2-GPI, can probably be compensated

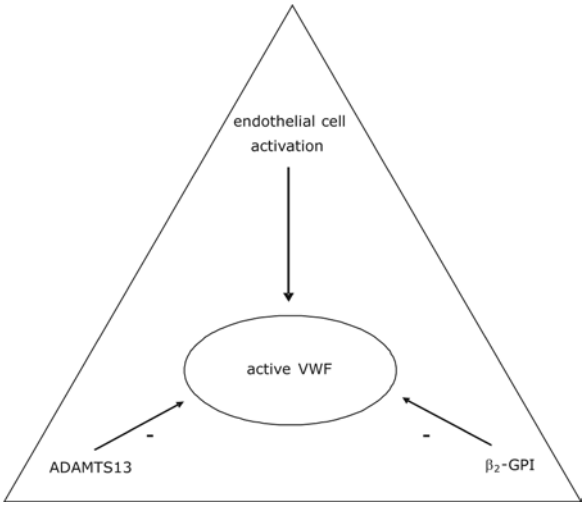

Figure 3: The regulating "triangle". Three components are thought to influence the activity of VWF: (1) The source of active VWF: endothelial cell activation, resulting in secretion of UL-VWF multimers from the Weibel-palade bodies and the regulatory factors: (2) proteolysis of VWF multimers by ADAMTS13 and (3) $ß_2$-GPI mediated inhibition of the function of active VWF.

by other factors. However, when one of the inhibitory factors is decreased, inhibited or deficient, a second hit may induce severe syndromes such as TTP, HELLP or the antiphospholipid syndrome. Imbalance in the system could also have implications for the development of other diseases associated with thrombocytopenia.

We found that increased amounts of active VWF in the antiphospholipid syndrome are associated with thrombosis (Chapter 4). In addition, it has been reported that thrombocytopenia is an independent risk factor for thrombo-embolic disease in the antiphospholipid syndrome.[43] Preeclampsia and HELLP syndrome are thought to be predictors of cardiovascular disease later in life.[44-46] The mechanisms underlying preeclampsia and HELLP syndrome, dysfunctional endothelium and inflammation, are also important in the onset of atherosclerosis.[47] Therefore, it is now assumed that preeclampsia, HELLP syndrome and atherosclerosis are manifestations of the same metabolic syndrome that predisposes women for these diseases. Increased levels of VWF have been described as a risk factor for cardiovascular disease.[48] However, a possible role for active VWF in the onset of cardiovascular disease has not been investigated in large epidemiologic studies yet. Considering our data, it might be

worthwhile to study the levels of active VWF and β_2-GPI in healthy individuals and patients with cardiovascular disease. Abnormal levels or a decreased ratio between β_2-GPI and active VWF, may indicate an increased risk for cardiovascular disease.

Reference List

(1) Huizinga EG, Tsuji S, Romijn RA et al. Structures of glycoprotein Ibalpha and its complex with von Willebrand factor A1 domain. Science. 2002;297:1176-1179.

(2) Nurden AT. Qualitative disorders of platelets and megakaryocytes. J Thromb Haemost. 2005;3:1773-1782.

(3) Ruggeri ZM, Pareti FI, Mannucci PM, Ciavarella N, Zimmerman TS. Heightened interaction between platelets and factor VIII/von Willebrand factor in a new subtype of von Willebrand's disease. N Engl J Med. 1980;302:1047-1051.

(4) Ruggeri ZM, Zimmerman TS. von Willebrand factor and von Willebrand disease. Blood. 1987;70:895-904.

(5) Dumas JJ, Kumar R, McDonagh T et al. Crystal structure of the wild-type von Willebrand factor A1-glycoprotein Ibalpha complex reveals conformation differences with a complex bearing von Willebrand disease mutations. J Biol Chem. 2004;279:23327-23334.

(6) Ulrichts H, Udvardy M, Lenting PJ et al. Shielding of the A1 domain by the D'D3 domains of von Willebrand factor modulates its interaction with platelet glycoprotein Ib-IX-V. J Biol Chem. 2006;281:4699-4707.

(7) Sugimoto M, Dent J, McClintock R, Ware J, Ruggeri ZM. Analysis of structure-function relationships in the platelet membrane glycoprotein Ib-binding domain of von Willebrand's factor by expression of deletion mutants. J Biol Chem. 1993;268:12185-12192.

(8) Emsley J, Cruz M, Handin R, Liddington R. Crystal structure of the von Willebrand Factor A1 domain and implications for the binding of platelet glycoprotein Ib. J Biol Chem. 1998;273:10396-10401.

(9) Dong JF, Moake JL, Nolasco L et al. ADAMTS-13 rapidly cleaves newly secreted ultralarge von Willebrand factor multimers on the endothelial surface under flowing conditions. Blood. 2002;100:4033-4039.

(10) Arya M, Anvari B, Romo GM et al. Ultralarge multimers of von Willebrand factor form spontaneous high-strength bonds with the platelet glycoprotein Ib-IX complex: studies using optical tweezers. Blood. 2002;99:3971-3977.

(11) Bowen DJ. An influence of ABO blood group on the rate of proteolysis of von Willebrand factor by ADAMTS13. J Thromb Haemost. 2003;1:33-40.

(12) Nishio K, Anderson PJ, Zheng XL, Sadler JE. Binding of platelet glycoprotein Ibalpha to von Willebrand factor domain A1 stimulates the cleavage of the adjacent domain A2 by ADAMTS13. Proc Natl Acad Sci U S A. 2004;101:10578-10583.

(13) Furlan M, Robles R, Lamie B. Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. Blood. 1996;87:4223-4234.

(14) Tsai HM. Physiologic cleavage of von Willebrand factor by a plasma protease is dependent on its conformation and requires calcium ion. Blood. 1996;87:4235-4244.

(15) Obert B, Tout H, Veyradier A et al. Estimation of the von Willebrand factor-cleaving protease in plasma using monoclonal antibodies to vWF. Thromb Haemost. 1999;82:1382-1385.

(16) Gerritsen HE, Turecek PL, Schwarz HP, Lammle B, Furlan M. Assay of von Willebrand factor (vWF)-cleaving protease based on decreased collagen binding affinity of degraded vWF: a tool for the diagnosis of thrombotic thrombocytopenic purpura (TTP). Thromb Haemost. 1999;82:1386-1389.

(17) Bohm M, Vigh T, Scharrer I. Evaluation and clinical application of a new method for measuring activity of von Willebrand factor-cleaving metalloprotease (ADAMTS13). Ann Hematol. 2002;81:430-435.

(18) Studt JD, Bohm M, Budde U et al. Measurement of von Willebrand factor-cleaving protease (ADAMTS-13) activity in plasma: a multicenter comparison of different assay methods. J Thromb Haemost. 2003;1:1882-1887.

(19) Tripodi A, Chantarangkul V, Bohm M et al. Measurement of von Willebrand factor cleaving protease (ADAMTS-13): results of an international collaborative study involving 11 methods testing the same set of coded plasmas. J Thromb Haemost. 2004;2:1601-1609.

(20) Kokame K, Nobe Y, Kokubo Y, Okayama A, Miyata T. FRETS-VWF73, a first fluorogenic substrate for ADAMTS13 assay. Br J Haematol. 2005;129:93-100.

(21) Mahdian R, Rayes J, Girma JP et al. Comparison of FRETS-VWF73 to full-length VWF as a substrate for ADAMTS13 activity measurement in human plasma samples. Thromb Haemost. 2006;95:1049-1051.

(22) Kremer Hovinga JA, Mottini M, Lammle B. Measurement of ADAMTS-13 activity in plasma by the FRETS-VWF73 assay: comparison with other assay methods. J Thromb Haemost. 2006;4:1146-1148.

(23) Shelat SG, Smith P, Ai J, Zheng XL. Inhibitory autoantibodies against ADAMTS-13 in patients with thrombotic thrombocytopenic purpura bind ADAMTS-13 protease and may accelerate its clearance in vivo. J Thromb Haemost. 2006;4:1707-1717.

(24) Wada H, Kaneko T, Ohiwa M et al. Plasma cytokine levels in thrombotic thrombocytopenic purpura. Am J Hematol. 1992;40:167-170.

(25) Levy GG, Nichols WC, Lian EC et al. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. Nature. 2001;413:488-494.

(26) Sporn LA, Marder VJ, Wagner DD. Inducible secretion of large, biologically potent von Willebrand factor multimers. Cell. 1986;46:185-190.

(27) Sporn LA, Marder VJ, Wagner DD. von Willebrand factor released from Weibel-Palade bodies binds more avidly to extracellular matrix than that secreted constitutively. Blood. 1987;69:1531-1534.

(28) Bernardo A, Ball C, Nolasco L, Moake JF, Dong JF. Effects of inflammatory cytokines on the release and cleavage of the endothelial cell-derived ultralarge von Willebrand factor multimers under flow. Blood. 2004;104:100-106.

(29) Brenner B. Haemostatic changes in pregnancy. Thromb Res. 2004;114:409-414.

(30) Redman CW, Sacks GP, Sargent IL. Preeclampsia: an excessive maternal inflammatory response to pregnancy. Am J Obstet Gynecol. 1999;180:499-506.

(31) Furlan M, Lammle B. Aetiology and pathogenesis of thrombotic thrombocytopenic purpura and haemolytic uraemic syndrome: the role of von Willebrand factor-cleaving protease. Best Pract Res Clin Haematol. 2001;14:437-454.

(32) Motto DG, Chauhan AK, Zhu G et al. Shigatoxin triggers thrombotic thrombocytopenic purpura in genetically susceptible ADAMTS13-deficient mice. J Clin Invest. 2005;115:2752-2761.

(33) de Laat HB, Derksen RH, Urbanus RT, Roest M, de Groot PG. beta2-glycoprotein I-dependent lupus anticoagulant highly correlates with thrombosis in the antiphospholipid syndrome. Blood. 2004;104:3598-3602.

(34) Lin F, Murphy R, White B et al. Circulating levels of beta2-glycoprotein I in thrombotic disorders and in inflammation. Lupus. 2006;15:87-93.

(35) Sheng Y, Reddel SW, Herzog H et al. Impaired thrombin generation in beta 2-glycoprotein I null mice. J Biol Chem. 2001;276:13817-13821.

(36) Yasuda S, Tsutsumi A, Chiba H et al. beta(2)-glycoprotein I deficiency: prevalence, genetic background and effects on plasma lipoprotein metabolism and hemostasis. Atherosclerosis. 2000;152:337-346.

(37) Banno F, Kokame K, Okuda T et al. Complete deficiency in ADAMTS13 is prothrombotic, but it alone is not sufficient to cause thrombotic thrombocytopenic purpura. Blood. 2006;107:3161-3166.

(38) Wada H, Kaneko T, Ohiwa M et al. Plasma cytokine levels in thrombotic thrombocytopenic purpura. Am J Hematol. 1992;40:167-170.

(39) Vince GS, Starkey PM, Austgulen R, Kwiatkowski D, Redman CW. Interleukin-6, tumour necrosis factor and soluble tumour necrosis factor receptors in women with pre-eclampsia. Br J Obstet Gynaecol. 1995;102:20-25.

(40) Madazli R, Aydin S, Uludag S, Vildan O, Tolun N. Maternal plasma levels of cytokines in normal and preeclamptic pregnancies and their relationship with diastolic blood pressure and fibronectin levels. Acta Obstet Gynecol Scand. 2003;82:797-802.

(41) Schinco P, Borchiellini A, Tamponi G et al. Lupus anticoagulant and thrombosis: role of von Willebrand factor multimeric forms. Clin Exp Rheumatol. 1997;15:5-10.

(42) de Groot PG, Derksen RH. Pathophysiology of the antiphospholipid syndrome. J Thromb Haemost. 2005;3:1854-1860.

(43) Hasselaar P, Derksen RH, Blokzijl L et al. Risk factors for thrombosis in lupus patients. Ann Rheum Dis. 1989;48:933-940.

(44) Smith GC, Pell JP, Walsh D. Pregnancy complications and maternal risk of ischaemic heart disease: a retrospective cohort study of 129,290 births. Lancet. 2001;357:2002-2006.

(45) Irgens HU, Reisaeter L, Irgens LM, Lie RT. Long term mortality of mothers and fathers after pre-eclampsia: population based cohort study. BMJ. 2001;323:1213-1217.

(46) Agatisa PK, Ness RB, Roberts JM et al. Impairment of endothelial function in women with a history of preeclampsia: an indicator of cardiovascular risk. Am J Physiol Heart Circ Physiol. 2004;286:H1389-H1393.

(47) Roberts JM, Taylor RN, Musci TJ et al. Preeclampsia: an endothelial cell disorder. Am J Obstet Gynecol. 1989;161:1200-1204.

(48) Vischer UM. von Willebrand factor, endothelial dysfunction, and cardiovascular disease. J Thromb Haemost. 2006;4:1186-1193.

Chapter 7

Nederlandse samenvatting

In ons bloed circuleren verschillende cellen, zoals rode en witte bloedlichaampjes en cellen zonder kern, die bloedplaatjes genoemd worden. Bloedplaatjes spelen een belangrijke rol bij het stelpen van een bloeding door op de plaats van een wond te hechten aan de beschadigde vaatwand. Hier vormen ze een propje dat de bloeding stopt.

Normaal hebben mensen tussen de 115 en 300 miljard bloedplaatjes per liter bloed. Als dit aantal sterk daalt en onder de 10 miljard per liter komt, ontstaat een groot risico op spontane bloedingen. Een daling in bloedplaatjesaantal kan ontstaan als er in korte tijd heel veel bloedplaatjes gebruikt worden.

In dit proefschrift hebben we vier verschillende ziekten bestudeerd, die allemaal gekenmerkt worden door trombocytopenie (een te laag bloedplaatjesaantal):

- Von Willebrand ziekte (VWD) type 2B
- trombotische trombocytopenische purpura (TTP)
- het syndroom van hemolyse, verhoogde leverenzymen en lage bloedplaatjesaantallen (HELLP-syndroom)
- antifosfolipiden-syndroom

In het bloed van patiënten met een van deze vier ziekten hebben we gekeken wat een mogelijke oorzaak was van de daling in bloedplaatjesaantal. We hebben gevonden dat een eiwit dat in het bloed circuleert, Von Willebrand Factor (VWF), hier een belangrijke rol bij speelt.

Von Willebrand Factor

Von Willebrand Factor (VWF) is een extreem lang eiwit (figuur 1, hoofdstuk 1) dat in ons bloed circuleert. Normaal is dit eiwit opgevouwen tot een soort bolletje, totdat het een beschadiging in de vaatwand tegenkomt. Op zo'n beschadigde vaatwand gaat het eiwit vastzitten en verandert het van een bolletje in een lange sliert. Deze sliert kan de langsstromende bloedplaatjes 'vangen' en vasthouden op de plaats waar ze nodig zijn. Hierna gaan de bloedplaatjes steviger hechten en kunnen ze een bloedpropje vormen, waardoor het bloeden stopt.

Bloedplaatjes en VWF circuleren normaal dus samen zonder met elkaar in contact te komen, totdat er een bloeding is die moet worden gestelpt. Pas als VWF ontvouwen is, kan het bloedplaatjes binden. Deze ontvouwing noemen we activering van VWF. Voor dit promotieonderzoek hebben we een methode ontwikkeld om specifiek het ontvouwen, actieve VWF aan te tonen in bloed van patiënten. In **hoofdstuk 2** is de ontwikkeling en toepassing van deze methode beschreven. De methode is gebaseerd op een antistof die ontwikkeld is in lama's. Hiervoor zijn lama's ingespoten met een stukje van het ontvouwen VWF. Omdat dit stukje VWF afkomstig is van een mens en dit dus een vreemd eiwit is voor de lama, maakt het dier afweerstoffen aan om het eiwit zo snel mogelijk uit de circulatie te kunnen verwijderen (zoals wij afweerstoffen maken tegen een griepvirus). De antistoffen van de lama hebben we vervolgens gezuiverd en met behulp van deze antistof hebben we gemeten hoeveel actief VWF er aanwezig is in het bloed van gezonde vrijwilligers en patiënten. In het bloed van gezonde vrijwilligers was, zoals verwacht, nauwelijks actief VWF aanwezig.

Von Willebrand ziekte type 2B

Vervolgens hebben we de hoeveelheid actief VWF bepaald in bloed van patiënten met Von Willebrand ziekte (VWD) type 2B. In deze ziekte, die in 1926 werd ontdekt door de Finse wetenschapper Erik Adolf von Willebrand, is het VWF gemuteerd, dat wil zeggen een klein beetje veranderd. Hierdoor is het VWF altijd ontvouwen en dus actief. Zoals gezegd is VWF een extreem lang eiwit. In de circulatie wordt VWF in stukjes geknipt, waardoor verschillende lengtes VWF circuleren. De langste VWF-strengen zijn het meest kleverig, en het gemakkelijkst te ontvouwen. Bij patiënten met VWD type 2B plakken die lange ontvouwen VWF-strengen dan ook het snelst aan bloedplaatjes. Hierdoor ontstaan er klompjes bloedplaatjes met VWF als lijm ertussen. Om te voorkomen dat dit trombose veroorzaakt, is er een opruimmechanisme in het lichaam dat deze klompjes zo snel mogelijk uit de circulatie verwijdert. Dit heeft tot gevolg dat patiënten met VWD type 2B de langste VWF-strengen missen. Omdat nu alleen nog de kortere, minder kleverige strengen overblijven, is het vangen van bloedplaatjes op de plek van een wond moeilijker. Hierdoor hebben patiënten met VWD type 2B sneller bloedingen en blauwe plekken.

Met behulp van onze nieuwe methode om actief VWF in bloed aan te tonen, hebben we de hoeveelheid actief VWF in bloed van patiënten met VWD type 2B bepaald. Het percentage actief VWF ligt gemiddeld twaalf keer zo hoog bij patiënten met deze ziekte. Bovendien hebben we een sterke relatie gevonden tussen de hoeveelheid actief VWF en het aantal bloedplaatjes. Hoe meer actief VWF er in het bloed aanwezig was, hoe minder bloedplaatjes er nog circuleerden. Dit geeft aan dat bloedplaatjes bij deze patiënten verbruikt worden doordat ze aan het actieve VWF binden.

Trombotische trombocytopenische purpura

Een andere ziekte die gekenmerkt wordt door lage bloedplaatjesaantallen is trombotische trombocytopenische purpura (TTP). TTP is een relatief zeldzaam ziektebeeld, dat in Nederland slechts bij 30-50 patiënten per jaar wordt geconstateerd. In het bloed van patiënten met TTP zijn ultra-lange (UL) VWF-strengen gevonden. Normaal worden deze UL-strengen geknipt door een protease (een soort schaartje) dat bekend staat als ADAMTS13 (A Disintegrin And Metalloprotease with ThromboSpondin motif; figuur 2, hoofdstuk 1). In TTP is dit enzym gemuteerd (veranderd) zodat het niet meer kan knippen, of patiënten zijn per ongeluk afweerstoffen gaan produceren tegen hun eigen eiwit, waardoor ADAMTS13 niet meer kan knippen of versneld uit de circulatie wordt verwijderd (figuur 4, hoofdstuk 1). De meeste patiënten met TTP maken autoantistoffen, TTP is dan een auto-immuunziekte. Doordat ADAMTS13 in TTP patiënten kapot is, of geremd wordt door de antistoffen, worden de extreem lange en zeer kleverige strengen VWF niet meer geknipt tot kleinere, minder plakkerige stukken. De kleverige VWF-strengen gaan spontaan aan bloedplaatjes plakken in de circulatie. In tegenstelling tot VWD type 2B worden de nu gevormde klompjes van bloedplaatjes en VWF niet opgeruimd, waarschijnlijk omdat hiervoor ook een werkzaam ADAMTS13 nodig is. De klompjes lopen vast in de kleine haarvaten die organen van zuurstof voorzien. Hierdoor krijgen patiënten met TTP onder andere nierproblemen. Verder hebben TTP-patiënten vaak hemolyse (het kapot gaan van de rode bloedcellen, die zich door de dichtgeslibde haarvaatjes proberen te wurmen), lage bloedplaatjesaantallen door het verbruik van bloedplaatjes, koorts en neurologische problemen.

In **hoofdstuk 2** van dit proefschrift hebben we de hoeveelheid actief VWF bepaald in het bloed van TTP patiënten. We vonden dat bij patiënten met de verworven vorm van TTP, de vorm waarin mensen antistoffen maken tegen hun eigen ADAMTS13, de hoeveelheid actief VWF tweemaal zo hoog was als bij gezonde vrijwilligers. Bij patiënten met de erfelijke vorm van TTP, waarin ADAMTS13 gemuteerd is en dus niet meer kan knippen, vonden we zelfs een achtvoudige verhoging van de hoeveelheid actief VWF in het bloed.

HELLP-syndroom

Veel ziekten lijken erg op TTP, zeker als er alleen gekeken wordt naar de symptomen waarmee een patiënt zich bij een arts meldt (tabel II, hoofdstuk. 1). Een van de ziekten die erg op TTP lijkt is het syndroom van hemolyse, verhoogde leverenzymen en lage bloedplaatjesaantallen (HELLP-syndroom). Dit syndroom is een ernstige vorm van zwangerschapsvergiftiging, waarbij de endotheelcellen (de bekleding van de vaatwand) beschadigd of geïrriteerd zijn geraakt. Patiënten met HELLP-syndroom hebben een verhoogd bloedplaatjesverbruik, waarbij bloedplaatjesaantallen kunnen dalen tot onder de 100 miljard per liter. Verder zijn er veel eiwitten in het bloed aanwezig die normaal te vinden zijn als er ergens in het lichaam een ontsteking is. Het is nog onduidelijk hoe endotheelcelschade bij patiënten met HELLP-syndroom kan leiden tot een verlaagd bloedplaatjesaantal.

Endotheelcellen produceren VWF. Deels wordt dit direct uitgescheiden in het bloed en deels wordt het opgeslagen als UL-strengen VWF. Als endotheelcellen gestimuleerd worden of beschadigd raken, worden deze UL-strengen, die zeer plakkerig zijn, direct in het bloed uitgescheiden. Als deze strengen niet zo snel mogelijk onschadelijk worden gemaakt, zouden ze kunnen binden aan bloedplaatjes, met een daling in bloedplaatjesaantallen tot gevolg. Om te onderzoeken of deze theorie klopt, hebben we eerst de hoeveelheid actief VWF in het bloed van gezonde zwangere vrijwilligers, patiënten met een gewone zwangerschapsvergiftiging en patiënten met het HELLP-syndroom gemeten **(hoofdstuk 3)**. Tijdens een gezonde zwangerschap is er meer VWF in het bloed aanwezig dan normaal. Dit VWF is ook iets actiever dan normaal, waarschijnlijk om veel bloedverlies bij de bevalling te voorkomen. De hoeveelheid VWF en de hoeveelheid actief VWF blijven op hetzelfde

niveau tijdens zwangerschapsvergiftiging. Alleen in het bloed van patiënten met de zeer ernstige vorm van zwangerschapsvergiftiging, het HELLP-syndroom, is de hoeveelheid VWF nog extra verhoogd. Bovendien is een veel hoger percentage van dit VWF in een actieve vorm aanwezig. Dit betekent dat de absolute hoeveelheid actief VWF bij patiënten met HELLP-syndroom zelfs vier maal zo hoog ligt als tijdens een gezonde zwangerschap.

Bij de productie van VWF in endotheelcellen wordt een klein stukje van het VWF afgesplitst, waarna de rest van het VWF aan andere strengen VWF gekoppeld wordt om de hele lange strengen VWF te vormen. Het kleine stukje dat wordt afgeknipt wordt samen met het VWF opgeslagen in de cel. VWF en dit kleine stukje, dat het propeptide genoemd wordt, worden opgeslagen in de verhouding 1:1. Bij stimulatie of beschadiging van de endotheelcellen komen deze eiwitten dan ook in een 1:1 verhouding in het bloed. Omdat het kleine propeptide binnen twee uur de circulatie weer verlaat en VWF pas na twaalf uur, is de normale ratio van deze twee eiwitten in het bloed 0.13 (de verhouding van de hoeveelheid propeptide ten opzichte van de hoeveelheid VWF). Als er een acute stimulatie van endotheelcellen plaatsvindt, verandert de verhouding van de twee eiwitten in het bloed en wordt de ratio tijdelijk hoger. Als deze stimulatie lang voortduurt en chronisch wordt, ontstaat er een nieuw evenwicht tussen aanmaak en verwijdering van de eiwitten en wordt de verhouding weer 0.13. Door de verhouding propeptide / VWF te meten in het bloed van patiënten, kun je dus zien of er sprake is van een acute stimulatie van endotheelcellen. Bij gezonde zwangeren en bij patiënten met zwangerschapsvergiftiging was deze ratio niet verhoogd: 0.10 (0.08-0.12) bij gezonde zwangeren en 0.12 (0.04-0.17) bij zwangerschapsvergiftiging. Bij zwangerschapsvergiftiging is wel sprake van schade aan de endotheelcellen, maar dit is chronisch en er heeft zich een nieuw evenwicht ingesteld. Bij patiënten met HELLP-syndroom werd wel een verhoogde ratio propeptide / VWF gevonden (0.17 (0.10-0.27)). Dit geeft aan dat een acute stimulatie van endotheelcellen waarschijnlijk de bron is voor het extreem actieve VWF dat in het bloed van deze patiënten te vinden is. Om dit verder te bewijzen hebben we gekeken of er een direct verband bestond tussen de ratio propeptide / VWF en de hoeveelheid actief VWF in de bloedcirculatie. In **hoofdstuk 3** (figuur 3C, hoofdstuk 3) laten we zien dat er een sterk verband bestaat tussen deze twee waarden. Dit ondersteunt de hypothese dat acute stimulatie van endotheelcellen een verhoging van de hoeveelheid actief VWF in de circulatie veroorzaakt. ADAMTS13 zou het

actievere VWF kunnen knippen tot minder actieve kleine strengen VWF. Daarom hebben we ook de activiteit van dit protease bepaald. De ADAMTS13-activiteit was weliswaar verlaagd bij patiënten met HELLP-syndroom, maar bleef wel binnen de normale waarden.

Antifosfolipiden-syndroom

Sommige symptomen van het antifosfolipiden-syndroom lijken ook op symptomen die voorkomen bij TTP, waaronder trombocytopenie en hemolyse. De belangrijkste kenmerken van het antifosfolipiden-syndroom zijn echter trombose en herhaalde miskramen. Bij patiënten met het antifosfolipiden-syndroom zijn bepaalde antistoffen aanwezig. Vroeger werd verondersteld dat die antistoffen gericht waren tegen fosfolipiden (bestanddeel van het membraan, de buitenste schil van een cel), vandaar de naam antifosfolipiden-syndroom. Tegenwoordig weet men dat de antistoffen eiwitten herkennen die voorkomen in het bloed en die normaal gesproken aan fosfolipiden kunnen binden. Een van de eiwitten die herkend wordt door antifosfolipiden antistoffen is β_2-glycoproteïne I (figuur 5, hoofdstuk 1).

Het is nog altijd niet duidelijk hoe antistoffen tegen dit eiwit trombose kunnen veroorzaken. Men denkt dat een complex van antistoffen en β_2-glycoproteïne I aan cellen kan binden en deze kan activeren. Op deze manier zouden cellen zoals bloedplaatjes en endotheelcellen kunnen worden gestimuleerd, met trombose als gevolg. Hoewel in het laboratorium deze activering kan worden aangetoond, is nog niet bewezen dat het ook in het lichaam zo werkt. Verder is nog altijd niet bekend wat de normale functie is van β_2-glycoproteïne I in het bloed.

In **hoofdstuk 4** hebben we geprobeerd de fysiologische functie van β_2-glycoproteïne I te vinden. Eerder onderzoek heeft aangetoond dat β_2-glycoproteïne I aggregatie (het aan elkaar klonteren van bloedplaatjes) kan remmen. Wij hebben gevonden dat dit alleen kan als VWF wordt gebruikt als lijm tussen de plaatjes en niet als we de bloedplaatjes op een andere manier stimuleren om bloedpropjes te vormen. Verder hebben we gevonden dat remming van β_2-glycoproteïne I met behulp van antistoffen bloedplaatjesklontering aan VWF bevordert. Na toevoeging van extra β_2-glycoproteïne I bleek het juist moeilijker om bloedplaatjes te laten aggregeren aan VWF. Het effect van β_2-glycoproteïne I op VWF-gemedieerde bloedplaatjesklontering kan worden

nagebootst met antistoffen tegen VWF of tegen GpIb (de bindingsplaats voor VWF op bloedplaatjes), wat suggereert dat $ß_2$-glycoproteïne I binding van bloedplaatjes aan VWF remt. We hebben dit effect van $ß_2$-glycoproteïne I ook gevonden in een meer fysiologisch systeem, waarin bloedplaatjes, al dan niet in aanwezigheid van $ß_2$-glycoproteïne I, over VWF worden geleid. In dit systeem wordt eigenlijk de vaatwand nagemaakt door, in dit geval, VWF op een oppervlak vast te plakken. Daarna wordt het systeem ingesteld zodat de bloedplaatjes met een bepaalde snelheid over dit oppervlak stromen. Ook in dit systeem hebben we gezien dat aanwezigheid van $ß_2$-glycoproteïne I hechting van de bloedplaatjes aan VWF kan remmen. Antistoffen die $ß_2$-glycoproteïne I herkennen heffen deze remming op.

Om te kijken of er een directe interactie was tussen $ß_2$-glycoproteïne I en VWF hebben we bindingsexperimenten gedaan. $ß_2$-glycoproteïne I bleek inderdaad aan VWF te kunnen binden, maar dan wel bij voorkeur aan VWF in de ontvouwen, actieve vorm, de vorm die ook bloedplaatjes kan binden. Deze binding werd verstoord door de aanwezigheid van anti-$ß_2$-glycoproteïne I antistoffen. De data suggereren dat $ß_2$-glycoproteïne I een natuurlijke remmer is van actief VWF.

Om te bevestigen dat dit ook belangrijk is in het menselijk lichaam, hebben we gekeken naar de hoeveelheid actief VWF in het bloed van patiënten met het antifosfolipiden-syndroom. Bij een speciale groep patiënten met het antifosfolipiden-syndroom, namelijk diegene met antistoffen tegen $ß_2$-glycoproteïne I, hebben we verhoogde hoeveelheden actief VWF gevonden. Dit suggereert dat een verlies van de remmende werking van $ß_2$-glycoproteïne I ook daadwerkelijk de hoeveelheid actief VWF in de circulatie beïnvloedt.

TTP-achtige ziekten

Zoals gezegd zijn er verschillende ziekten die op basis van de symptomen gemakkelijk te verwarren zijn met TTP (tabel II, hoofdstuk 1). Omdat TTP een dodelijke ziekte is wanneer die niet behandeld wordt, is het belangrijk om snel de juiste diagnose te stellen. Aan de andere kant kan behandeling van TTP door het vervangen van het plasma van de patiënt voor donorplasma ook verschillende bijwerkingen hebben. Daarom is het ook belangrijk deze behandeling achterwege te laten als de patiënt geen TTP heeft.

Op grond van de symptomen is het vaak erg moeilijk om de verschillende ziekten te onderscheiden van TTP. Wel zijn er meerdere publicaties die laten zien dat afwezigheid van ADAMTS13-activiteit specifiek is voor TTP. Daarom is er in de afgelopen jaren hard gewerkt aan methoden om de aanwezigheid en activiteit van ADAMTS13 te meten. Recentelijk is er een nieuwe methode ontwikkeld waarmee de activiteit van ADAMTS13 kan worden bepaald. Deze methode, de FRETS-assay is veel sneller dan de oude methoden (een uur vs. drie dagen), waardoor sneller uitsluitsel verkregen kan worden. In **hoofdstuk 5** hebben we deze nieuwe methode vergeleken met de oude methode, die op veel laboratoria werd gebruikt. Onze studie, zoals weergegeven in **hoofdstuk 5**, en andere studies die de nieuwe methode met andere methoden hebben vergeleken, geven aan dat de nieuwe methode zeer gevoelig en specifiek is voor TTP. Bovendien zijn de data binnen een laboratorium goed reproduceerbaar. Natuurlijk moet in de toekomst blijken of de reproduceerbaarheid ook goed is tussen verschillende laboratoria.

Een kleine kanttekening bij deze methode is dat er alleen statisch, oftewel zonder stroming, gemeten wordt. Eventuele effecten van stoffen die de binding van ADAMTS13 aan VWF beïnvloeden in de bloedcirculatie zouden dus gemist kunnen worden.

Discussie

Actief VWF speelt een belangrijke rol bij het ontstaan van trombocytopenie in VWD type 2B, TTP, HELLP-syndroom en het antifosfolipiden-syndroom. De hoeveelheid actief VWF wordt bepaald door ADAMTS13, de aanwezigheid van endotheelcelschade of -irritatie, en wordt beïnvloed door de aanwezigheid van β_2-glycoproteïne I. Speciale muizen die geen ADAMTS13 of geen β_2-glycoproteïne I maken hebben geen TTP of antifosfolipiden-syndroom. Dit duidt erop dat de eiwitten apart van elkaar misbaar zijn. Waarschijnlijk is er een natuurlijke balans tussen β_2-glycoproteïne I, ADAMTS13 en endotheelcelactivatie, zodat de hoeveelheid actief VWF en de mate van activiteit strikt gereguleerd kan worden. Verlies van een van de componenten kan wellicht door de andere factoren worden opgevangen.

Het is bekend dat muizen die geen ADAMTS13 maken wel TTP krijgen als ze een infectie doormaken. Verder kan zwangerschap bij mensen TTP induceren. Tijdens de zwangerschap of een infectie circuleren speciale eiwitten in het bloed, de zogenaamde inflamatiemarkers. IL-6 is zo'n marker. Van IL-6 is bekend dat het de activiteit van ADAMTS13 in de bloedstroom kan remmen, een effect dat niet kan worden gevonden met de statische methoden die in de meeste diagnostische laboratoria worden gebruikt. Onlangs is gepubliceerd dat β_2-glycoproteïne I daalt vanaf de achtste week van de zwangerschap. Verhoging van IL-6 en daling van β_2-glycoproteïne I zouden redenen kunnen zijn dat zwangerschap TTP kan induceren.

De balans in de driehoek zoals weergegeven in figuur 3 van **hoofdstuk 6** zou de verschillen tussen patiënten kunnen verklaren. VWD type 2B patiënten met dezelfde mutatie in VWF kunnen bijvoorbeeld sterk verschillende bloedplaatjesaantallen hebben. Dit zou kunnen komen doordat de ene patiënt meer β_2-glycoproteïne I heeft dan de ander, waardoor er minder VWF is dat gaat plakken aan de bloedplaatjes.

Sommige patiënten hebben geen ADAMTS13-activiteit en toch ook geen TTP-aanval. Dit zou kunnen komen doordat β_2-glycoproteïne I VWF-geïnduceerde bloedplaatjesklontering voorkomt. Een extra verstoring van de balans door zwangerschap of een infectie zou alsnog een aanval kunnen induceren.

Niet alle patiënten met zwangerschapsvergiftiging ontwikkelen HELLP-syndroom. Dit zou kunnen komen door het verschil in de hoeveelheid β_2-glycoproteïne I in het bloed. Verder zou ADAMTS13 een belangrijke rol kunnen spelen. Bij patiënten met HELLP-syndroom wordt vaak een verhoogde IL-6 spiegel gemeten. Dit zou ADAMTS13-activiteit in de circulatie kunnen remmen, waardoor de extreem lange VWF-strengen afkomstig uit beschadigde endotheelcellen, niet kunnen worden geknipt.

Regulatie van de hoeveelheid actief VWF is dus een balans tussen verschillende factoren. Verlies van de balans kan ernstige ziekten veroorzaken, zoals TTP en HELLP-syndroom. Aanwezigheid van actief VWF in het antifosfolipiden-syndroom lijkt bovendien samen te hangen met de aanwezigheid van trombose. Tegenwoordig wordt gedacht dat zwangerschapsvergiftiging en HELLP-syndroom voorspellers zijn van hart- en vaatziekten in een latere fase van het leven. Verhoging van de hoeveelheid VWF in de bloedcirculatie is dikwijls beschreven als een risicofactor voor

hart- en vaatziekten. In het licht van onze data is het interessant te onderzoeken wat de rol is van actief VWF in hart- en vaatziekten. Afwijkende hoeveelheden β_2-glycoproteïne I of (actief) VWF, of een verlaagde ratio tussen β_2-glycoproteïne I en (actief) VWF, zouden een indicatie kunnen zijn voor een verhoogd risico op hart- en vaatziekten.

Dankwoord

Meestal beginnen dankwoorden met de zin: “Zo, klaar”, of iets dergelijks. Natuurlijk voelt het ook voor mij zo, maar toch heb ik het gevoel dat het moeilijkste van alles nu nog moet: mijn dankwoord. Het meest gelezen hoofdstuk van een proefschrift, en bovendien het hoofdstuk waar veel mensen hoge verwachtingen van hebben. Nieuwsgierig en altijd met de hoop dat er ook over jou iets leuks en persoonlijks gezegd wordt, scant iedereen zo’n dankwoord. Erg vervelend als je dan vergeten bent. Daarom wil ik maar beginnen met IEDEREEN te bedanken. Jullie hebben allemaal wel een steentje bijgedragen. Hoe groot of klein dat steentje ook was, zonder jullie was dit proefschrift nooit geworden wat het nu is, en was ik ook niet geworden wie ik nu ben. Bedankt voor alles!!!

Natuurlijk wil ik ook wat mensen persoonlijk bedanken. Allereerst mijn promotor. Beste Flip, in het begin vond ik het best lastig. Moet ik nu u of jij zeggen en mag ik wel zomaar binnenlopen? Maar in de loop van de jaren werd wel duidelijk dat je altijd tijd voor iemand maakt en werd de drempel aanzienlijk lager. Je had steeds weer nieuwe ideeën en oplossingen voor het onderzoek. Bedankt dat je me de kans gaf te groeien.

Rob, jij weet echt alles te verkopen. Vandaar natuurlijk dat ik als een blok viel voor jouw onderwerp: TTP. Bedankt voor je bijna niet te remmen enthousiasme. Zelfs als niemand er meer iets in zag, vond jij altijd een lichtpuntje. Bedankt ook voor onze huisgenoot, we hebben erg veel plezier van haar en zullen haar voorlopig dan ook niet opeten :) .

Al dat enthousiasme moest door iemand in balans gebracht worden. Peter, door jouw realisme konden we af en toe ook weleens nee zeggen tegen Rob. En dat is best moeilijk… Persoonlijk ben ik gegroeid door jouw begeleiding: ‘Luctor et emergo’. Als Zeeuws meisje spreekt me dat natuurlijk wel aan.

Carina, ook al kwam je pas toen ik al een jaar bezig was, ik heb het gevoel dat je er steeds bij bent geweest. We hebben heel wat uurtjes onder het genot van een kopje thee alle facetten van ons onderzoek besproken. Bedankt dat ik altijd op je kon rekenen, en bedankt ook dat je zelfs in de laatste fase nog naar me wilde luisteren

toen mijn hoofd helemaal vol zat van dit proefschrift. Wat een geduld! En ook nu kan ik weer op je rekenen en sta je naast me tijdens het hoogtepunt van vier jaar promotieonderzoek. Nog even doorzetten, jij bent er ook bijna!

Thessa, wij delen al veel langer alle ups en downs. Jij hebt me laten geloven in mijzelf en in mijn talenten. Bedankt voor je vertrouwen en steun. Als bekroning op onze vriendschap is nu ONS proefschrift af. Je hebt er heel wat uurtjes inzitten, ook al had je het al zo druk. Ik hoop dat ik jou bij de moeilijke beslissingen die je nu moet gaan nemen, maar ook bij de mooie dingen die in het verschiet liggen, net zo kan steunen als jij mij in de afgelopen negen jaar.

Humor is erg belangrijk, ook in de wetenschap. Bas, wij hebben dit samen bewezen in hoofdstuk 4 van dit proefschrift. Waar een grapje in de AIO-kamer al niet toe kan leiden! Het was leuk om met iemand samen te werken die zijn hart zo bij het onderzoek heeft en zo enthousiast is. Soms zelfs zo erg dat mijn data al bij Flip lagen voordat ik ze zelf gezien had. Marieke, bedankt voor het bijhouden van Bas' agenda. Als ik jou mail weet ik tenminste zeker dat het voor elkaar komt.

Astrid, jij stond en staat altijd voor me klaar. Of het nu over werk of privé ging, altijd had je een luisterend oor en goede raad. Jouw openheid en spontaniteit zorgden ervoor dat ik me al snel thuis voelde op mijn nieuwe plekje op lab 3. Samen hebben we alle moeilijkheden van het vinden van een nieuwe baan doorstaan. Een trouwere vriendin kun je je niet wensen. Ik hoop dat er nog veel wandelingen en kopjes thee mogen volgen.

Mijn eerste en enige student Marieke. Zo'n student wil iedereen wel! Alles wat jij aanraakte veranderde in goud. Niet veel mensen kunnen zeggen dat ze hun student moesten afremmen. Het liefst wilde je alle ideeën voor proeven op één dag uitvoeren. Je kreeg het niet gemakkelijk met een begeleider die nog nauwelijks iets over APS en β_2-GPI wist, maar samen hebben we ons er goed doorheen geslagen. Veel succes met je nieuwe stage, afstuderen en plannen maken voor de toekomst.

Chantal, Hanneke, Bernard, Evelyn en later ook Quirijn: Robs groepje. Chantal en Hanneke, jullie waren er van het begin af aan en hebben me geholpen mijn plek te vinden op lab 1. Chantal, jouw gevoel voor controles was onovertroffen en heeft me echt geholpen goede experimenten te leren opzetten. Bedankt allemaal voor de ontspanning bij de etentjes, waarbij het altijd weer spannend was of Rob ook zou

komen. Bernard, veel succes met je eigen promotie en opleiding en veel geluk met je kersverse gezinnetje. Quirijn, hoewel je er alleen op vrijdagen bent, ben je al snel een onmisbaar onderdeel geworden van Robs groep. Bedankt voor de gesprekken over van alles en nog wat. Evelyn, veel succes met je promotieonderzoek en alle dingen die daaraan vastzitten.

Dromen zijn eigenlijk het mooist. Ton, blijf dromen van je wijngaard, eigen wijn en eindeloze lunches. Mocht het ooit realiteit worden dan reken ik op een uitnodiging :) . Eén van je dromen komt in elk geval al snel dichterbij. Veel succes in Groningen.

Maarten en Rolf. Samenwerking in de diepste betekenis van het woord. Bedankt voor bergen plasma-$ß_2$-GPI, antistoffen en goede raad. Veel succes met het afronden van jullie promotieonderzoek.

Sandra, ik kon me altijd even omdraaien voor een kletspraatje, of soms ook een diep gesprek. Bedankt voor de steun bij het realiseren van mijn droom… klinisch chemicus worden. Bedankt ook dat je altijd van die handige spulletjes had liggen die ik gemakkelijk kon 'lenen'. Er gaat heel wat veranderen voor jou in de nabije toekomst. Veel succes, plezier en geluk daarmee.

Alle dames van het ASL en ex-ASLers; jullie deur is echt altijd open, of het nu voor samples of NPP was of gewoon voor een praatje. Er is heel wat gebeurd in de afgelopen jaren. Daardoor zijn jullie een heel bijzondere groep geworden. Heel veel sterkte en succes in de toekomst.

Lucy, eerst ASL en toen lab 2. Het was geweldig nieuws toen we hoorden dat je bij ons lab kwam werken. Jammer dat het maar zo kort geduurd heeft. Je was echt het zonnetje van lab 2. Bedankt dat je altijd een luisterend oor had en ook een flinke dosis humor om al onze probleempjes mee weg te lachen. Succes in Amsterdam.

Wegens bezuinigingen heb ik tijdens congressen met verschillende AIO's het bed gedeeld. Bedankt dat jullie allemaal niet snurkten. Menno, ook in je slaap blijf je geluiden maken, maar gelukkig heel wat zachter dan overdag. Reizen met jou is wel een hele opgave. Waarom ben je niet standaard geleverd met routeplanner? Ingrid, we hebben allemaal respect voor je gekregen. Ronan, your alarm clock is ringing

forever! You really have a hard time coming out of your bed. Lucky that research is so flexible. Good luck with your last months. Just take it day by day and remember that there is an end to it.

Marjolijn, zonder jouw SMS was dit proefschrift er zeker niet geweest. Bedankt dat je meteen aan mij dacht toen je hoorde van deze AIO-plek. Samen met Patricia hebben we wat af gepraat en ook al lagen onze promotieonderwerpen best ver uit elkaar, ik heb ook voor dit proefschrift heel wat gehad aan jullie verhalen en adviezen.

Enthousiasme en geluk zijn ontzettend belangrijk tijdens een promotie. Monique, jij bent bijna letterlijk het zonnetje op het Celbio-lab. Ondanks al je tegenslag blijf je erin geloven en straal je energie uit over iedereen die in je buurt komt. Bedankt, want dat had ik af en toe ook echt nodig. Succes met jouw laatste maanden. Het gaat je lukken.

Ik kan wel bezig blijven, over iedereen is wel iets bijzonders of grappigs te zeggen. Maar om dit hoofdstuk ook nog leesbaar te houden, ga ik nu toch over op het bedanken van hele labs tegelijk. Lab 1, mijn thuisbasis en zo is het altijd blijven voelen. Bedankt voor alle steun en hulp die ik van jullie heb gekregen om mijn onderzoek uit te voeren. Martin, nog even speciaal bedankt voor de eindeloze rij perfusies die je hebt gedaan.

Lab 2. Ook al kwam ik niet zo heel vaak bij jullie, toch heb ik mogen genieten van de sfeer op jullie lab en van Hazes en andere 'grote' artiesten die jullie luid en duidelijk lieten horen. Inmiddels is het een stuk rustiger geworden bij jullie, maar de gezelligheid is wel gebleven.

Lab 3. Mijn vaste honk in de laatste jaren. Ik heb van jullie veel nieuwe dingen geleerd en ben blij dat ik altijd aan kon kloppen met (al dan niet domme) vragen over aggregaties, bloedplaatjes enzovoort, enzovoort. Bedankt ook voor de gezelligheid. Ik hoop dat jullie niet helemaal gek van me werden als ik tijdens het schrijven wat afleiding zocht en jullie dan lekker van het werk kwam houden.

Verder wil ik de patiënten van de TTP-patiëntenvereniging, alle behandelend artsen, de Noaber Foundation en de Hoge Dennen Stichting, bedanken. Dankzij jullie betrokkenheid en financiële steun konden wij een grote database opzetten met plasma van TTP-patiënten. Zonder deze bijdrage had ik dit onderzoek niet kunnen

verrichten. Het was heel bijzonder om de patiëntendagen mee te mogen maken. Ik heb gezien hoe TTP het dagelijkse leven beïnvloedt en dat heeft me zeker gemotiveerd om door te gaan. Hopelijk zal al het onderzoek resulteren in een verbeterde behandeling voor TTP.

Verder wil ik mijn familie bedanken voor hun interesse voor mijn onderzoek. Het was soms moeilijk maar altijd leuk om jullie in te wijden in mijn wereldje. Opa en oma, het is heel bijzonder dat jullie dit allemaal nog mogen meemaken. Het is fijn om te merken hoe jullie meeleven en trots zijn. Dat heeft me zeker vooruit geholpen.

Mijn schoonouders en zwager Marnix, ik wil jullie bedanken voor alle steun. Jullie doorzettingsvermogen en onuitputtelijke hoop hebben mij geholpen mijn 'problemen' te relativeren en in te zien dat er veel belangrijkere dingen zijn in het leven.

Arne, Linda en Tirza, jullie zijn een geweldig gezinnetje! Het zien van Tirza, maar ook even ouderwets melig zijn, of een gezellig babbeltje en een kopje thee bleek altijd voldoende om even alles te vergeten.

Pappa en mamma, ondanks dat het onderzoek heel wat moeilijke woorden en begrippen in huis heeft gebracht, zijn jullie altijd erg betrokken geweest. Mamma, bedankt dat je tijd nam voor de soms eindeloze telefoongesprekken en dat je me altijd weer wist te motiveren door te zetten als ik het even niet meer zag. Pappa, bedankt voor je geduld als mamma weer eens uren aan de telefoon zat met mij. Wij krijgen steeds meer raakvlakken in ons werk en ik vind het heel bijzonder dat we dat met elkaar kunnen delen.

Wilmer… er zijn haast geen woorden te vinden voor wat jij allemaal hebt gedaan en hebt betekend. Met eindeloos geduld hoorde je mijn verhalen aan die varieerden van Chinees voor beginners tot allerlei details over mensen die je nog nooit had gezien. Sorry dat ik soms zo ratel en nauwelijks tijd nam voor jouw verhalen. Elke emotie heb je met me gedeeld, je bent boos en verdrietig geweest met mij, maar we hebben het ook samen gevierd als er een succesje werd geboekt. Uiteindelijk was het echt onze promotie!

Janine

Curriculum Vitae

De schrijfster van dit proefschrift werd geboren op 3 mei 1979 te 's-Hertogenbosch. Na het behalen van het Gymnasiumdiploma aan het Buys Ballot College te Goes, studeerde zij vanaf 1997 Medische Biologie aan de Universiteit Utrecht. In de laatste fase van deze studie werd een onderzoeksstage voltooid bij de afdeling Medische Oncologie, onder begeleiding van Ir. A. Reijerkerk, Dr. M.F.B.G. Gebbink en Prof. Dr. E.E. Voest en bij de afdeling Celbiologie onder begeleiding van Ir. J. Schantl en Prof. Dr. G.J. Strous. In 2002 werd het doctoraal examen behaald.

Van november 2002 tot november 2006 was zij werkzaam als assistent in opleiding bij de afdeling Klinische Chemie en Haematologie van het Universitair Medisch Centrum Utrecht. Het in die periode uitgevoerde onderzoek is in dit proefschrift beschreven en werd uitgevoerd onder leiding van Dr. R. Fijnheer, Dr. P.J. Lenting en Prof. Dr. Ph.G. de Groot. In november 2006 is zij begonnen aan een opleiding tot klinisch chemicus in het Jeroen Bosch Ziekenhuis te 's-Hertogenbosch onder begeleiding van Dr. G.C.M. Kusters en Dr. P. van 't Sant.

List of publications

Full papers

1. Acute activation of the endothelium results in increased levels of active Von Willebrand Factor in HELLP syndrome.
   Hulstein JJ, van Runnard Heimel PJ, Franx A, Lenting PJ, Bruinse HW, Silence K, de Groot PG, Fijnheer R.
   J Thromb Haemost. 2006 DOI: 10.1111/j.1538-7836.2006.02205.x

2. FRETS-VWF73: a rapid and predictive tool for thrombotic thrombocytopenic purpura.
   Groot E, Hulstein JJ, Rison CN, de Groot PG, Fijnheer R.
   J Thromb Haemost. 2006 Mar;4(3):698-9

3. A novel nanobody that detects the gain-of-function phenotype of Von Willebrand Factor in ADAMTS13 deficiency and Von Willebrand disease type 2B.
   Hulstein JJ, de Groot PG, Silence K, Veyradier A, Fijnheer R, Lenting PJ.
   Blood. 2005 Nov 1;106(9):3035-42.

4. The spacer domain of ADAMTS13 contains a major binding site for antibodies in patients with thrombotic thrombocytopenic purpura.
   Luken BM, Turenhout EA, Hulstein JJ, Van Mourik JA, Fijnheer R, Voorberg
   J Thromb Haemost. 2005 Feb;93(2):267-74.

5. Afwezige activiteit van Von Willebrand factor splitsend eiwit (ADAMTS-13) diagnostisch voor primaire en zwangerschapsgerelateerde trombotische trombocytopenische purpura.
   Hulstein JJ, Rison CN, Kappers-Klunne MC, Hene RJ, Franx A, De Groot PhG, Brand A, Fijnheer R.
   Ned Tijdschr Geneeskund. 2004 2 okt; 148(40): 1972-1976

Other publications

1. Acute activation of endothelium induces circulation of active Von Willebrand Factor in HELLP syndrome.
   Hulstein JJ, Van Runnard Heimel PJ, Franx A, Lenting PJ, Bruinse HW, Silence K, De Groot PhG, Fijnheer R.
   Br Med J. April 2006; 133 (1):88 (223), Poster presentation, Poster-award 2006

2. Acute activation of endothelium induces circulation of active Von Willebrand Factor in HELLP syndrome.
   Hulstein JJ, Van Runnard Heimel PJ, Franx A, Lenting PJ, Bruinse HW, Silence K, De Groot PhG, Fijnheer R.
   Blood (ASH Annual Meeting Abstracts), Nov 2005; 106: 2658, Poster presentation

3. Active Von Willebrand Factor is present in plasma of patients with Von Willebrand Disease type 2B or ADAMTS-13 deficiency.
   Hulstein JJ, Lenting PJ, Silence K, Fijnheer R, de Groot PG
   J Thromb Haemost. 2005; Volume 3, Supplement 1: OR028, Oral presentation

4. The sensitivity and specificity of ADAMTS13 activity for primary thrombotic thrombocytopenic purpura.
   Hulstein JJ, Rison C, Hene R, De Groot PhG, Romijn R, Fijnheer R.
   J Thromb Haemost. 2003; 1 Supplement 1 July: P0327, Poster Presentation