Active Von Willebrand Factor in Thrombotic Thrombocytopenic Purpura and Malaria

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Active Von Willebrand Factor in Thrombotic Thrombocytopenic Purpura and Malaria

Actief Von Willebrand Factor in Trombotische Trombocytopenische Purpura en Malaria

(met een samenvatting in het Nederlands)

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Nobody said it was easy…

Coldplay
The Scientist

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Chapter 1

The presence of active von Willebrand factor under various pathological conditions

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Introduction

Von Willebrand factor (VWF) is a multimeric protein that contributes to the recruitment of platelets to the injured vessel wall [1]. Participation of circulating VWF in this process requires conversion of VWF from a latent into an active conformation, that is a state in which the binding site for its platelet-receptor glycoprotein-Ibα (GpIbα) is exposed. Normally, this conversion is well-regulated in that only binding of VWF to exposed subendothelial matrix leads to formation of active VWF. Unfortunately, undesired activation of VWF may also occur under certain pathological conditions, which subsequently results in VWF-platelet aggregates that are associated with thrombocytopenia, thrombotic complications or both.

In search for a tool to specifically detect active VWF in the circulation, we have recently developed a recombinant llama-derived antibody fragment [2]. This nanobody (designated AU/VWFa-11) preferentially recognizes the active conformation of VWF, and has been applied in an immunosorbent-based assay to quantify active VWF levels in plasma samples of various patient populations.

In the current review, we will discuss mechanisms that regulate the balance between latent and active VWF, and we will outline several pathological conditions in which a disturbed balance is leading to increased levels of active VWF.

Platelet-binding properties of freshly secreted VWF

VWF is produced in endothelial cells and megakaryocytes as a single-chain pro-subunit with a distinct domain structure: D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK [3,4]. These subunits are first coupled into pro-VWF dimers via carboxy-terminal linkage. Further processing subsequently involves multimerization via intermolecular cystine-bonding within the D'-D3 domains, producing a heterologous pool of differentially sized multimers, which can be a large as 30-mers. Limited proteolysis then separates propeptide from the mature VWF-multimer, followed by targeting of both proteins to storage organelles [5]. In megakaryocytes, VWF and its propeptide are stored in granules [6], while in endothelial cells they are targeted to endothelial-specific Weibel-Palade bodies [7]. Endothelial cells are the dominant source of VWF that is found in subendothelial tissue and in plasma.

VWF stored in endothelial cells or platelets is dissimilar to VWF that circulates in plasma. The most striking example that illustrates this dissimilarity is that freshly-secreted VWF is able to interact with the platelet GpIb-IX-V complex, whereas circulating VWF (under normal physiological conditions) is not. A first clue that such difference exists came forward in an elegant study by André *et al*., in which it was demonstrated that endothelial-derived VWF is able to mediate GpIbα-dependent platelet adhesion to stimulated mouse endothelium *in vivo* [8]. It should be noted that platelet adhesion was transient, with a decline in the number of adhering platelets within several minutes after stimulation. Also in *in vitro* studies it has been firmly established that stimulation of endothelial cells results in the release of platelet-binding VWF molecules [9-12].

What makes freshly released VWF different from circulating VWF, allowing it to interact spontaneously with platelets? One point of difference concerns O-linked glycosylation of VWF with sialylated T-antigen, which is reduced in stored VWF compared to circulating VWF [13]. However, there are currently no data available indicating that differences in O-linked glycosylation endow the platelet-binding capacity. A more significant difference relates to the presence of VWF molecules in storage organelles that can be qualified as being unusually large or ultra large (UL), representing a molecular weight that is normally not seen in plasma [14-18]. An important breakthrough in understanding the properties of these UL-VWF molecules was achieved when Arya *et al*. were able to characterize the interaction between purified UL-VWF and GpIbα using optical tweezers [19]. From these studies it became apparent that UL-VWF multimers expose high-affinity binding sites for GpIbα, whereas these sites are unavailable in plasma-derived VWF. As a consequence, UL-VWF multimers are able to interact spontaneously with platelets without the need for high-shear stress or modulators such as ristocetin or botrocetin. The molecular basis of why the GpIbα binding sites are accessible in UL-VWF but not in plasma-derived VWF is unclear and remains to be elucidated.

Inactivation of active VWF: Contribution of thrombospondin-1 and ADAMTS13

The immediate availability of platelet-binding VWF molecules may be advantageous at moments when platelet recruitment is needed at sites of vascular injury. Its continuous presence, however, may lead to undesired formation of platelet-rich thrombi that occlude the vasculature. Thus, regulatory mechanisms must exist that convert UL-VWF multimers into derivatives that are unable to bind platelets spontaneously. It is assumed that this process involves the conversion of UL-multimers into smaller sized fragments. One potential mechanism that controls multimeric size has been described by Xie *et al.* and involves thrombospondin-1 [20]. *In vitro*, thrombospondin-1 has the capacity to induce new thiols in VWF, and to reduce VWF multimer size. It was surprising therefore that the average size of VWF multimers proved smaller in thrombospondin-1 deficient mice than in control mice [21]. In contrast, the size of platelet-derived multimers was increased in these deficient mice. This suggests that thrombospondin-1 contributes to the regulation of platelet-derived multimers rather than reducing the size of UL-VWF multimers released from endothelial cells.

The reduction of multimeric size can also be obtained via limited proteolysis. Indeed, circulating VWF consists of distinct proteolytic fragments [22]. Although for several years it was known that a specific VWF-cleaving metalloproteinase was responsible for the generation of these fragments [23,24], it was in 2001 that this enzyme was identified as being a member of the ADAMTS-family (A Disintegrin and Metalloprotease with ThromboSpondin motif), that is ADAMTS13 [25-27]. This protease cleaves VWF at a distinct site within the A2 domain between residues Tyr1605 and Met1606, thereby reducing the average size of VWF multimers. Deficiency of ADAMTS13 in humans and mice is characterized by the presence of an increased average multimeric size, reinforcing the *in vivo* relevance of this enzyme [16,28-31].

Does ADAMTS13-mediated proteolysis coincides with the disappearance of spontaneous platelet VWF interactions? Dong and coworkers demonstrated that endothelial-derived UL-VWF multimers form long strings (up to several millimeters!) along the surface of stimulated endothelial cells under conditions of flow, and that these strings become filled with platelets [11]. More recently, preliminary data by Huang *et al*. suggest that these strings are composed of multiple, intertwined VWF multimers [32]. Only in the presence of ADAMTS13 these platelet-rich strings disappear rapidly. These findings have been shown to occur *in vivo* as well: platelet-rich strings persist for a prolonged period of time at the surface of stimulated mouse endothelium provided that no functional ADAMTS13 is available [31,33]. Of note, VWF appears also susceptible to ADAMTS13-dependent proteolysis when incorporated within the thrombus. Indeed, ADAMTS13 was found to promote thrombus dissolution in injured arterioles in *in vivo* experiments [34]. Moreover, *in vitro* experiments showed that ADAMTS13 regulates size of VWF-mediated platelet thrombi in flowing blood [35].

Having identified ADAMTS13 as a key element in the inactivation of VWF, it leaves but speculation how ADAMTS13-mediated cleavage results in the inaccessibility of the GpIbα-binding sites. Is it simply the reduction of multimeric size, or do other more complex molecular reorganisations take place? The GpIbα-binding site is located within the A1 domain of VWF [36]. One possible consequence of ADAMTS13 cleavage is that the A1 domain becomes buried within the VWF molecule. It has been reported not only that individual VWF molecules may self-associate via homotypic interactions [32,37], but also that inter-domainal interactions may occur within a single VWF subunit. For example, Ulrichts *et al*. found that the GpIbα-binding site is covered by the D'-D3 region [38], while Nakayama *et al*. showed that the peptide regions adjacent to the A1 domain modulate the interaction with GpIbα [39]. It is tempting to speculate that homotypic and inter-domainal interactions are controlled by ADAMTS13-cleavage in the A2 domain, providing a means to indirectly regulate platelet-VWF interactions.

Reactivation of VWF

The following step in the sequence of events is that VWF regains its platelet-binding capacity after being released into the circulation as an inactive molecule. Following vascular injury, circulating VWF multimers bind to exposed subendothelial matrix components, such as collagen [1,40]. Although collagen binding under static conditions in vitro is sufficient to open the GpIbα-binding site, it is assumed that this process is facilitated when VWF is exposed to shear. In fact, conditions of high shear rates alone (with a threshold of approximately 10 000 s^{-1}) are able to induce VWF-platelet interactions [41]. High shear likely affects the exposure of the active A1 domain in multimeric VWF rather than contributing to the stability of the VWF-GpIbα complex and therefore both multimeric size (and thus the number of available A1 domains) and shear stress play an important role in the contribution of VWF to thrombus formation.

Detection of active VWF

Under normal physiological conditions, the presence of the above-mentioned regulatory mechanisms should be sufficient to prevent active VWF from being present in the circulation, thereby avoiding the formation of unwanted VWF-platelet aggregates.

However, a number of pathological conditions have been described that are characterized by spontaneous VWF-platelet interactions, such as von Willebrand disease (VWD)-type 2B and thrombotic thrombocytopenic purpura (TTP). These conditions suggest that active forms of VWF should be present in plasma. But how to detect these active VWF molecules? Possible assays may be based on the direct interaction between GpIbα (either recombinant or platelet-exposed) and VWF. However, these assays are characterized by a low sensitivity, probably due to the high dissociation rate constant of the VWF-GpIbα interaction, which is amplified by introducing washing-steps in such assays. We have recently developed a recombinant llama-derived antibody (designated nanobody AU/VWFa-11), which is able to distinguish between the active and latent form of VWF by displaying preferential binding to the GpIbα-binding conformation of the VWF A1 domain [2]. Importantly, this nanobody is unable to interfere with the VWF-GpIbα interaction, indicating that the nanobody binds to a site distinct from the GpIbα interactive site. Nevertheless, from the data that are currently available, it appears that AU/VWFa-11 has the potential to be used as a tool to detect the presence of active VWF in plasma.

Active VWF under pathological conditions: VWD-type 2B

VWD can be categorized by quantitative (VWD-type 1 or VWD-type 3) or qualitative (VWD-type 2) deficiencies [42]. Qualitative deficiencies generally originate from suboptimal VWF-platelet interactions. However, the VWD-type 2B subtype is characterized by heightened interactions between VWF and platelets, resulting from gain-of-function mutations within the VWF A1 domain [3,42]. VWD-type 2B patients lack high multimers and have reduced platelet counts due to the formation of VWF-platelet complexes. We have used nanobody AU/VWFa-11 to investigate whether active VWF indeed circulates in plasma of these patients. It was not surprising that levels of active VWF were increased in type 2B patients compared to healthy controls [2]. However, the unexpected observation in this exercise was that the levels of active VWF varied considerably amongst patients, between 2- and 15-fold. Further analysis revealed that there was an inversed correlation between the amounts of active VWF and the platelet count in these patients. Apparently, active VWF levels determine the extent of VWF-platelet complex formation, and thereby the extent of thrombocytopenia. Why levels of active VWF vary between VWD-type 2B patients is yet unclear, but may relate to the underlying mutation.

Active VWF under pathological conditions: Thrombotic thrombocytopenic Purpura

Another candidate disorder where active VWF might be present in the circulation is thrombotic thrombocytopenic purpura (TTP), a life threatening disease associated with thrombocytopenia, haemolytic anaemia and vascular microangiopathy [43]. TTP is caused by a genetic or autoimmune-based deficiency or dysfunction of ADAMTS13. Since this protease is critical for the "inactivation" of UL-VWF multimers, its absence results in continuous release of active VWF molecules into the circulation. Indeed, levels of active VWF were increased 2 to 12-fold in patients with acquired and congenital TTP, respectively, and no overlap in levels of active VWF were found between both patient groups [2]. The samples that were analyzed were taken upon hospitalization,

thus in an acute phase of the disease. At this stage, a considerable part of the active molecules is likely to be caught into the VWF-rich platelet aggregates, which may explain the variance in active VWF levels between patients. On the other hand, the lack of overlap between acquired and congenital TTP patients with regard to active VWF levels may provide an opportunity to use the AU/VWFa-11-based immunosorbent assay to distinguish between both disorders.

Active VWF under pathological conditions: HELLP

The identification of active VWF in plasma of patients with VWD-type 2B or TTP encouraged us to analyse other conditions with suspected VWF-dependent platelet aggregates. One such condition is HELLP (Hemolysis, Elevated Liver enzymes, Low Platelets), a severe complication of preeclampsia during pregnancy [44]. This syndrome displays a number of clinical manifestations (e.g. low platelets and microthrombopathy) similar to those in TTP [45,46]. Like for TTP, HELLP plasma samples were characterized by the presence of elevated amounts of active VWF (3-fold compared to healthy pregnant women) [47]. To understand the origin of active VWF in these patients, a number of additional tests were performed. First, levels of ADAMTS13 activity were determined. In line with a previous report [48], ADAMTS13 levels were reduced to a limited but significant extent (about 75 % compared to normal pooled plasma). On the other hand, VWF antigen levels were strongly increased. Moreover, ratios of propeptide over VWF were increased 3-fold in HELLP-patients but not in pregnant controls or preeclampsia patients [47], pointing to an acute endothelial activation in HELLP-patients [49]. It seems conceivable that the combination of reduced ADAMTS13 with increased levels of freshly released VWF is associated with the escape of some VWF molecules from ADAMTS13-mediated inactivation. Whether these increased levels of active VWF contribute to the microthrombotic pathology of the HELLP syndrome remains to be examined.

Active VWF under pathological conditions: Malaria

Another pathological condition that has drawn our attention is parasitic malaria. Again, advanced stages of malaria are characterized by thrombocytopenia and cerebral thrombotic events. A recent study by Hollestelle *et al*. demonstrated the presence of increased levels of acutely released VWF in the plasma of these patients [50]. In a preliminary analysis, we also found increased levels of active VWF in these plasmas (unpublished observations). To gain more insight into the development of thrombocytopenia during the pathogenesis of malaria, we have initiated a study in which volunteers are experimentally infected with *Plasmodium falciparum* [51]. Daily analysis of platelet counts revealed that a reduction in platelet counts occurs early during infection, even before the appearance of a positive blood smear. VWF levels (both total antigen and active VWF) increased complementary to the drop in platelet counts. Apparently, the release of merozoites from hepatocytes coincides with both an increase of endothelial-derived active VWF and decreased platelet count. Again, further studies are needed to investigate (1) mechanisms that trigger the release of VWF from the endothelium so early in the disease, and (2) if active VWF is responsible for the platelet drop. Another intriguing aspect that deserves further study is whether or not such correlation is specific for malaria, or a more common phenomenon following pathogen infection. Indeed, several infections are manifested by thrombocytopenia, and Othman and colleagues recent-

ly demonstrated that the thrombocytopenia that occurred upon adenovirus infection was in part mediated by VWF [52].

Active VWF under pathological conditions: Antiphospholipid syndrome

Another look-alike of TTP is the antiphospholipid syndrome, in which thrombocytopenia and microthrombopathy are common clinical manifestations [46]. The antiphospholipid syndrome is an autoimmune disease manifested by thrombotic complications and/or recurrent pregnancy loss that occur in combination with the persistent presence of autoantibodies against a variety of (mostly phospholipid-binding) proteins, including ß2-glycoprotein I (ß2-GPI) [53]. Antibodies against these proteins may be monitored via a number of different laboratory assays, the most commonly used of which is the phospholipid-based coagulation assay, Lupus anticoagulans (LAC). The LAC-test may for instance be used to determine if the prolongation of the clotting time in this test is dependent on the presence of antibodies against ß2-GPI or against other proteins, like prothrombin. It is important to make this distinction, because a ß2-GPI dependent LAC is associated with a more than 40-fold increased risk for thrombotic complications, whereas this is but 2-fold for ß2-GPI independent LAC [54]. When we analysed samples of antiphospholipid-syndrome patients, we were surprised to detect active VWF in patients with ß2-GPI dependent LAC (about 2-fold compared to normal controls) but not in those with a ß2-GPI independent LAC [55]. In search for an explanation for these slightly, but significantly increased levels of active VWF, a number of possibilities were addressed. This revealed that the presence of active VWF was not due to (i) an acute activation of the endothelium; (ii) reduced activity of ADAMTS13, or (iii) the presence of autoantibodies that drive VWF in its active conformation. We subsequently challenged the hypothesis that ß2-GPI itself influences active VWF levels via direct interactions. Indeed, ß2-GPI was found to interact with VWF, albeit rather weakly, but binding was enhanced several-fold upon conversion of VWF into its active GpIbα-binding conformation [55]. Interestingly, ß2-GPI binds to the VWF A1-domain, and interferes with the VWF-GpIbα interaction, as is illustrated by the inhibitory effect of ß2-GPI on VWF-dependent platelet agglutination and adhesion. This inhibitory effect could be neutralized by the addition of anti- ß2-GPI antibodies, either monoclonal or derived from antiphospholipid-syndrome patients. This neutralizing effect of anti- ß2-GPI antibodies may provide an explanation for the selective presence of active VWF in those patients who have a ß2-GPI-dependent LAC. Moreover, these findings suggest the presence of a previously unrecognised mechanism that regulates the availability of active VWF: ß2-GPI preferentially recognizes active VWF, and might therefore act as a weak, but natural inhibitor of active VWF. As such, ß2-GPI may prevent small amounts of active VWF to react prematurely with circulating platelets.

Conclusion

The above described findings have provided new insight into the presence of active VWF under various pathological conditions. Currently, studies in our laboratory and of others are ongoing to expand the list of conditions in which the presence of active VWF may contribute to the pathophysiology of the disease. These include myocardial infarction, disseminated intravascular coagulation and malignant hypertension. Besides knowing whether active VWF levels are increased or not, it is also of importance to address the

origin of active VWF in such conditions (see Table 1). By doing so, we have uncovered a novel ß2-GPI dependent mechanism that may contribute to the regulation of VWF-platelet interactions. Nevertheless, additional studies are needed to further unravel the fundamental aspects of what makes VWF active and what determines it to bind to platelets.

Table 1. Pathological conditions that are characterized by the presence of circulating active VWF

Disease	Origin active VWF	Active VWF levels (relative to controls)	Reference
Von Willebrand disease type 2B	Gain-of-function mutation in VWF-A1 domain results in increased affinity for GpIbα	2-15 fold increase	[2]
Congenital/ acquired TTP	Absence of ADAMTS13 prevents inactivation of freshly secreted VWF	2-12 fold increase	[2]
HELLP syndrome	Endothelial activation and mild decrease in ADAMTS13	3-fold increase	[47]
Malaria	Endothelial activation?	Up to 7-fold increase	unpublished
Antiphospholipid syndrome	Anti-ß2-glycoprotein I auto-antibodies prevent shielding of active VWF	2-fold increase	[55]

GpIbα, glycoprotein Ibα; TTP, thrombotic thrombocytopenic purpura; ADAMTS, a disintegrin and metalloprotease with thrombospondin motif; HELLP, hemolysis, elevated liver enzymes, low platelets.

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Chapter 2

Outline of this thesis

Outline of this thesis

Thrombotic thrombocytopenic purpura (TTP) and malaria are two diseases of distinct origin. TTP is a rare disorder caused by a deficiency of the von Willebrand factor (VWF) cleaving protease ADAMTS13. Malaria is a poverty-related disease caused by protozoan parasites from the genus *Plasmodium*. TTP and malaria share several clinical symptoms including intravascular platelet agglutination with thrombocytopenia, haemolytic anemia, neurological symptoms and fever.

VWF secreted from endothelial cells displays spontaneous platelet-binding properties in the absence of ADAMTS13. ADAMTS13-mediated proteolysis reduces the accessibility of platelet adhesion sites by converting VWF from an active into a latent conformation. Aberrations in VWF have been described for both TTP and malaria. In acute TTP, when patients suffer from thrombosis and require plasma exchange, absence of ADAMTS13 results in the presence of active VWF multimers in the circulation. Consequently, microthrombi are being formed that cause occlusion of the microvasculature and organ damage. With respect to remission, when patients do not longer require plasma exchange to prevent thrombosis, no information is available about active VWF. For malaria, elevated VWF-antigen levels have been reported in field studies, but the eventual role of active VWF in the development of platelet-clumping and thrombocytopenia is unknown.

This thesis aims to provide more insight into the origin of active VWF and the role of active VWF in TTP and malaria. Chapter 3 focuses on endothelial cells and reports studies on the origin of active VWF. Chapter 4 describes VWF- and ADAMTS13-related features in a cohort of TTP patients in remission and gives insight in the composition of active VWF multimers. Chapter 5 addresses the relevance of an ADAMTS13 activity assay, based on fluorescence resonance energy transfer, with regard to TTP diagnosis. Chapters 6 and 7 describe the relation between (active) VWF, ADAMTS13 and thrombocytopenia in an experimental human infection malaria study involving healthy volunteers (chapter 6) and in a field study on the Indonesian island Sumba (chapter 7). In the last chapter (chapter 8), the findings from the preceding chapters are taken together and discussed.

2

Chapter 3

Regulation of von Willebrand factor activity during release from endothelial cells

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SUMMARY

Von Willebrand factor (VWF) can exist in two different conformations. The most common conformation is the plasma form, in which VWF is unable to interact with the platelet receptor glycoprotein (Gp)-Ib (the resting conformation). A second form of VWF, as expressed in von Willebrand disease (VWD) type 2B or thrombotic thrombocytopenic purpura is able to interact directly with GpIb (the active conformation). In this study we have used AU/VWF-a11, a nanobody that recognizes only the active conformation, to study the fate of VWF during storage and release from endothelial cells. VWF inside the Weibel-Palade bodies of endothelial cells is stored in a resting conformation. Upon secretion under static conditions, VWF switches to a conformation that binds AU/VWF-a11 but is unable to support platelet deposition. VWF, secreted under conditions of flow, is in its active conformation and arranges as thing strings to which platelets adhere. Under these conditions, not all platelet-binding sites are accessible as 3-fold more platelets adhere to ristocetin activated strings or strings secreted from von Willebrand disease type 2B cells. Osteoprotegerin (OPG), an important inhibitor of osteoclastogenesis, has been shown to co-localize with VWF in the Weibel-Palade bodies and to remain in complex after secretion. We showed by immunofluorescence that OPG remains associated to VWF strings under conditions of flow. In addition, platelet adhesion to a VWF-coated surface was reduced by 40% in the presence of OPG. OPG is thus a novel inhibitor of VWF function by interfering with VWF-dependent platelet adhesion.

INTRODUCTION

Von Willebrand factor (VWF) plays an essential role in the adhesion of platelets to the injured vessel wall. At higher shear rates, it not only functions as a molecular glue between platelets and the vessel wall, but it also mediates platelet-platelet interaction. VWF is a high molecular weight multimeric molecule that is build up out of identical units that are multimerized by disulphide bonds. Each unit consists of a series of structural domains (D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK) of which the A-domains are of particular importance for platelet deposition to the vessel wall. VWF from endothelial cells is either constitutively secreted into the circulation or targeted to rod-shaped storage organelles called Weibel-Palade bodies. Several other proteins have been identified to co-localize with VWF inside the Weibel-Palade bodies and co-trafficking of such a protein to these organelles relies on its interaction with VWF. Upon secretion, some proteins remain in complex with VWF, among which osteoprotegerin (OPG). OPG is a decoy receptor that functions in the inhibition of osteoclast differentiation and bone resorption. Zannettino and colleagues demonstrated the presence of OPG in the Weibel-Palade bodies of endothelial cells and its physiological association with VWF in plasma [1]. Biochemical characterization revealed that OPG binds to the VWF A1 domain, the domain being crucial for interaction with platelets [2,3].

Under conditions of flow, stimulated endothelial cells secrete long stretches of VWF molecules that can interact spontaneously with glycoprotein (Gp)Ibα at the platelet surface [4-6]. VWF in the Weibel-Palade bodies may thus be readily stored in its active, platelet-binding conformation. Alternatively, VWF could be stored in a resting conformation and become activated upon secretion, for example by shear stress. In the present paper we have used AU/VWF-a11, a nanobody that specifically recognizes the GpIb-binding conformation of VWF [7], to show that VWF in the Weibel-Palade bodies of endothelial cells is stored in a resting conformation. Under conditions of flow, secreted VWF undergoes a conformational change that allows platelets to adhere. However, shear stress might not the only factor involved as more platelets can adhere to ristocetin activated VWF strings or strings released from von Willebrand disease (VWD) type 2B endothelial cells. Immunofluorescence microscopy revealed that OPG decorated the VWF strings. Subsequently, the role of OPG in platelet adhesion to VWF was investigated.

MATERIALS AND METHODS

Proteins and antibodies

Llama derived nanobody AU/VWF-a11 is directed against the VWF A1 domain and specifically recognizes the platelet-binding conformation of VWF [7]. Nanobody AU/VWF-C37H is directed against the VWF A3 domain and recognizes the platelet-binding as well as the non-binding conformation. This nanobody was developed and characterized in our lab and has not been described elsewhere. The nanobodies were conjugated to Alexa Fluor dyes 488 or 568 (Invitrogen). Polyclonal anti-VWF antibody (DakoCytomation) binding was visualized by a TRITC-conjugated goat-anti-rabbit antibody from Jackson Immunoresearch Europe Ltd. FITC-conjugated polyclonal goat-anti-llama antibody was obtained from Bethyl laboratories. Plasma-derived VWF was purified

from cyoprecipitate (Haemate P 250 IE, Behringwerke AG, Marburg, Germany) as described [8]. Recombinant human OPG (TNFRSF11B) and a monoclonal antibody against OPG (MAB805) were purchased from R&D systems.

Cell culture

Human umbilical vein endothelial cells (HUVEC) from healthy newborns and from a newborn with von Willebrand disease (VWD) type 2B (mutation arginine to glutamine at position 1306) were isolated as described [9] and cultured in endothelial basal medium (EBM-2) supplemented with EGM-2 bulletkits (Lonza).

Static stimulation

HUVECs were grown to confluence in 24-wells plates on glass coverslips. After removing the medium, cells were washed for 1 hour in serum-free medium containing 2 mM L-Glutamine (Gibco) and 1% bovine serum albumin (Sigma GmbH). VWF secretion was induced by incubation with 20 ng/mL phorbol-12-myristate-13-acetate (PMA) in serum-free medium containing 2 mM L-Glutamine (Gibco) and 1% bovine serum albumin for 60 minutes. After stimulation, supernatant was collected for quantification of active VWF or cells were fixed in 4% paraformaldehyde for 30 minutes for immunostaining experiments. To study whether VWF secreted under static conditions supports platelet adhesion, HUVECs were stimulated with PMA for 60 minutes after which fixed platelets were added for 30 minutes. Cells and eventual adhered platelets were fixed in 4% paraformaldehyde for 30 minutes for immunostaining experiments.

Preparation of washed platelets

Blood from healthy donors was collected in 3.1% sodium citrate. Platelet-rich plasma (PRP) was prepared by centrigugation (156 x *g*, 15 minutes). After addition of acid citrate dextrose (0.1% of the PRP volume), PRP was centrifuged 15 minutes at 330 x *g*. The platelet pellet was resuspended in HEPES-Tyrode buffer pH 6.5 (10 mM HEPES, 137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH_2PO_4, 1.7 mM $MgCl_2$, 11.9 mM $NaHCO_3$) supplemented with prostaglandin (10 ng/mL). Platelets were centrifuged again 15 minutes at 330 x *g*. Then the platelet pellet was resuspended in HEPES-Tyrode buffer pH 7.3. All donors gave written informed consent. Approval was obtained from the institutional review board of the University Medical Centre Utrecht (Utrecht, The Netherlands).

Perfusion

HUVECs were grown on glass coverslips till confluence. Prior to perfusion, coverslips were washed once in Medium 199 (Hyclone). Platelets (150.000/µL) were perfused over the HUVECs at a shear rate of 300 s^{-1}. Formation of VWF strings was visualized using an Axio Observer microscope (Zeiss). After 2,5 minutes of perfusion, a snapshot was taken of every experiment for quantification of the amount of platelets that adhered to a VWF string. In other experiments, the perfused coverslips were fixed under flow (4% paraformaldehyde for 5 minutes, 300 s^{-1}), followed by static fixation for 30 minutes in 4% paraformaldehyde. These fixed coverslips were subsequently used for immunostaining with anti-VWF and anti-OPG antibodies as described below.
Perfusions over VWF-coated glass coverslips were performed 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%. Glass coverslips were coated

with VWF (10 µg/mL). Prior to perfusion, coverslips were incubated with recombinant human OPG (10 µg/mL in 20 mM Bis-Tris, 10 mM $CaCl_2$, pH 7.3 buffer) for 1 hour at room temperature. As a control, VWF-coated coverslips were incubated in the same buffer without OPG. Perfusion experiments were performed at a shear rate of 300 s^{-1}. After perfusion, the coverslips were washed, fixed and stained with May-Grühnwald and Giemsa after which platelet adhesion was evaluated using OPTIMAS 6.0 software (Dutch Vision System, Breda, The Netherlands). Perfusions were performed with blood from 3 different donors.

Immunostaining experiments

For immunostaining experiments, HUVECs were grown to confluency on glass coverslips and fixed in 4% paraformaldehyde for 30 minutes at room temperature. They were washed 3 times in PBS and permeabilized in 0.5% Triton X-100 for 5 minutes. After blocking with 1% BSA in PBS (10 minutes), cells were incubated with Alexa Fluor 488-conjugated AU/VWF-a11 and Alexa Fluor 568-conjugated AU/VWF-C37H for 30 minutes at 37 ºC. Cells were washed again in PBS and embedded in FluorSave (Calbiochem).

In stimulation and flow experiments, cells were not permeabilized in order to study the presence of extracellular VWF. To stain extracellular VWF, initial staining procedures were similar as described for the non-stimulated HUVECs, but incubation with AU/VWF-a11 was followed a FITC-conjugated secondary antibody to amplify the fluorescent signal of the thin VWF strings. In these experiments, polyclonal rabbit-anti-VWF antibody followed by TRITC-conjugated secondary antibody incubation was used instead of AU/VWF-C37H as the small llama nanobodies (15 kDa) can partly penetrate paraformaldehyde fixed cells without permeabilization.

For detection of OPG, HUVECs were fixed in methanol which did not require permeabilization prior to antibody incubation. After blocking in 1% BSA in PBS (10 minutes), cells were incubated with mouse anti-OPG followed by a FITC-conjugated secondary antibody (30 minutes at 37 ºC). For these experiments, HUVECs were double-stained with an anti-OPG antibody followed by a FITC-conjugated secondary antibody and an anti-VWF antibody followed by a TRITC-conjugated secondary antibody (30 minutes at 37 ºC).

Quantification of active VWF levels

Active VWF levels were determined using an immunosorbent assay based on llama nanobody AU/VWF-a11 as described [7]. The term VWF activation factor was used to express the relative amount of VWF that circulates in its active conformation. VWF activation factor of NPP was referred to as 1.

Statistical analysis

Analyses of data were performed using GraphPad Prism software (GraphPad Prism version 4.0, GraphPad Software, San Diego, CA). All data were expressed as mean ± SD. The *t* test for paired observations was used to compare data obtained in perfusion experiments. *P*-values below 0.05 were considered significant.

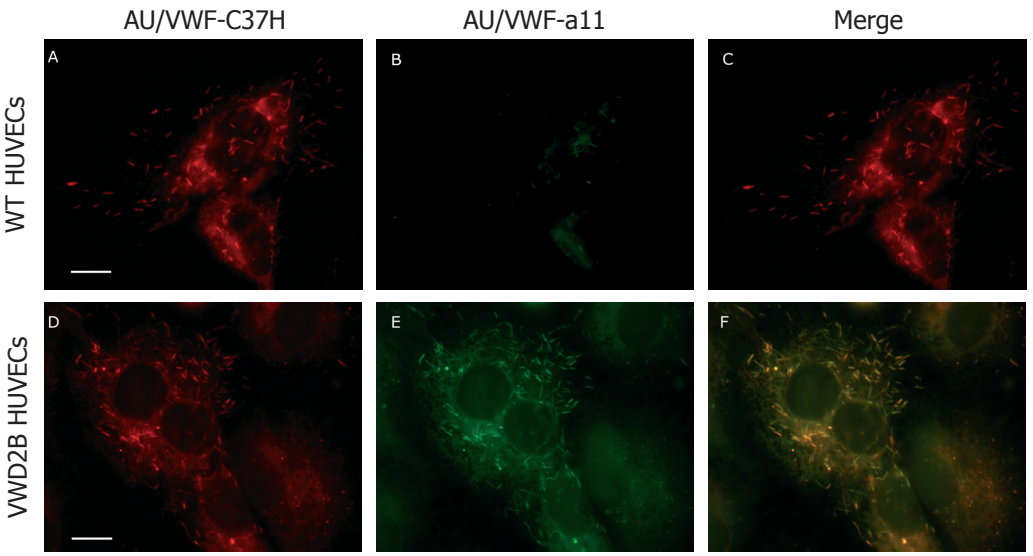

Figure 1. Von Willebrand factor (VWF) is stored in its resting conformation in the Weibel-Palade bodies of human umbilical vein cells (HUVECs). *Cultured wildtype (WT) HUVECs (A-C) and von Willebrand disease (VWD) type 2B HUVECs (D-F) were permeabilized and stained with Alexa Fluor 568-conjugated AU/VWF-C37H (A,D) and Alexa Fluor 488-conjugated AU/VWF-a11 (B,E). Merged images are shown in C and F. AU/VWF-C37H binds VWF in the Weibel-Palade bodies of WT and VWD-type 2B HUVECs (A,D). AU/VWF-a11 staining was negative in WT HUVECs (B). In VWD type 2B HUVECs, AU/VWF-a11 fluorescence was observed in the Weibel-Palade bodies (E) which co-localized with AU/VWF-C37H staining (F). Bars represent 10 micron.*

RESULTS

VWF within the Weibel-Palade bodies does not possess platelet-binding capacities

To study whether VWF in the Weibel-Palade bodies of endothelial cells is stored in a GpIb-binding conformation, fixed HUVECs were permeabilized and double-stained with Alexa Fluor-conjugated nanobodies AU/VWF-a11 and AU/VWF-C37H. AU/VWF-a11 is directed against the VWF A1 domain and specifically recognizes the GpIb-binding conformation of VWF. AU/VWF-C37H is directed against the VWF A3 domain and recognizes both the active and the resting conformation of VWF. AU/VWF-C37H distinctly stained the Weibel-Palade bodies of WT HUVECs (Figure 1A), while no fluorescence was observed for AU/VWF-a11 (Figure 1B). Apparently, VWF in the Weibel-Palade bodies is stored in a resting conformation. VWD type 2B patients are characterized by high active VWF levels in plasma due to a gain-of-function mutation in the VWF A1 domain [7]. To show that AU/VWF-a11 is able to recognize active VWF in the Weibel-Palade bodies,

immunofluorescence experiments were also performed on HUVECs of a newborn with VWD type 2B. In contrast to WT HUVECs, AU/VWF-a11 fluorescence was found present in the Weibel-Palade bodies of the VWD-type 2B HUVECs where it co-localized with AU/VWF-C37H (Figure 1D-F).

Stimulated HUVECs secrete VWF in its active conformation

Next we investigated whether VWF exposes its platelet-binding sites after secretion from endothelial cells. WT HUVECs were stimulated under static conditions with PMA for 15 minutes to induce VWF secretion. After stimulation, the cells were fixed and double-stained with AU/VWF-a11 and a polyclonal anti-VWF antibody. Results are shown in Figure 2. Polyclonal antibody staining revealed that the HUVECs secreted irregularly shaped linear VWF (Figure 2A). These VWF-structures were not observed on non-stimulated cells. AU/VWF-a11 staining revealed co-localization with the polyclonal anti-VWF antibody

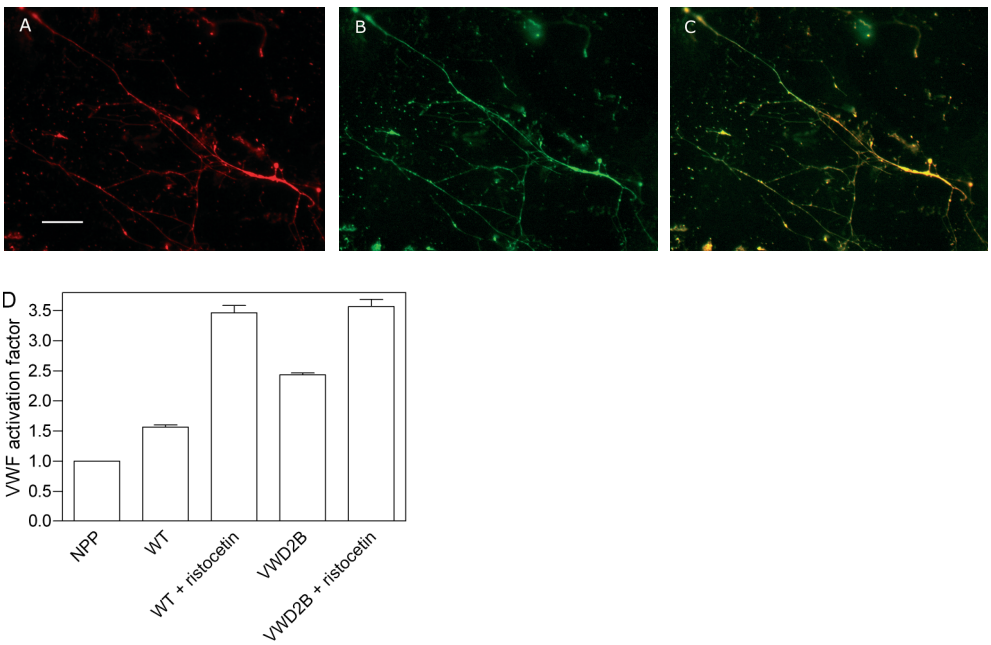

Figure 2. Stimulated human umbilical vein endothelial cells (HUVECs) secrete von Willebrand factor (VWF) that binds AU/VWF-a11 but not platelets. *Confluent HUVECs were stimulated with 20 nM phorbol-12-myristate-13-acetate (PMA) for 1 hour. Cells were either fixed in 4% paraformaldehyde for immunofluorescence experiments (A-C) and supernatant was collected for analysis in immunosorbent assays (D). Extracellular VWF was double stained with an anti-VWF antibody followed by a TRITC-conjugated secondary antibody (A) and Alexa Fluor 488-conjugated AU/VWF-a11 followed by a FITC-conjugated secondary antibody (B). Merged image is shown in panel C. Bar represents 10 micron. (D) Microtiter wells immobilized with AU/VWF-a11 were incubated with normal pooled plasma (NPP) or supernatant from stimulated wildtype or von Willebrand disease type 2B HUVECs. HUVEC-VWF was incubated both in the absence and presence of ristocetin. Bound VWF was detected using horse radish peroxidase-conjugated polyclonal anti-VWF antibody. The relative amount of active VWF was compared to NPP and was expressed as VWF activation factor.*

(Figure 2B,C). Apparently, HUVEC-VWF is readily secreted in its active conformation under these conditions. Surprisingly, incubation of these stimulated cells with fixed platelets did not result in adherence of the platelets to these VWF structures (data not shown).

Supernatants from the stimulation experiments were measured for the amount of active VWF in the AU/VWF-a11 based ELISA (Figure 2D). VWF secreted from WT HUVECs displayed a VWF activation factor of 1.6 which is comparable to values found in acute TTP patients. The VWF activation factor of VWF secreted from the VWD type 2B HUVECs was found to be higher, namely 2.4. Upon addition of ristocetin, VWF activation factors of both WT- and type 2B-VWF increased to a similar extent, as VWF activation factors were respectively 3.3 and 3.4. Apparently, VWF secreted from endothelial cells is more active compared to VWF in plasma but can be activated further.

HUVECs secrete active VWF strings under flow

Platelets readily bind to VWF secreted from endothelial cells and form platelet-decorated strings under conditions of flow [6]. We therefore assumed that these VWF strings would be recognized by nanobody AU/VWF-a11. In order to investigate this, we used a perfusion model based on the experiments described by Dong et al and combined this with AU/VWF-a11-based immunofluorescence experiments. Fixed platelets were perfused over a monolayer of HUVECs at a shear rate of 300 s^{-1}. Fixed platelets adhere normally to VWF and were used instead of washed platelets to ensure that the stained

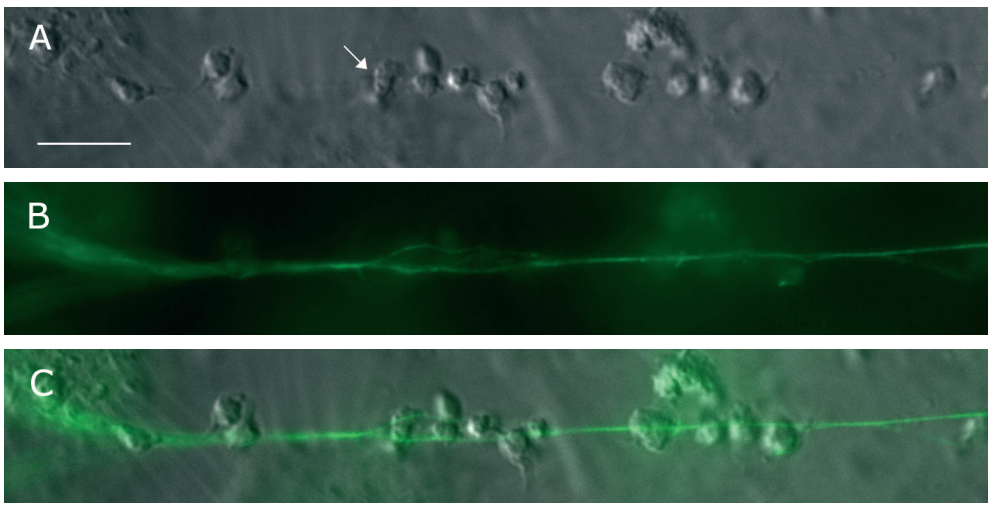

Figure 3. Human umbilical vein endothelial cells (HUVECs) secrete active von Willebrand factor (VWF) strings under flow*. Fixed platelets were perfused over wildtype HUVECs at a shear rate of 300 s^{-1}. After perfusion, coverslips were fixed in 4% paraformaldehyde under flow and incubated with Alexa Fluor 488-conjugated AU/VWF-a11 followed by FITC-conjugated secondary antibody. Differential interference contrast microscopy revealed adherence of platelets to secreted VWF. The arrow points at platelets bound to two branches of a bifurcating VWF string (A). AU/VWF-a11 staining was found as a thin string interconnecting the platelets (B). Merged image is shown in panel C. Bar represents 10 micron.*

VWF was exclusively derived from the endothelial cells and not from the platelets. When compared, we found that fixed and washed platelets adhered similarly to the strings. Upon exposure to laminar flow, HUVECs secreted long strings of adhesive VWF-multimers to which fixed platelets adhered (Figure 3A). After perfusion, the coverslips were fixed under flow and stained afterwards with AU/VWF-a11. As expected, the strings were visible as thin lines interconnecting the adhering platelets (Figure 3B). Platelets usually arranged alongside each other but occasionally seemed to stack on top of each other (see arrow in Figure 3A). In fact, this appeared to be binding of platelets to two branches of a bifurcating VWF string (Figure 3C).

VWF strings can be further activated

Although AU/VWF-a11 recognized the whole VWF string, platelets did not completely cover the string. This suggested that not all platelet-binding sites along the VWF strings were available for platelets. To investigate whether the VWF strings could be further activated, we tested the influence of ristocetin on the adhesion of platelets to the strings. Fixed platelets, either or not mixed with ristocetin, were perfused over a monolayer of HUVECs at a shear rate of 300 s^{-1}. Of each perfusion experiment, a real-time snapshot was taken after 2,5 minutes of perfusion and the number of platelets that adhered to a string was counted. Representative images of perfusion experiments with normal platelets and platelets mixed with ristocetin are shown in Figure 4A and 4B. Mean platelet coverage was calculated by dividing the number of platelets that adhered to a VWF string by the length of that string. In the absence of ristocetin, the mean platelet coverage (± SD) based on 6 independent experiments was 0.15 ± 0.03 platelets/µm VWF string (Figure 4A,D). Addition of ristocetin significantly increased the mean platelet coverage to 0.43 ± 0.06 platelets/µm VWF string (Figure 4,D; n = 6; $P < 0.0005$). VWF strings that were activated with ristocetin were fully covered with platelets, in contrast to what was seen in the absence of ristocetin. To investigate whether we could mimic the effect of ristocetin in a more physiological way, perfusion experiments were performed on HUVECs from a newborn with VWD type 2B. Perfusion of normal platelets over the VWD type 2B HUVECs resulted in an adhesion pattern that was similar to what was seen for WT HUVECs after activation by ristocetin (Figure 4C). Apparently, when exposed to laminar flow, WT VWF strings expose only part of their platelet-binding sites as more platelets can adhere to ristocetin-activated strings or strings released from VWD type 2B HUVECs.

OPG remains associated with VWF under conditions of flow

We next hypothesized that adherence of platelets to WT VWF strings might be influenced by binding of certain proteins also present in the Weibel-Palade bodies. OPG is a likely candidate as it was shown to (i) co-localize with VWF in the Weibel-Palade bodies, (ii) remain associated with VWF after secretion and (iii) interact with VWF via the VWF A1 domain [3]. To investigate whether OPG also remains associated with VWF under conditions of flow, HUVEC-coated coverslips were fixed after perfusion with washed platelets, and double stained with antibodies against OPG and VWF (Figure 5A-C). Co-localization was observed along the strings, indicating that OPG remains associated with secreted VWF under conditions of flow.

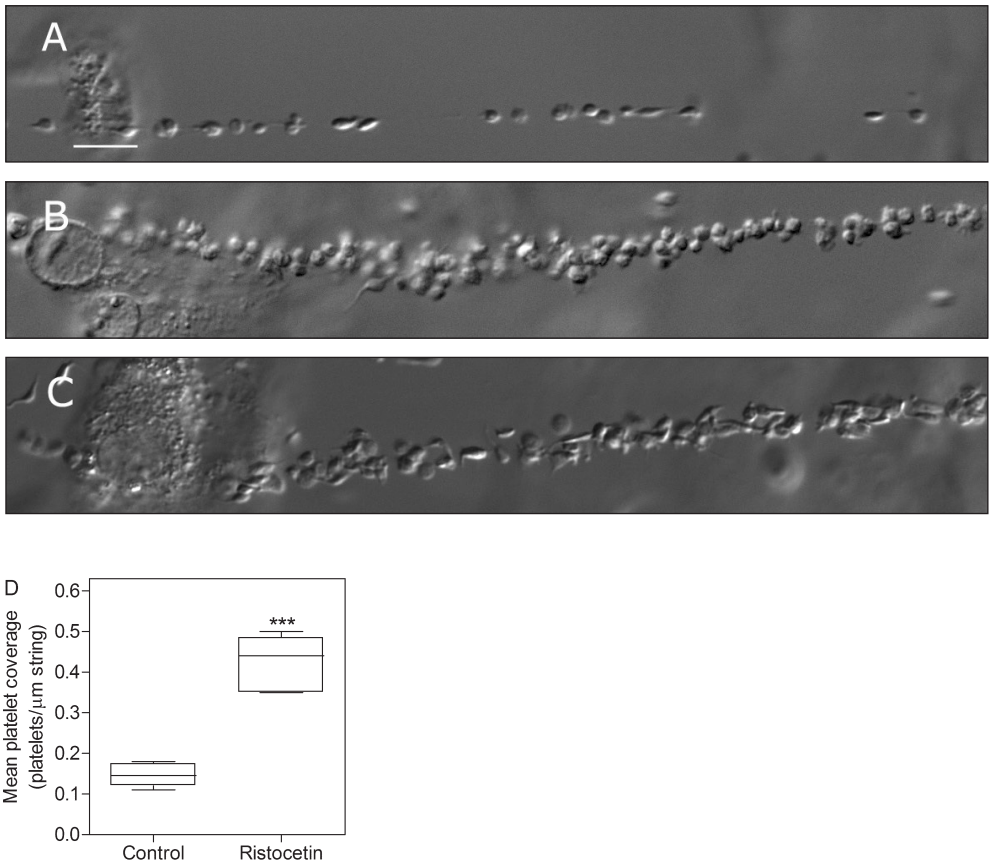

Figure 4. Von Willebrand factor (VWF) strings can be further activated. *Fixed platelets in the absence (A) or presence (B) of ristocetin were perfused over a monolayer of wildtype (A,B) or von Willebrand disease type 2B HUVECs (C) at a shear rate of 300 s^{-1}. Snapshots were taken after 2,5 minutes of perfusion over wildtype HUVECs in the presence and absence of ristocetin and the mean platelet coverage was calculated by dividing the number of adhered platelets by the length of a string. Results were plotted in a box and whiskers graph (D). Addition of ristocetin significantly increased the mean platelet coverage (*** P < 0.0005). Data represent the mean (± SD) of 6 independent experiments.*

OPG inhibits platelet adhesion to VWF under conditions of flow

We next investigated the effect of OPG on platelet adhesion to immobilized VWF in a perfusion system. Prior to perfusion, plasma-derived VWF-coated glass coverslips were incubated with recombinant OPG or buffer. Reconstituted blood was perfused over the coverslips at a shear rate of 300 s^{-1}. Platelet coverage to VWF incubated with buffer in the absence of OPG was set at 100% (Figure 6,C). Pre-incubation of the VWF-coated surface with OPG resulted in a 40 ± 18% decrease in platelet coverage compared to incubation with buffer ($P < 0.0001$; n=3) (Figure 6B,C).

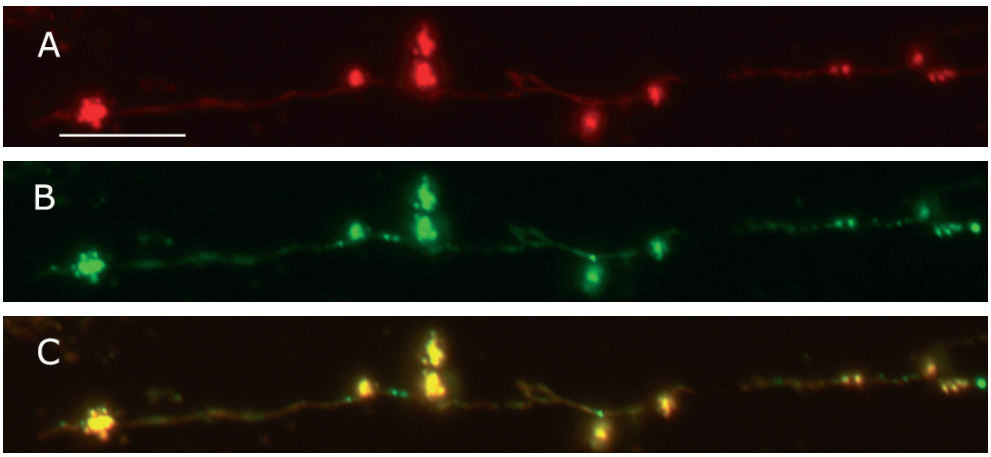

Figure 5. Osteoprotegerin (OPG) remains associated with von Willebrand factor (VWF) under conditions of flow. *Washed platelets were perfused over wildtype human umbilical vein endothelial veins (HUVECs) at a shear rate of 300 s^{-1}. After perfusion, coverslips were fixed in methanol and double-stained with an anti-VWF antibody followed by a TRITC-conjugated secondary antibody (A) and an anti-OPG anti-OPG antibody followed by a FITC conjugated secondary antibody (B). Merged image is shown in panel C. Bar represents 10 micron.*

DISCUSSION

VWF is mainly synthesized in endothelial cells. It is stored in the Weibel-Palade bodies from where it is released upon stimulation. Here we show that VWF is stored inside the Weibel-Palade bodies in its resting conformation, unable to directly interact with platelets. Upon stimulation, VWF is not only released into the medium but also deposited onto the cells. The release of VWF from the Weibel-Palade bodies coincides with a conformational change because both VWF in the medium and VWF deposited on the cells can be recognized by AU/VWF-a11, a nanobody that specifically recognizes VWF in its active conformation. However, both VWF in the medium and VWF anchored to the cell membrane are not able to bind platelets spontaneously. Only after release of VWF from the cells under conditions of flow, long strings of VWF are formed with platelets

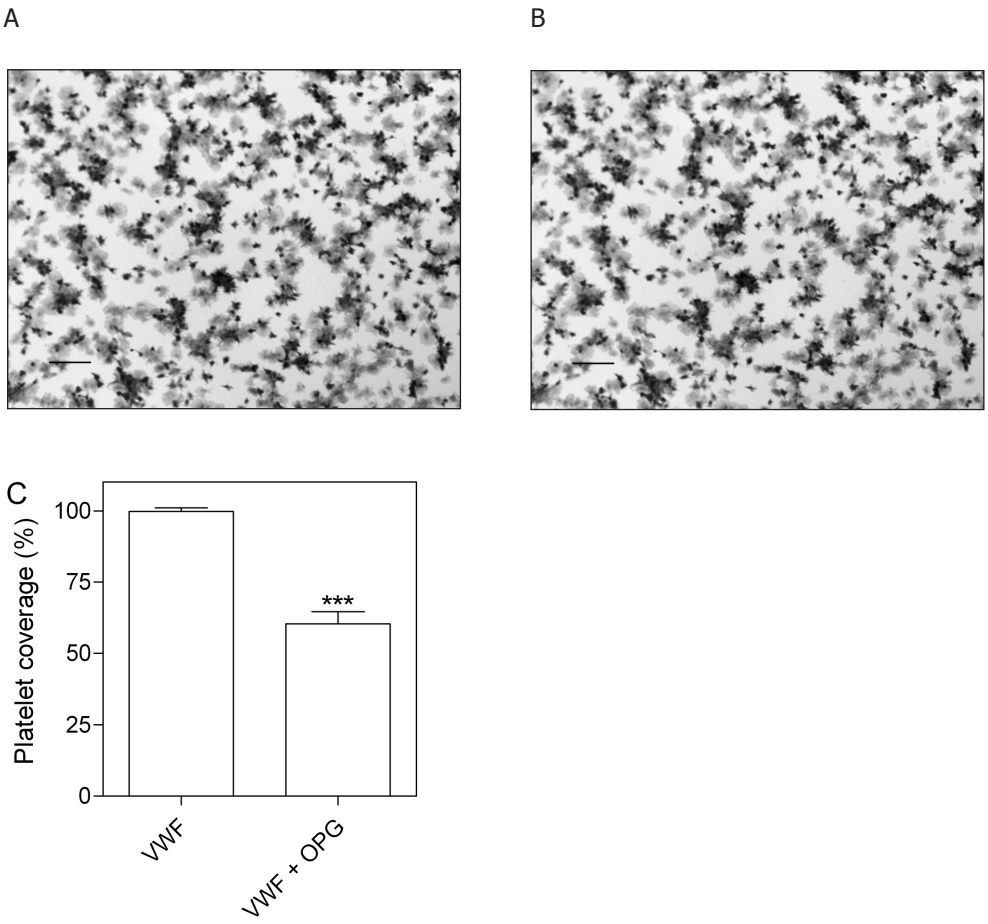

Figure 6. Osteoprotegerin (OPG) inhibits platelet adhesion to von Willebrand factor (VWF) under conditions of flow. *Glass coverslips were coated with VWF and subsequently incubated with buffer (A) or OPG (B). Reconstituted blood was then perfused over these coverslips at a shear rate of 300 s^{-1}. After perfusion, coverslips were fixed in 0.5% glutaraldehyde, dehydrated in methanol and stained with May-Grünwald and Giemsa. Adhered platelets were visualized using light microscopy. Dark regions represent platelet aggregates. Platelet adhesion was evaluated using computer-assisted analysis and expressed as the percentage of surface covered with platelets (C). To compare experiments, platelet coverage on VWF + buffer was set at 100% and relative coverage was calculated for VWF + OPG. Data represent the mean (± SD) of 3 experiments. *** P < 0.0001. Bar represents 20 micron.*

adhering to them. Experiments in which ristocetin is added show that not all potential platelet binding sites are occupied with platelets. An explanation for the restricted platelet adhesion could be that together with VWF, OPG is released from the cells and stays attached to the A1 domain of VWF. Indeed, addition of OPG inhibits the binding of platelets to VWF in *in vitro* models. The presence of OPG seems to prevent released VWF under conditions of flow of becoming fully active.

VWF is tightly packaged into ordered tubules in the Weibel-Palade bodies of endothelial cells. Here, AU/VWF-a11 does not recognize VWF. This indicates that VWF in the Weibel-Palade bodies is stored in its resting conformation, a conformation that does not support platelet adhesion. Upon stimulation under static conditions, VWF undergoes a conformational change, as was shown by AU/VWF-a11 binding. However, platelets did not interact with VWF in the medium or with VWF that remained anchored to the cell membrane. These data were in line with the findings of de Groot and colleagues who showed that VWF secreted from normal endothelial cells does not induce platelet aggregation in the absence of ristocetin, while VWF secreted from VWD type 2B endothelial cells induced spontaneous platelet aggregation [10]. Nanobody AU/VWF-a11 does not inhibit platelet binding to the VWF A1 domain, indicating that AU/VWF-a11 and GpIb recognize a different epitope on A1. There are several possible explanations of why VWF released from endothelial cells under static conditions is recognized by AU/VWF-a11, but not by platelets. Firstly, only a few A1 domains within a VWF multimer are active. It is likely that a platelet needs several activated A1 domains in order to bind to VWF. If only a few A1 domains of secreted VWF are active, this might be enough for visualisation of AU/VWF-a11 binding, but not enough for a platelet to bind. Secondly, activation of VWF can take place in different steps. Secretion under static conditions might lead to exposure of the binding site for AU/VWF-a11, but not yet for platelets. In the next step of activation, VWF further unfolds and becomes fully activated, allowing platelets to bind. This second step in VWF activation could be induced by shear stress [11]. Thirdly, it is possible that under static conditions, platelet adhesion is inhibited by the presence of proteins that are bound to VWF and shield the platelet binding sites. So far, only one inhibitor of VWF function has been described. ß2-glycoprotein I (ß2-GPI), the major antigenic target of autoantibodies in the antiphosholipid syndrome, has been shown to function as a physiological inhibitor of the VWF-GpIb interaction [12]. However, ß2-GPI is not synthesized in endothelial cells and could thus not be responsible for the inhibition of platelet adhesion to VWF secreted from endothelial cells. Alternatively, platelet adhesion to VWF could be inhibited by proteins that co-localize with VWF in the Weibel-Palade bodies, for example VWF propeptide, interleukin-8, CD63, P-selectin, angiopoietin-2 and OPG. Here we have shown that OPG functions as an inhibitor of platelet adhesion to VWF. Future protein binding studies are necessary to address the questions whether (i) AU/VWF-a11 can bind VWF in the presence of OPG and (ii) OPG can still bind VWF in its platelet-binding conformation. If AU/VWF-a11 and OPG bind different epitopes on VWF A1, it is possible that AU/VWF-a11 can bind VWF secreted under static conditions, while binding of platelets under these conditions is inhibited by OPG. If dissociation of OPG from VWF is the consequence of VWF activation, for example by flow, it is to be expected that OPG will bind to normal VWF, but not to ristocetin activated VWF.

OPG plays a central role in bone turnover through the inhibition of osteoclastogenesis [13]. As part of the OPG/ receptor activator of NF-κB (RANK)/ receptor activator of NF-κB ligand (RANKL) triad, OPG regulates the critical balance between bone formation (osteoblasts) and bone resorption (osteoclasts). OPG acts as a soluble decoy receptor competing for RANKL, thereby inhibiting osteoclast differentiation. The importance of OPG in bone turnover is illustrated by the decreased bone density in OPG deficient mice [14] and in Paget's disease in humans [15]. Besides regulating bone mass, OPG has been suggested to play a role in vascular disease. Initial evidence was based on the observation that OPG knockout mice exhibit vascular calcification [14]. Over recent years, several clinical studies reported elevated serum OPG levels in a range of cardiovascular pathologies. Moreover, Zannettino et al showed that OPG not only circulates as a complex together with VWF, but that it also binds the VWF reductase thrombospondin-1 (TSP-1) [1]. Our data provide further evidence for a role of OPG in vascular disease, as it was shown to directly inhibit platelet binding to VWF, a first step in thrombus formation.

In conclusion, VWF in the Weibel-Palade bodies of endothelial cells is stored in a resting conformation. Upon secretion, VWF undergoes a conformational change but platelets can only adhere after exposure to flow. OPG was identified as a novel physiological inhibitor of VWF function and its binding to VWF strings seems to prevent VWF of being fully active.

ACKNOWLEDGEMENTS
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Chapter 4

The active conformation of von Willebrand factor in patients with thrombotic thrombocytopenic purpura in remission

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SUMMARY

Background: Functional deficiency of ADAMTS13 in thrombotic thrombocytopenic purpura (TTP) patients is associated with circulating ultra-large von Willebrand factor (UL-VWF) molecules that display spontaneous platelet-binding capacities. Upon remission however, ADAMTS13 activity does not always return to baseline. *Objective:* To study ADAMTS13 and VWF-related features in TTP-patients in remission on long-term prospect. *Methods:* ADAMTS13 activity, anti-ADAMTS13 antibodies, VWF antigen, UL-VWF and levels of VWF that circulate in a glycoprotein (Gp)Ibα-binding conformation were determined in plasma samples of 22 acquired TTP-patients in remission between 1 month and 6 years after achieving remission. Composition of active multimers was investigated via a novel immunoprecipitation assay based on monoclonal antibody AU/VWF-a12 that specifically recognizes the active conformation of VWF. *Results:* ADAMTS13 activity was undetectable in 23% of the patients even for years after achieving remission and was associated with increased active VWF levels and the presence of UL-VWF multimers. Active VWF levels and UL-VWF were also associated with blood-groups. Results from immunoprecipitation experiments revealed the full range of multimer distribution to be present. *Conclusion:* ADAMTS13 deficiency and the concurrent presence of UL-VWF and increased active VWF levels can be detected in TTP-patients even after years of achieving remission. Immunoprecipitation results suggest that the active conformation of VWF may be present in the lower molecular weight multimers, but future studies are necessary to confirm our findings.

INTRODUCTION

Von Willebrand factor (VWF) contributes to platelet recruitment at sites of vascular injury. Produced as a monomeric subunit consisting of distinct domains (D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK), VWF undergoes dimerization and subsequently multimerization. VWF is either directly released from endothelial cells in a constitutive or basal manner or stored in the alpha-granules of platelets or in the Weibel-Palade bodies of endothelial cells. The term basal secretion was recently used to describe the release of VWF from post-Golgi compartments of non-stimulated cells, a process distinct from constitutive secretion [1]. Stimulated endothelial cells secrete long stretches of VWF molecules that can interact spontaneously with GpIbα at the platelet surface [2-4]. Plasma VWF however, does not display this spontaneous platelet-binding property. It is generally assumed that binding of the VWF/A3-domain to exposed subendothelial collagen re-installs the platelet-binding properties [5]. Activation of VWF can also be induced by high shear [6,7]. *In vitro*, VWF can adopt its active conformation upon immobilisation or upon addition of ristocetin or botrocetin [8,9].

We have described several pathological conditions in which a disturbed balance between activation and inactivation has led to increased levels of active VWF and thrombotic complications [10]. One of these conditions is thrombotic thrombocytopenic purpura (TTP), a rare life-threatening disease, the clinical features of which include thrombocytopenia, haemolytic anemia, fever and neurological dysfunction. The pathogenesis of TTP relates to the absence of ADAMTS13 activity (either genetic or auto-antibody mediated) and is associated with the formation of platelet-rich aggregates that can occlude microvasculature. We have previously shown that at least part of the VWF molecules circulate in an active, platelet-binding conformation in TTP patients. An assay, based on a llama-derived recombinant antibody (i.e. AU/VWF-a11), was developed allowing us to distinguish between the active and the latent conformation of the VWF/A1-domain [11]. Using this assay, it was shown that levels of circulating active VWF were increased about 2-fold in patients with acquired TTP and 2 to 12-fold in congenital TTP-patients.

The beneficial effect of plasma exchange therapy for TTP-patients is thought to be due to removal of anti-ADAMTS13 inhibitory antibodies and/or the supply of donor ADAMTS13. Remarkably, patients in remission do not always display normalized ADAMTS13 levels. Undetectable ADAMTS13 activity has even been described to persist for several months without development of a new episode [12,13]. Moreover, UL-VWF multimers have also been reported to circulate in patients in remission [13,14]. UL-VWF is generally assumed to be the culprit of thrombosis in acute TTP and therefore its presence in patients in remission is poorly understood. In the current study, we have studied these UL multimers in more detail. We analyzed a cohort of 22 acquired TTP-patients between 1 month and 6 years after achieving remission. We focussed on levels of active VWF, the presence or absence of UL-VWF and the eventual relation between both parameters. From our studies we conclude that ADAMTS13 deficiency and the concurrent presence of active VWF and UL-VWF can be detected in TTP-patients even years after achieving remission without any clinical signs of a thrombotic microangiopathy.

MATERIALS AND METHODS

Patients/ plasma samples

Plasma samples from healthy individuals, VWD-type 2B patients and acquired TTP-patients in remission were collected using 3.1% sodium citrate Vacutainer systems. To obtain normal pooled plasma (NPP), platelet-poor-plasma from 160 healthy individuals was mixed, aliquoted and stored at -80°C. NPP was calibrated against WHO-standards. Plasma samples of TTP-patients were obtained between 1 month and 6 years after they experienced their latest episode. Patients in remission had no clinical signs of microangiopathy and all but one had normal platelet counts (>150x10^9/ml). All donors and patients gave written informed consent. Approval was obtained from the review board of our institute.

Production and selection of recombinant nanobodies

Llama antibodies AU/VWF-a11 and AU/VWF-a12 were both raised by immunization with wt-VWF and selected for their specific binding to the active VWF/A1-domain. Production and selection methods were similar for both nanobodies and have been extensively described [11]. AU/VWF-a11 was used in immunosorbent assays to quantify active VWF levels in plasma and AU/VWF-a12 was used to precipitate and visualize active multimers (see below).

Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords from healthy individuals as described [15]. HUVECs were maintained in EBM-2 Basal Medium supplemented with EGM-2 SingleQuots (Clonetics). First passage cells were used. VWF secretion was induced by stimulation with phorbol-12-myristate-13-acetate (Sigma) for 6 hours. Supernatant was collected for analysis.

Laboratory assays

VWF antigen (VWF:Ag) was quantified as described [16], using NPP as a reference. Active VWF was determined using an AU/VWF-a11 based immunosorbent assay [11]. VWF activation factor was used to express the relative amount of VWF that circulates in its active conformation. VWF activation factor of NPP was referred to as 1. ADAMTS13 activity was measured using fluorescent substrate FRETS-VWF73 (Peptides International, Inc., USA). Presence of auto-antibodies (IgG) against ADAMTS13 were determined using ADAMTS13-INH ELISA (Technoclone GmbH). Briefly, recombinant ADAMTS13 was immobilized on a microtiter plate and wells were incubated with plasma. Bound anti-ADAMTS13 antibodies were detected using HRP conjugated anti-human antibody using TMB as a substrate. Amount of anti-ADAMTS13 antibodies is expressed in units/ml. According to the manufacturer, negative samples contain <12 U/ml, borderline samples 12-15 U/ml and positive samples >15 U/ml antibodies. Specificity and sensitivity of the assay are warranted by the manufacturer. Multimeric composition of VWF was analyzed using 2% SDS/agarose gel-electrophoresis, followed by immunoblotting [17]. After incubation with HRP-conjugated anti-VWF antibody (Dakocytomation), separated multimers were visualized using 3,3' diaminobenzidine tetrachloride (Sigma) as a substrate. Multimeric bands were numbered starting with the smallest sized band

(band 1, 2, 3 etcetera). Based on this counting, patients were categorized in normal sized multimers, intermediate large (IL) multimers or ultra large (UL) multimers, which respectively corresponds to 20, 21-25 and >25 bands.

Specific binding of active VWF to nanobodies

Microtiter wells (Maxisorb) were coated overnight at 4°C with 5 µg/ml AU/VWFa-11 or AU/VWF-a12. After blocking with PBS/3% BSA/0.1% Tween-20 (30 minutes 37°C), wells were washed with PBS/0.1% Tween-20 and incubated with NPP or VWD-type 2B plasma for 1 hour at 37°C. Wells were washed again and incubated with an HRP-conjugated anti-VWF antibody (1.5 µg/ml) for 1 hour at 37°C to detect bound VWF. Wells were washed and binding was detected by measuring HRP activity using OPD as a substrate (Merck).

Nanobody based immunoprecipitation

In precipitation experiments, 3.5 µg biotinylated (Sulfo-NHS-LC-biotin, Pierce) nanobody AU/VWF-a12 was added to that volume of NPP, patient plasma or supernatant from stimulated HUVECs that contained 650 ng VWF. Where indicated, ristocetin was added at 1 mg/ml. Final volume was adjusted to 650 µl with PBS. Nanobody-VWF complex formation was allowed for one hour at 4°C, after which 1 mg of streptavidin-conjugated magnetic beads (Dynabeads M280, Dynal) was added for another hour at 4°C. Magnetically precipitated beads were washed three times in PBS after which they were resolved in sample buffer. Precipitated VWF multimers were analyzed using 2% SDS/agarose gel electrophoresis.

Data analysis and statistics

Statistical analysis was performed using Graphpad v 4.0. Pearson-correlation was used to analyze the correlation between ADAMTS13 activity and active VWF levels. Unpaired T-tests were used to evaluate significant differences between healthy individuals and TTP-patients, patients with IL-VWF and UL-VWF and patients with different blood groups. *P*-values below 0.05 were considered significant.

RESULTS

VWF and ADAMTS13 in TTP-patients in remission

From a cohort of 22 acquired TTP-patients in remission, blood samples were drawn between 1 month and 6 years after their latest episode. Details are summarized in Table 1. Four patients relapsed between 1 and 6 months after blood sampling. Platelet count was normal (>150x10^9/ml) in all but one patient. This patient relapsed one month after blood sampling. ADAMTS13 activity was undetectable in five patients, two of which relapsed between one and four months after blood sampling. Low to normal ADAMTS13 activity was detected in 17 patients (39 ± 28% activity; range 9 - 88%) of which 2 patients relapsed between 1 and 6 months after blood sampling. Mean ADAMTS13 activity in 29 healthy individuals was 95% ± 15% (range 73 - 127%). Anti-ADAMTS13 antibodies were detected in 6 patients, evenly distributed over patients with and without ADAMTS13 activity. These anti-ADAMTS13 antibodies were found in three of four relapsing patients.

Table 1. Biological and clinical features of 22 TTP patients in remission.

Patient	Blood group	Months after latest TTP episode	Months prior to new TTP episode	Platelet count, 10^9/ml	ADAMTS13 activity, (%)	Anti-ADAMTS13 anti-bodies (U/ml)	VWF:Ag, U/ml	Multi-meric analy-sis	VWF activa-tion factor
1	A	1	-	367	0	15	1.5	UL	2.4
2	A	6	-	205	0	101	1.8	UL	2.4
3	A	39	-	308	0	5	1.0	UL	1.5
4	A	41	1	310	0	25	0.8	UL	1.4
5	B	29	4	177	0	29	1.5	UL	2.3
6	O	32	6	254	9	7	0.9	UL	1.3
7	AB	8	-	346	13	12	1.3	UL	1.7
8	A	35	-	188	14	13	1.5	UL	2.0
9	A	7	-	282	14	5	1.5	UL	2.5
10	B	31	-	199	17	13	1.0	UL	2.1
11	AB	72	-	224	17	3	2.4	UL	2.6
12	AB	28	-	260	19	20	1.1	UL	1.9
13	A	9	1	140	19	23	1.7	UL	#1.9
14	AB	21	-	274	25	8	1.0	UL	2.3
15	O	23	-	169	37	12	1.5	UL	1.4
16	O	17	-	208	43	8	0.8	IL	2.1
17	O	142	-	202	57	5	1.4	IL	1.6
18	O	35	-	233	61	5	0.5	IL	1.2
19	O	25	-	256	67	5	1.7	IL	1.0
20	O	114	-	236	77	1	1.7	IL	1.3
21	O	31	-	220	84	80	0.7	IL	0.8
22	A	24	-	269	88	6	1.4	IL	1.3

Multimers consisting of 20-25 or >25 bands were classified respectively as intermediate large (IL) or ultra-large (UL).

VWF: Ag levels were 1.3 ± 0.4 U/ml, which was similar to levels found in 29 healthy individuals (1.2 ± 0.4; Figure 1A). In contrast, active VWF levels were significantly increased (1.8 ± 0.5) compared to healthy individuals (1.1 ± 0.3; $P < 0.0001$; Figure 1B). A strong inverse correlation between ADAMTS13 activity and active VWF levels was found in TTP-patients (Pearson r=0.64, $P = 0.001$; Figure 2A) but not in healthy individuals (Figure 2B).

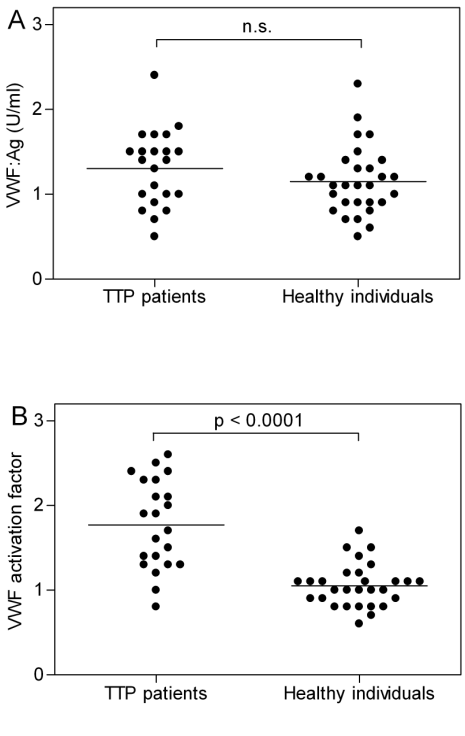

Figure 1. Active VWF but not VWF antigen levels are increased in TTP-patients in remission*. VWF:Ag (A) and active VWF levels (B) were measured in 22 TTP-patients in remission and compared to 29 healthy individuals. Results were plotted in a scatter plot.*

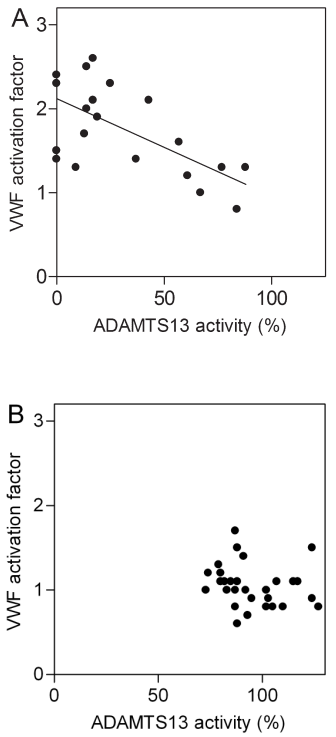

Figure 2. Active VWF levels are associated with a decrease in ADAMTS13 activity in TTP. *From 22 TTP-patients in remission (A) and 29 healthy individuals (B), VWF activation factor was plotted against ADAMTS13 activity. A significant correlation was found in TTP (Pearson r=0.64; P=0.001) but not in healthy individuals.*

Analysis of VWF multimers in TTP-patients in remission

Patient plasma samples were analyzed for multimeric composition of VWF and compared to NPP and VWF secreted from HUVECs. Figure 3A shows multimeric blots of representative samples. All 22 patients had VWF multimers that were larger than what was observed in NPP (20 multimeric bands). Intermediate large (IL) multimers (21-25 bands) were found in 7 patients. UL-VWF (>25 bands) was detected in 15 patients,

though these multimers were not as large as VWF secreted by cultured HUVECs. Mean ADAMTS13 activity was significantly lower in patients with UL-VWF compared to patients with IL-VWF (respectively 12 ± 11% and 68 ± 16%; $P < 0.0001$; Figure 3B). Consequently, active VWF levels were significantly higher in patients with UL-VWF compared to patients with IL-VWF multimers (2.0 ± 0.4 versus 1.3 ± 0.4; $P < 0.005$; Figure 3C). ABO blood-groups are known to influence ADAMTS13-mediated cleavage of VWF. Classification of the 17 patients with residual ADAMTS13 activity by blood-group revealed higher prevalence of UL-VWF in patients with blood-group non-O (93%) compared to blood-group O (25%). Patients with blood-group non-O were characterized by significantly higher active VWF levels than patients with blood-group O (2.0 ± 0.4 versus 1.3 ± 0.4; $P < 0.005$; Figure 3D). Adjustment for ADAMTS13 activity decreased the relationship between active VWF and blood-group and active VWF and UL-VWF. Apparently, these associations can be at least partly explained by ADAMTS13 activity. Active VWF levels in healthy individuals were similar between both blood-groups (1.1 ± 0.2 versus 1.0 ± 0.3; Figure 3E).

Active conformation of VWF is not restricted to UL-VWF

To investigate which multimers are actually in an active conformation, a panel of nanobodies was examined for their specificity towards active VWF and their suitability in an assay in which active multimers were precipitated and analyzed for their multimeric composition. Nanobody AU/VWF-a11, used for the immunosorbent assay, specifically recognizes active VWF but proved to be unsuitable for the immunoprecipitation approach in preliminary experiments (data not shown). The screen revealed an appropriate candidate, i.e. AU/VWF-a12. Specificity of AU/VWF-a12 towards active VWF was compared to AU/VWF-a11 in an immunosorbent assay. Microtiter wells, coated with AU/VWF-a12 (Figure 4A) or AU/VWF-a11 (Figure 4B), were incubated with different concentrations of NPP or VWD-type 2B plasma. Bound VWF was detected with an HRP-conjugated anti-VWF antibody. VWF from VWD-type 2B plasma but not from NPP was readily recognized by AU/VWF-a11 and AU/VWF-a12, indicating similar specificity of both nanobodies. Different association and dissociation rate constants were observed for AU/VWF-a11 and AU/VWF-a12 in surface plasmon resonance experiments. AU/VWF-a11 has a low Koff, appropriate for ELISA, while AU/VWF-a12 has a higher Kon, appropriate for pull-down experiments (data not shown).

AU/VWF-a12 was subsequently applied in an immunoprecipitation assay to visualize the multimeric composition of active VWF. Plasma (NPP, TTP in remission and VWD-type 2B) or HUVEC-supernatant was incubated with biotinylated AU/VWF-a12. Complexes were precipitated by streptavidin-coated magnetic beads and analyzed for their multimeric composition. Representative examples are shown in Figure 4C and 4D. Multimeric analysis (Figure 4C) revealed the presence of UL-VWF in HUVEC supernatant (lane 2) and in plasma from TTP patients #1, #2 and #11 (lane 3-5; see also Table 1). Patient #22 displayed IL-VWF (lane 6). Highest multimers were absent in VWD-type 2B (lane 7). Little, if any active multimers were precipitated from NPP, whereas all multimers were precipitated upon addition of ristocetin (Figure 4D, lane 1 and 2). Of note, intensity of the lower molecular weight multimers of ristocetin-activated NPP was less strong compared to its higher multimers. All multimers could be precipitated from VWF secreted by stimulated HUVECs, ranging from the UL-VWF molecules to the dimers (lane 3). Results

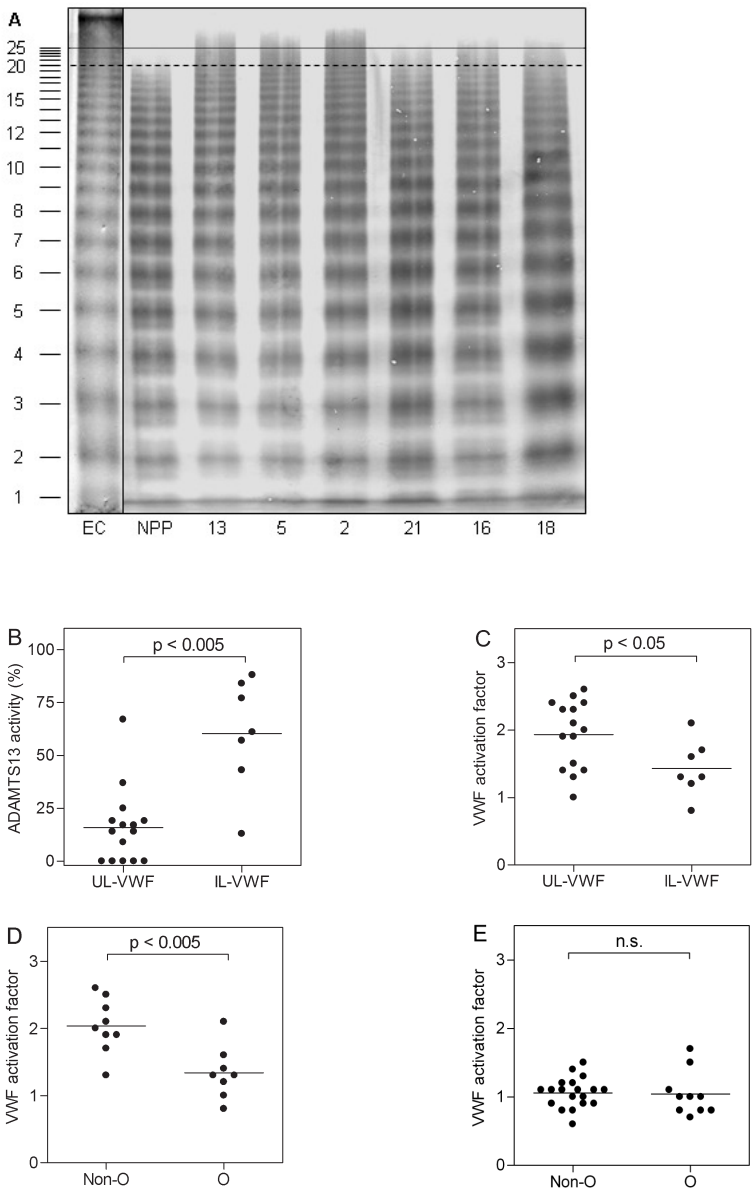

Figure 3. UL-VWF is frequently observed in TTP-patients in remission and is associated with low ADAMTS13 activity and increased active VWF levels. *(A) Supernatant from stimulated endothelial cells (EC), NPP or plasma from TTP-patients in remission was separated on a 2% SDS/agarose gel. After immunoblotting, VWF was detected with an HRP-conjugated polyclonal anti-VWF antibody. Patients 13, 5 and 2 are representative for UL-VWF (defined as >25 bands; indicated by the black line), and patients 21, 16 and 18 for IL-VWF (defined as 21-25 bands; indicated by the dashed line). ADAMTS13 activity (B) and active VWF levels (C) were plotted for TTP-patients with UL-VWF and IL-VWF. Active VWF levels were compared between blood-group O and non-O in TTP-patients (D) and healthy individuals (E).*

from TTP patients with UL-VWF and thus increased active VWF levels (lane 4-6) were similar to ristocetin-activated NPP: the full range of multimers was precipitated although intensity of the lower bands was less strong compared to the higher molecular weight multimers. From patients with normal active VWF levels (and IL-VWF), no multimers could be precipitated (lane 7). In VWD-type 2B patients, a gain-of-function mutation in the VWF/A1-domain causes a permanent active state of each multimeric subunit. Indeed, all multimers from VWD-type 2B plasma were precipitated (lane 8).

DISCUSSION

The majority of TTP-patients lack ADAMTS13 activity at presentation. Upon remission, ADAMTS13 activity does not always return to baseline [12,18,19]. Consequently, long stretches of VWF multimers can persist in the circulation that have the potential to spontaneously interact with platelets. Quantification of the amount of VWF circulating in a platelet-binding or active conformation was enabled by the development of an immunosorbent assay based on antibody AU/VWF-a11. Using this assay it was shown that active VWF levels are 2-fold increased in TTP-patients in the acute phase compared to healthy individuals [11]. So far it was unknown how levels of active VWF develop during remission. Here we followed 22 patients with a history of acquired TTP up to 6 years after their latest episode. ADAMTS13 activity remained undetectable in 23% of the patients and was low to normal in the other patients. Previous reports have shown that low or absent ADAMTS13 activity upon remission is a risk factor for the development of a new episode [20,21]. Indeed, three of the four patients of our cohort that relapsed had low or absent ADAMTS13 activity. Levels of active VWF were significantly higher in the cohort compared to healthy individuals, which could be explained by the strong inverse correlation between ADAMTS13 activity and active VWF levels. Apparently, ADAMTS13 activity is needed to optimally inactivate VWF.

Undetectable ADAMTS13 activity and increased active VWF levels were suggestive for the presence of UL-VWF. The term UL-VWF is generally used for VWF that is secreted from the endothelium that has not yet been proteolyzed by ADAMTS13. This type of VWF displays a molecular weight that is much higher than VWF from NPP. However, no actual definition exists for UL-VWF, which is probably due to a lack in sensitivity of the techniques used to visualize VWF multimers. By refining VWF multimer analysis, we can now detect VWF molecules over 25 multimeric bands. Multimers of all TTP-patients were larger than NPP (20 multimeric bands), but smaller than VWF from ECs. More specifically, about one third of the TTP-patients had multimers between 21 and 25 bands, while two third of the patients had multimers that were even larger than 25 bands. Based on these findings, we re-classified VWF to normal sized VWF, intermediate large VWF and UL-VWF.

Multimeric analysis does not only provide information about the multimeric size of VWF, it also enables visualization of eventual VWF satellite bands. The typical triplet structure of satellite bands surrounding a center band has been reported to be the consequence of proteolysis by ADAMTS13 [22,23]. The presence of satellite bands was therefore only expected in patients with detectable ADAMTS13 activity. However, satellite bands were clearly observed in all patients on both normal multimeric blot and in precipitated samples, even in those patients without detectable ADAMTS13 activity. As expected,

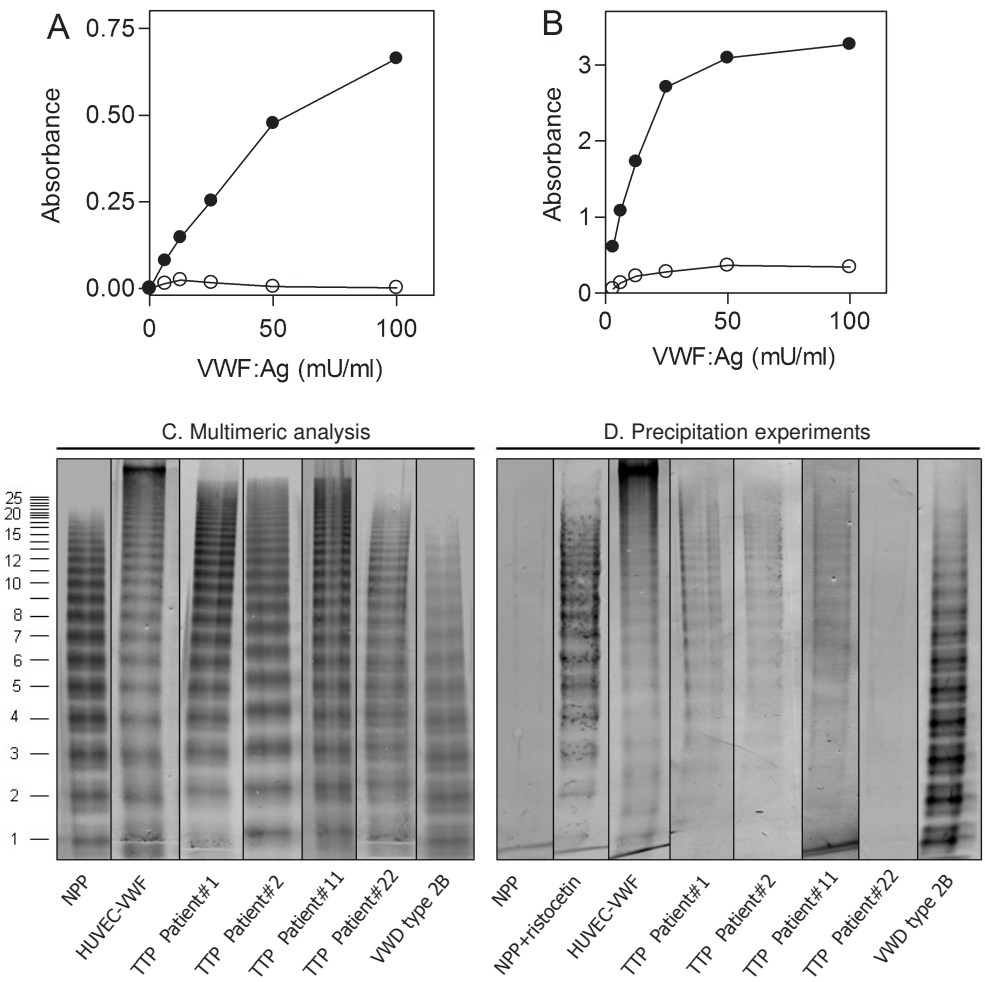

Figure 4. Full range of multimers is precipitated from patients with increased levels of active VWF. *Microtiter wells immobilized with AU/VWF-a12 (A) or AU/VWF-a11 (B) were incubated with NPP (open circles) or VWD-type 2B plasma (closed circles) in a concentration range up to 100 mU/ml. Bound VWF was detected with HRP-conjugated polyclonal anti-VWF antibody. (C) NPP (lane 1), supernatant from stimulated HUVECs (lane 2) or plasma from TTP-patients in remission (lane 3-6) and VWD-type 2B patients (lane 7) were separated on a 2% SDS/agarose gel to analyse multimeric composition. (D) Active multimers from HUVEC supernatant, NPP and patient plasma samples were immunoprecipitated using an AU/VWF-a12 based immunoprecipitation assay and subsequently separated on a 2% SDS/agarose gel.*

HUVEC-VWF multimers did present as single bands on multimeric blot. Possibly, resi-dual ADAMTS13 levels not detectable by our assay are able to proteolyze VWF. Alternatively, VWF is subjected to cleavage by other proteases.

Classification of the cohort on multimeric size revealed lower ADAMTS13 activity and higher active VWF levels in the UL-VWF group compared to the IL-VWF group.

Multimeric size of VWF might not only be influenced by ADAMTS13 plasma levels but also by the potential of ADAMTS13 to cleave VWF from different blood-groups. ADAMTS13 more efficiently cleaves VWF from individuals with blood-group O than with non-O [24]. Indeed, patients with blood-group non-O had a more than 3-fold higher incidence of UL-VWF than patients with blood-group O. This would implicate that the combination of low ADAMTS13 and blood-group non-O is least favourable for a patient with respect to VWF processing. This could have consequences on the response of acute TTP patients with different blood-groups to plasma exchange.

It has been generally assumed that UL-VWF multimers are the most active towards platelets [25-27]. For example, Arya *et al* demonstrated that VWF secreted from stimulated HUVECs has the potential to interact spontaneously with platelets. Comparison of HUVEC-derived VWF and plasma-VWF revealed a difference in multimeric composition in that HUVEC-VWF was enriched in UL-multimers but lacked smaller sized multimers. This led them to conclude that UL-multimers selectively displayed platelet-binding capacity. We and others however, show that HUVEC-VWF does not solely consist of higher multimers, but that the smaller multimers are also present [28,29]. To investigate whether these smaller multimer could also adopt an active conformation, we developed an immunoprecipitation assay that enabled specific precipitation and consequent visualisation of the active multimers. This assay was based on monoclonal antibody AU/VWF-a12. Nanobodies AU/VWF-a11 and AU/VWF-a12 were selected in the same screen for their specificity towards active VWF. AU/VWF-a11 was used in immunosorbent assays to quantify active VWF levels in plasma while AU/VWF-a12 was shown to be more suitable for pull-down experiments. Results from the immunoprecipitation assay suggested that the active conformation may be present not only in the UL-multimers but also in the smaller multimers. Immunoprecipitation experiments on plasma from TTP-patients further confirmed our findings: the whole spectrum of multimers was present in the precipitate of the patients. Of note, intensity of the smallest multimers was lower compared to the higher multimers. It is likely that only part of the VWF/A1-domains of a multimer will be in an active conformation. By chance, the nanobody will more often precipitate a larger than a smaller multimer, since the former has a larger probability to contain an active VWF/A1-domain. Therefore, the amount of precipitated high multimers will exceed that of the amount of low multimers. The exact ratio of active over latent VWF/A1-domains of each multimer remains unclear, except for VWD-type 2B patients where every VWF/A1-domain is in its active conformation due to a gain-of-function mutation. Indeed, multimers from VWD-type 2B samples were all precipitated to the same extent. Although our results suggest that smaller multimers can also adopt an active conformation, it should be noted that these results could be the consequence of an artefact of the experimental procedure. For instance, the antibody may recognize ultra-large multimers but which are integrated with smaller multimers that are therefore also pulled down. Future studies incorporating alternative approaches are required to confirm our findings that all multimers can adopt an active conformation.

Our results suggest that VWF secreted from endothelial cells is inherently active and that the presence of active VWF multimers in plasma from TTP-patients is mainly due

to the absence of the inactivating machinery ADAMTS13. Literature however, also provides alternative explanations of how ADAMTS13 deficiency may lead to accumulation of active VWF molecules in TTP. As advocated by Tsai [30], endothelial-derived VWF is not inherently active but just becomes activated by circulatory shear stress. This process could occur to both larger and smaller VWF multimers, as long as the size is sufficiently large to be flexible and responsive to shear stress. Shear-unfolded VWF will be rapidly cleaved by ADAMTS13 to generate globular, less active molecules, a process that appears absent in TTP. As a consequence, the unfolded active forms of VWF can persist in the circulation and bind platelets. Although our results from the precipitation experiments on HUVEC-VWF suggest that freshly secreted VWF is uniformly active in the absence of ADAMTS13 and shear stress, we cannot rule out the contribution of shear stress to the presence of active VWF in our TTP-patients.

Our data demonstrate that several characteristics, which are generally believed to be involved in the pathogenesis of acute TTP, are commonly found in TTP patients even after years of achieving remission. Although ADAMTS13 deficiency (and related to that UL-VWF which appeared to be uniformly active) in patients in remission seems to increase the risk for developing a new episode, these strong pro-thrombotic conditions do not directly lead to platelet aggregation and microthrombus formation. Or if these thrombi are being formed, they remain undetectable as patients do not present any clinical signs of a microangiopathy.

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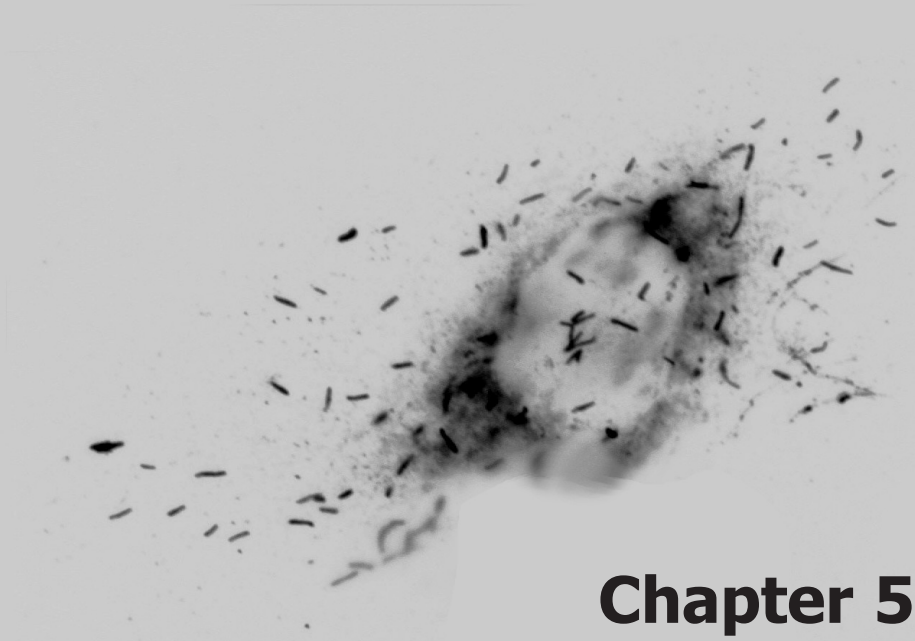

Chapter 5

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

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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 auto-antibodies against ADAMTS13. ADAMTS13 regulates the size of VWF and its deficiency results in unusually large multimers of VWF (ULVWF). These ULVWF 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 ultra-large VWF multimers are still visible after proteolysis and present if the ultra-large 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 hour [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 compared with 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

Table 1. 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

as primary TTP, five as pregnancy-related TTP, eight as HUS, 11 as TTP after bone marrow transplantation, four as sepsis, four as malignant hypertension, eight as haemolysis, elevated liver enzymes and low platelets (HELLP), seven as cancer, four as renal insufficiency and another six 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% to 75% in the FRETS-VWF73 assay. In the other 55 patients, where thrombocytopenia and hemolytic anemia were caused by the 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.

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Chapter 6

Thrombocytopenia and release of active von Willebrand factor during early *Plasmodium falciparum* malaria

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SUMMARY

Background: Thrombocytopenia occurs early during malarial infection, but its underlying mechanism is unclear. Secretion of von Willebrand factor (VWF) occurs on endothelial cell activation, and it plays an important role in platelet agglutination. *Methods:* In 14 healthy human volunteers who were experimentally infected with *Plasmodium falciparum*, we studied VWF secretion and proteolysis as well as the relationship between changes in circulating platelet numbers and plasma levels of VWF and active VWF. *Results:* Platelet numbers started to decrease between days 7 and 9 after infection, which corresponded to the earliest phase of blood-stage infection. With the decrease in platelet numbers, levels of VWF, VWF propeptide (markers of chronic and acute endothelial cell activation, respectively), and active VWF (exposing the glycoprotein-Ibα platelet-binding domain) increased proportionally. A strong, reciprocal relationship was observed between platelet numbers and levels of both VWF and active VWF. Activity of the VWF-cleaving protease ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) — a regulator of VWF activity — remained unchanged. *Conclusions: P. falciparum* induces systemic acute endothelial cell activation and release of active VWF immediately after the onset of blood-stage infection. The resulting platelet agglutination may result in early thrombocytopenia and may play a role in the pathogenesis of malaria.

INTRODUCTION

Clinical studies have shown the occurrence of thrombocytopenia in up to 80% of patients with malaria [1–3], and platelet numbers have been negatively correlated with both parasitemia [2] and disease severity [4]. We have also observed a significant drop in platelet numbers in healthy volunteers who were experimentally infected in our clinic with *Plasmodium falciparum*. Remarkably, this decline started very early during blood-stage infection — at the time when parasite densities were still at a submicroscopic detection level and before symptoms of malaria were present. Platelets may play a significant role in the pathogenesis of malaria. Platelet accumulation in the brain microvasculature has been described in children who died of cerebral malaria [5]. Intravascular platelet agglutination with increased platelet clearance from the circulation can be induced by active high-multimeric von Willebrand factor (VWF). Elevated levels of VWF have been reported in field studies of patients with malaria [6–8], but its role in malaria-induced thrombocytopenia and platelet clumping is not known.

VWF is a large, multimeric glycoprotein that functions as a carrier for factor VIII and as a bridging molecule for platelet adhesion and aggregation. It is predominantly produced in vascular endothelial cells, in which it undergoes extensive processing after synthesis, including dimerization, multimerization, and endoproteolytic cleavage of a propeptide. Large VWF multimers and VWF propeptide are stored in specialized granules called 'Weibel-Palade bodies' [9], which are released during endothelial cell activation. Therefore, both VWF and VWF propeptide serve as markers of endothelial cell activation, although VWF propeptide reflects acute endothelial cell perturbation because of its shorter half-life. After release from Weibel-Palade bodies, VWF multimers are rapidly proteolyzed to smaller and less active forms by the plasma metalloprotease ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) [10]. At least some released VWF is in an active conformation, which allows interaction with the platelet receptor glycoprotein (Gp)-Ibα [11]. Determination of active VWF levels recently became possible with the introduction of a novel, llama-derived nanobody (AU/VWFa-11) that specifically recognizes the VWF GpIbα–binding conformation [12]. Increased amounts of active VWF were subsequently demonstrated in the plasma of patients with thrombotic thrombocytopenic purpura (TTP) [12]. This disease shares some similarities with malaria: intravascular platelet agglutination with thrombocytopenia, nonimmune hemolysis, neurological symptoms, and fever.

Vascular endothelial cell activation is considered to be a common feature of malaria and plays an important role in the pathogenesis of malaria by increasing sequestration of parasitized red blood cells in the peripheral vasculature [6,13,14]. We hypothesized that endothelial cell activation with subsequent release of active VWF is an early event in malaria and is related to the development of early thrombocytopenia. Therefore, we studied the role played by endothelial cell–derived activate VWF in malaria-induced thrombocytopenia in healthy volunteers who were experimentally infected with *P. falciparum.*

SUBJECTS, MATERIALS, AND METHODS

Experimental malarial infection, study subjects, and blood sampling

Experimental human malarial infections are invaluable for phase 2a malaria-vaccine trials and are a powerful tool for the study of the pathophysiology and immunology of early malaria. By use of a stringent protocol, experimental human malarial infections have been proven to be reliable, safe, and generally well tolerated [15, 16]. In addition to malaria-induced thrombocytopenia, the current experimental infection was used to study immunological responses during early *P. falciparum* malaria. Fourteen healthy volunteers with no previous exposure to malaria were selected and infected with *P. falciparum* parasites as described by Verhage et al [16]. In short, *Anopheles stephensi* mosquitoes were maintained in the insectary at the animal house of the Radboud University Nijmegen Medical Centre and were infected with the NF54 strain of *P. falciparum*. Batches with 190% infected mosquitoes were used for the experimental human infection. Volunteers were infected by 5 *P. falciparum*–infected mosquitoes and were followed-up via thick blood smears and assessment of malarial symptoms twice a day from day 4 to 6 after infection. From day 6 until 3 days after initiation of anti-malarial treatment, these assessments were done 3 times a day. Serial blood samples were collected once daily in 4-mL Vacutainer glass tubes containing 48 μL of EDTA-K_3 (BD Diagnostics) at baseline, from day 4 after infection until 3 days after the start of anti-malarial treatment, and on days 21 and 42 after infection. After centrifugation, plasma was stored at -80 °C. All assays were performed on freshly thawed samples. Plasma from 40 healthy donors was used as a control (normal pooled plasma [NPP]). For the determination of ADAMTS13 activity, heparin plasma from baseline and from day 8 after infection was used. Treatment with a standard 6-dose curative regimen of artemether-lumefantrine was immediately initiated on microscopic detection of parasites in a thick blood smear. The Institutional Review Board of the Radboud University Nijmegen Medical Centre approved the protocol (CMO 2004/129), and volunteers provided written informed consent.

Quantitative nucleic acid sequence–based amplification (QT-NASBA) and VWF, VWF propeptide, active VWF, and ADAMTS13 activity

Three times daily, real-time QT-NASBA, as described by Schneider et al. [17], was used for retrospective determination of parasitemia. The sensitivity of this assay is 20 parasites/mL and is ~1000 times more sensitive than standard microscopy. VWF and VWF propeptide levels were determined at baseline and from the last day before the onset of bloodstage infection (as assessed by QT-NASBA) until 2 days after the start of anti-malarial treatment. VWF levels were determined as described elsewhere [18]. VWF propeptide levels were measured as described by Borchiellini et al. [19]. Levels of active VWF were measured using the AU/VWFa-11 nanobody, as described [12]. The relative amount of active VWF was determined by calculating the ratio of the absorbance slope of a plasma sample to the slope of the corresponding baseline sample and then correcting for the amount of VWF. ADAMTS13 activity was determined at baseline and on day 8 after infection by the FRETS-VWF73 assay (Peptides International) [20], in accordance with the manufacturer's protocol. NPP was used as a standard.

Statistical analysis

Platelet number and VWF, VWF propeptide, active VWF, and ADAMTS13 activity were expressed as percentage of baseline values, and each participant served as his or her own control. Means with corresponding 95% confidence intervals (CIs) or SDs were calculated for normally distributed data, whereas medians with ranges were determined for data with a skewed distribution. Changes in serial levels of VWF and VWF propeptide during the course of the infection were tested by repeated-measures analysis of variance, and the Wilcoxon matched-pairs test was used for active VWF. Relationships between the separate variables were examined by Pearson's correlation analysis or by the nonparametric Friedman test for non-normally distributed data. The sample size allowed detection of a minimal increase in active VWF levels of 50%, with a power of 180%. $P < 0.05$ was taken to indicate a significant difference. Statistical analyses were performed with GraphPad Prism for Windows (version 4.0; GraphPad Software).

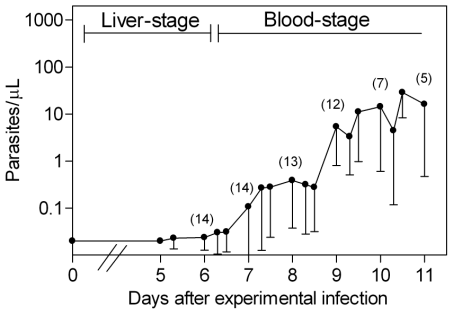

Figure 1. Kinetics of Plasmodium falciparum parasitemia before anti-malarial treatment. *Fourteen healthy volunteers were experimentally infected with P. falciparum. Data are values for parasitemia mean ± SD before anti-malarial treatment, as determined by real-time quantitative nucleic acid sequence–based amplification. Shown in parentheses are the nos. of volunteers tested at the indicated time points.*

RESULTS

Clinical course of volunteers and platelet number in time

After exposure to *P. falciparum*–infected mosquitoes, all 14 volunteers developed parasitemia. The QT- NASBA became positive after a median time of 7.0 days (range, 6.0 – 9.0 days) after infection and showed increasing parasitemia before the initiation of anti-malarial treatment (Figure 1). Treatment was started after a median time of 9.65 days (range, 7.3 – 11.3 days) immediately on microscopic detection of *P. falciparum* parasites in a thick blood smear. As shown in Figure 2, mean platelet numbers started to decrease almost immediately after the onset of blood-stage infection, reaching a nadir of 58.6% (95% CI, 46.8% – 70.4%) of the baseline value. Successful anti-malarial treatment was followed by a rebound thrombocytosis, reaching a mean peak level of 142% (95% CI, 130.2% – 154.4%) of the baseline value on day 21 after infection before returning to normal on day 42 (data not shown). Haemoglobin levels remained unchanged throughout the infection (data not shown).

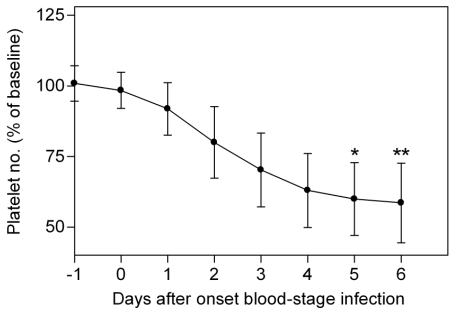

Figure 2. Kinetics of platelet nos. during early Plasmodium falciparum blood-stage infection. *Fourteen healthy volunteers were experimentally infected with P. falciparum. Data are values for relative mean ± SD platelet no. (expressed as the percentage of the baseline value) during early blood-stage infection. The mean absolute platelet no. at baseline was 249 x 10^9 platelets/L (95% confidence interval, 222 x 10^9 – 276 x 10^9 platelets/L). Changes in relative platelet nos. throughout the infection were significant (P < 0.0001, repeated-measures analysis of variance). * Mean for 10 volunteers; ** mean for 8 volunteers.*

Time course of VWF, VWF propeptide and active VWF levels

Figure 3A shows the kinetics of plasma VWF levels during early blood-stage infection in the 14 volunteers. Plasma VWF levels started to increase almost immediately after the onset of blood-stage infection, reaching a mean peak plasma level of 173% (95% CI, 116% – 230%) of the baseline value. The range of individual peak levels was 115% – 385% of the baseline value. VWF propeptide levels followed a similar kinetic pattern (Figure 3B), with a mean peak plasma level of 166% (95% CI, 133% – 200%) of the baseline value and with individual peak levels ranging from 129% to 340% of the baseline value. By use of AU/VWFa-11, a recently developed nanobody that specifically recognizes the GpIbα -binding conformation of the VWF A1 domain [12], we determined the amounts of active VWF present at 3 time points during the infection: (1) baseline, (2) the day of the first significant decrease in platelet number, defined as a decline of at least 20 x 10^9 platelets/L compared with the previous day; and (3) the day of platelet nadir. Figure 4 shows the change in the amount of active VWF in individual volunteers. On the day with the first significant decrease in platelet number, the median level of active VWF was 140% (range, 59% – 410%) of the baseline value, whereas on the day of platelet nadir it was 261% (range, 57% – 1370%) of the baseline value.

ADAMTS13 activity

To determine whether a decrease in ADAMTS13 activity could account for the increased levels of active VWF, we quantitatively measured ADAMTS13 activity at baseline and on day 8 after infection in the 14 volunteers. The mean ADAMTS13 activity on day 8 after infection was 99.9% (range, 83% – 127%) of preinfection activity (data not shown). Therefore, the elevated levels of active VWF could not be explained by decreased proteolysis of large VWF multimers.

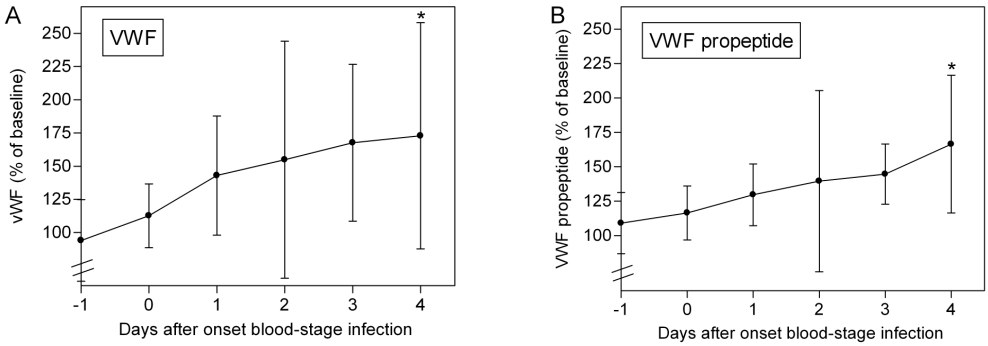

Figure 3. Kinetics of von Willebrand factor (VWF) and VWF propeptide during early Plasmodium falciparum blood-stage infection. *Fourteen healthy volunteers were experimentally infected with P. falciparum. Data are values for relative levels (expressed as the percentage mean ± SD of the baseline value) of VWF (A) and VWF propeptide (B) during early blood-stage infection. Mean absolute baseline levels of VWF and VWF propeptide were 35.3 nmol/L (95% confidence interval [CI], 26.5 – 44.0 nmol/L) and 5.8 nmol/L (95% CI, 5.1 – 6.6 nmol/L), respectively. Changes in both VWF and VWF propeptide during the course of infection were significant (P < 0.0001, repeated-measures analysis of variance). *Mean for 11 volunteers.*

Relationship between platelet numbers, parasitemia, and levels of active VWF

To examine whether levels of VWF and active VWF were related to the change in platelet numbers, we plotted the relative levels of these variables (expressed as a percentage of their corresponding baseline value) against each other. During the course of blood-stage infection, serial VWF levels showed a strong inverse relationship (Pearson's r = -0.62 [95% CI, -0.74 to -0.47]; $P < 0.0001$) with corresponding platelet numbers on that same day (Figure 5A). During platelet nadir, a similar strong association (Pearson's r = -0.61 [95% CI, -0.86 to -0.11]; $P = 0.021$) was found between relative VWF levels and corresponding platelet numbers (Figure 5B). Moreover, relative levels of active VWF were significantly related (Spearman's r = -0.75 [95% CI, -0.92 to -0.35]; $P < 0.002$) to platelet numbers on that day (Figure 5C). The degree of parasitemia (expressed as the log number of parasites per milliliter of blood) from the onset of parasitemia until the start of anti-malarial treatment correlated weakly (Pearson's r = 0.374 [95% CI, 0.13 to 0.58]; $P = 0.0035$) with corresponding relative VWF levels (data not shown).

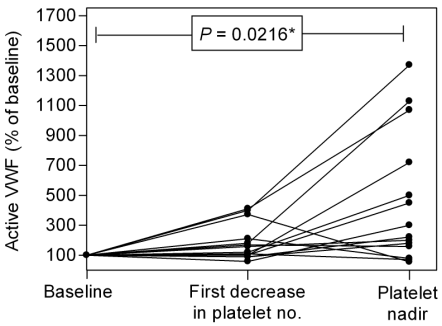

Figure 4. Levels of active von Willebrand factor (VWF) during early Plasmodium falciparum infection. *Fourteen healthy volunteers were experimentally infected with P. falciparum. Data are relative levels (expressed as the percentage of the baseline value) of active VWF at 3 time points: baseline, the day of the first significant decrease in platelet no. (defined as a decline of at least 20 x 10^9 platelets/L compared with the previous day), and the day of platelet nadir. *Wilcoxon matched-pairs test.*

DISCUSSION

In the present study, we demonstrated that VWF and VWF propeptide secretion were significantly increased at a very early stage in *P. falciparum* blood-stage infection, pointing at acute endothelial cell activation in malaria. Increased amounts of active VWF, exposing the GpIbα-binding site of VWF for platelets, were observed as well. Platelet numbers and levels of both VWF and active VWF showed a strong inverse correlation in our small group of volunteers. Active VWF may therefore be an important inducer of thrombocytopenia during early malaria and may as such contribute to the pathogenesis of malaria.

We are the first to demonstrate increased levels of active VWF in an infectious disease. Levels of active VWF in our volunteers were comparable to levels found during TTP and during the HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome [12,21]. These diseases are characterized by consumptive thrombocytopenia with intravascular platelet adhesion and clumping. Our present findings of a very early decrease in platelet number, together with elevated levels of active VWF, suggest that early intravascular platelet adhesion and clumping may also be a prominent and unique feature of malaria. Circumstantial evidence for this was provided by an autopsy study from Malawi that showed increased platelet accumulation in the brains of children who died of cerebral malaria, compared with that in children who died of non-malarial encephalopathy [5]. In addition, electron microscopy studies have also demonstrated platelet adherence to brain endothelial cells during both human and murine cerebral malaria [22,23]. Besides causing thrombocytopenia, VWF-mediated platelet clumping may contribute to the pathogenesis of malaria in several ways. First, adhering and aggregating platelets may cause organ perfusion failure and tissue hypoxia. Second, VWF may play a role in the cytoadherence of parasitized red blood cells to vascular

endothelium during the early stages of malaria. Multiple endothelial receptors are involved in this process, most notably CD36 and intercellular adhesion molecule–1. However, up-regulation of these endothelial receptors takes time, because they require de novo synthesis. In contrast, stored active VWF can immediately be secreted from endothelial cells with subsequent binding of platelets. It has recently been demonstrated that platelets may facilitate cytoadherence by acting as bridges between parasitized red blood cells and endothelial cells [24]. Last, VWF-mediated platelet adhesion may be important in brain microvessels that express only a little CD36 [14]. Platelets expressing high levels of CD36 could deliver the required CD36 in these situations.

We know of 3 cross-sectional studies that have previously analyzed VWF concentrations in patients with malaria, and all found elevated levels. In a report from 1985 [7], the inverse relationship between VWF levels and platelet numbers was demonstrated. The authors concluded that thrombocytopenia during malaria is not indicative of disseminated intravascular coagulation but may relate to endothelial damage. In the other two studies [6,8], the relationship between VWF and platelet number was not reported. Therefore, previous results are in line with our findings but, also because of the nature of these studies, fail to conceptualize the idea that VWF-mediated platelet clumping plays an important role in the development of thrombocytopenia and in the pathogenesis of malaria. The experimental human malarial infection model, however, enables comparison of the kinetics of these variables during the very early stages of malaria.

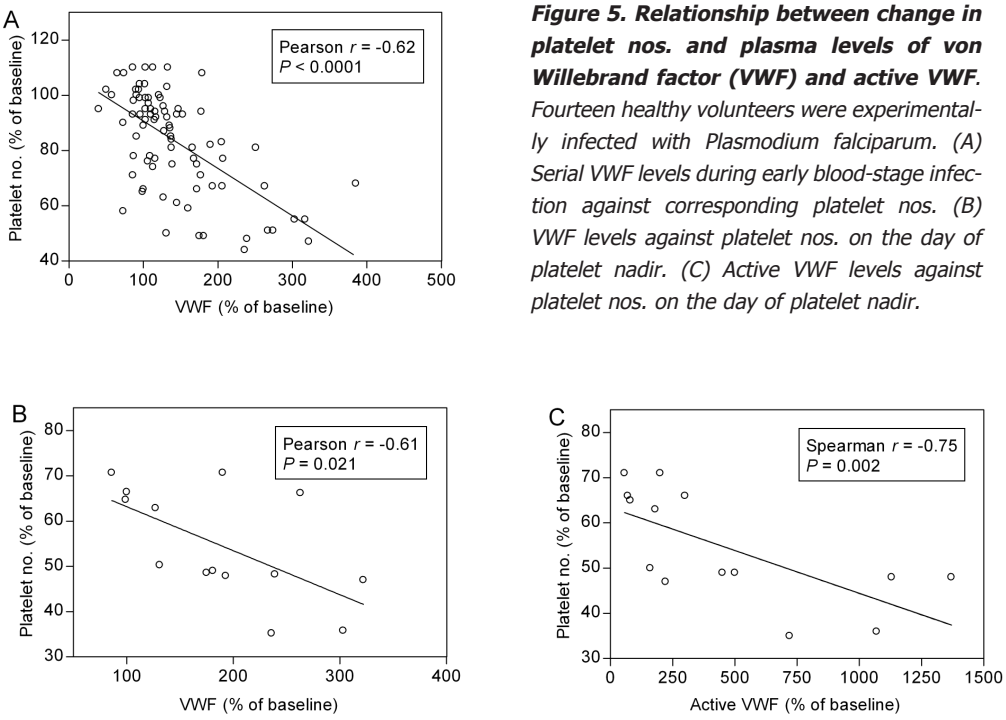

Figure 5. Relationship between change in platelet nos. and plasma levels of von Willebrand factor (VWF) and active VWF. *Fourteen healthy volunteers were experimentally infected with Plasmodium falciparum. (A) Serial VWF levels during early blood-stage infection against corresponding platelet nos. (B) VWF levels against platelet nos. on the day of platelet nadir. (C) Active VWF levels against platelet nos. on the day of platelet nadir.*

This allows a more reliable assessment of the direct effect of *P. falciparum* on endothelial cells and platelets. In addition, several other pathogenic mechanisms for malaria-induced thrombocytopenia during more advanced stages of malaria have been suggested, such as splenic sequestration [25], oxidative stress [26], platelet apoptosis [27], and antibody- and cell-mediated immunity [28–30]. In our volunteers, however, platelet numbers decreased at such an early time point during the infection that it is highly unlikely that these mechanisms could have played a significant role. Activation of the coagulation system may also cause platelet consumption. However, the coagulation is usually only mildly activated during malaria, and disseminated intravascular coagulation is rare, even during severe malaria [31].

Because both VWF and VWF propeptide are predominantly synthesized in vascular endothelial cells and are released on endothelial cell activation [32], the rise in circulating VWF and VWF propeptide levels in our volunteers, during or shortly after the release of malarial parasites from the liver, provides evidence for acute endothelial cell activation at this early time point. Although we use the term 'endothelial cell activation' to describe changes in endothelial cell phenotype that result in VWF release, we realize that endothelial cell pathophysiology is complex and that changes may encompass a spectrum ranging from simple perturbation to activation and even endothelial cell damage. Early endothelial cell activation may be critical during the early stages of malaria. Sequestration of parasitized red blood cells containing the more mature stages of the malarial parasite is important for parasite survival. We have previously demonstrated that the mean asexual life cycle of malarial parasites is 43.7 hours and have provided evidence that parasitized red blood cells indeed adhere to vascular endothelium within 2 days after release from the liver [33]. In this way, early removal and destruction by the spleen is avoided. Early endothelial cell activation probably facilitates this sequestration by, for example, up-regulating endothelial adhesion molecules and VWF release. The trigger for the early endothelial cell activation and VWF release in our volunteers is unclear. Inflammatory cytokines are known endothelium agonists [34] and have been shown to directly stimulate the release of VWF from endothelial cells or to inhibit ADAMTS13 [35]. We have shown in the past that levels of proinflammatory cytokines started to increase 1–2 days before treatment initiation during experimental malaria [36]. Alternative mechanisms may also be involved in the initiation of endothelial cell activation and VWF release. A still-undetermined factor released at the end of liver schizogony or, alternatively, malarial parasite products could be potential candidates. One of these parasite products, glycosylphosphatidylinositol anchors, has already been linked to endothelial cells by its potential to induce cytokine production and up-regulate the expression of endothelial adhesion molecules directly [37]. *P. falciparum* parasitized red blood cells also have the capacity to directly stimulate human endothelial cells [38]. However, it is important to realize that, at the time when levels of VWF and VWF propeptide started to rise, only a very small percentage of erythrocytes (estimated at < 0.001%) were infected and that malarial antigen levels, including of glycosylphosphatidylinositol, were probably still low.

Plasma concentrations of VWF are influenced by blood group. Subjects with blood group O have ~30% lower levels of VWF than do those with blood group A, B, or AB [39]. Subjects with blood group O are relatively resistant against severe malaria [40]. Our hypothesis that VWF is involved in the pathogenesis of malaria may thus offer an addi-

tional explanation for this phenomenon. In our small group of 14 volunteers, there was no significant difference in median VWF levels between the 7 volunteers with blood group O and the 7 with blood group non-O (data not shown).
In conclusion, our findings suggest that *P. falciparum* induces systemic acute endothelial cell activation and release of active VWF immediately after the onset of the blood stage of the infection. The phenomenon may be responsible for the early thrombocytopenia and may play a role in the pathogenesis of malaria by providing a link between parasitized red blood cells and endothelial cells.

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Chapter 7

ADAMTS13 deficiency with elevated levels of ultra-large and active von Willebrand factor in *P. falciparum* and *P. vivax* malaria

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SUMMARY

A deficiency in ADAMTS13 (a von Willebrand factor [VWF] cleaving protease) activity is associated with accumulation of prothrombogenic unusually large VWF multimers (UL-VWF) in plasma. We studied VWF release and proteolysis in patients with symptomatic *Plasmodium falciparum* or *P. vivax* malaria on the Indonesian island Sumba. Malaria patients had significantly lower platelet counts and higher VWF concentrations and active VWF levels than healthy hospital staff controls. The latter indicates that a higher amount of circulating VWF was in a conformation enabling spontaneous platelet binding. In addition, ADAMTS13 activity and antigen levels were reduced in both malaria groups, and this was associated with the presence of UL-VWF. The mechanism behind this reduction and the role in malaria pathogenesis needs to be further elucidated. In malaria, endothelial cell activation with increased circulating amounts of active and UL-VWF, together with reduced VWF inactivation by ADAMTS13, may result in intravascular platelet aggregation, thrombocytopenia and microvascular disease.

INTRODUCTION

Malaria remains an important cause of morbidity and mortality in the tropics with an estimated number of 500 million cases and 1–3 million deaths each year. Although the exact pathogenesis of malaria is still incompletely understood, it is well known that thrombocytopenia and endothelial cell activation are prominent features of clinical *Plasmodium falciparum* malaria and that platelet–endothelium interactions may play an important role in its complications, such as cerebral malaria [1–4].

We have previously shown in healthy volunteers participating in an experimental human *P. falciparum* infection that the decline in platelet numbers was associated with the onset of endothelial cell activation and with an increase in the amount of active von Willebrand factor (VWF) — i.e. the amount of VWF that has undergone a conformational change from a latent state to a state enabling spontaneous binding of platelets [5,6].

VWF is predominantly synthesized by endothelial cells and stored in specialized granules, called Weibel-Palade bodies. VWF mediates platelet adhesion and aggregation at sites of vessel injury. Both the multimeric size and the conformation of VWF determine its activity, whereby ultra-large (UL) and elongated VWF multimers interact best with platelets. Under normal conditions, ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin-1-like domains) rapidly cleaves UL and prothrombogenic VWF multimers [7]. UL-VWF accumulation caused by absent or markedly reduced ADAMTS13 activity is characteristic for the rare microangiopathic disease thrombotic thrombocytopenic purpura (TTP) [8], highlighting the physiologic importance of ADAMTS13 cleavage of UL-VWF in humans. In recent years, an acquired ADAMTS13 deficiency has also been described in various other pathologic conditions [9] such as sepsis and diffuse intravascular coagulation [10,11]. Mutations or polymorphisms in the *ADAMTS13* gene may also account for reduced plasmatic ADAMTS13 activity. A gene polymorphism (*P475S*; rs11575933) that impairs ADAMTS13 activity is common in Japan (allelic frequency 5.1%) [12] but rare in China (1.7%) and Europe (0.5%) [13,14]. No other common functional polymorphisms have been identified thus far.

In our experimental human *P. falciparum* malaria model in healthy Dutch volunteers, we found no changes in ADAMTS13 activity levels during the pre-clinical and early clinical stages of malaria, when parasitemia levels were still below microscopy detection level [5]. However, we hypothesized that secondary ADAMTS13 deficiency may occur in later stages of clinical malaria, in which they may contribute to the development of thrombocytopenia and organ dysfunction, like in other systemic microangiopathic diseases [9]. Moreover, although thrombocytopenia and organ dysfunction are also observed in *P. vivax* malaria [15–17], no data are available on VWF secretion and proteolysis in *P. vivax* malaria, whereas this infection is highly prevalent in Asia and South America and is responsible for considerable morbidity and mortality [18]. Therefore, the aim of this study was to determine levels of VWF, active VWF, and ADAMTS13 and its relation with thrombocytopenia in subjects with symptomatic *P. falciparum* or *P. vivax* malaria on the Indonesian island Sumba. These levels were compared with those of healthy controls. Factors associated with ADAMTS13 activity, such as the multimeric size of VWF, the presence of ADAMTS13 inhibitors, the relation with endothelial cell activation and inflammatory markers, and the occurrence of the *P475S* mutation and other mutations in the *ADAMTS13* gene on Sumba were also studied.

MATERIALS AND METHODS

Study area, study population, and ethics

This study was conducted from April to August 2007 at the Rumah Sakit Karitas Hospital in Weetabula, West Sumba, East Nusa Tenggara Province, Indonesia, an area of unstable *P. falciparum* and *P. vivax* malaria transmission [19]. Consecutive subjects presenting to hospital with clinical symptoms of malaria and a *P. falciparum* or *P. vivax* parasite density of at least 2,500 and 500 parasites/μL respectively, were enrolled. Healthy, asymptomatic Sumbanese controls with a negative blood slide were recruited among hospital staff from Weetabula, which can be characterized as a low malaria transmission area. In addition, a group of asymptomatic Sumbanese subjects with a negative malaria blood slide were selected from a remote village, located in a high malaria transmission area. To screen for the occurrence of common polymorphisms in the *ADAMTS13* gene in the Sumbanese population, DNA from 71 Sumbanese individuals was used. This group consisted of villagers participating in a cross-section malariometric survey and of the abovementioned asymptomatic Sumbanese controls and the hospital staff controls. The whole *ADAMTS13* gene was sequenced in five subjects from this group. Healthy Dutch controls were recruited among laboratory staff and students. Finally, plasma from five Dutch TTP patients was used. Diagnostic criteria for TTP were the presence of thrombocytopenia with microangiopathic hemolysis and no detectable ADAMTS13 activity. This study received ethical approval for the use of human subjects from the Eijkman Institute for Molecular Biology Research Ethics Committees (Jakarta, Indonesia) and of the medical ethical committee of the University Medical Center Utrecht for use of TTP patient plasma for research purposes. All study participants — or parent or guardian in case of children — gave written informed consent to participate in this study.

Sample collection

Venous blood was collected before administration of antimalarial drugs and/or any other treatment. Blood collected in EDTA tubes (Becton-Dickinson Vacutainer Systems, Rutherford, NJ) was used for determination of a full blood count; blood collected in CTAD tubes (Becton-Dickinson Vacutainer Systems; tubes containing citrate and the platelet stabilizing agents theophylline, adenosine, and dipyridamole) was used for coagulation and endothelial cell activation marker assays and determination of interleukin (IL)-6 concentration. Blood collected in heparin tubes was used for detection of anti-ADAMTS13 autoantibodies. All samples collected in the hospital were centrifuged at 3,500 rpm for 10 minutes and frozen at -20°C until further analysis. Double centrifugation to obtain platelet-poor plasma is not necessary with the CTAD system. For normal pooled plasma, platelet-depleted plasma of 40 healthy Dutch volunteers was pooled and stored in aliquots at -80°C.

Laboratory procedures

Determination of parasitemia and full blood count. Thick and thin blood smears were stained with Giemsa, and the number of parasites was quantified against 200 white blood cells. Parasite density was calculated assuming a white blood cell count of 8,000/μL. A full blood count was determined by a standard hematology analyzer (Arcus, Diatron, Vienna, Austria).

VWF and ADAMTS13 antigen and activity assays. VWF concentrations were determined by enzyme-linked immunosorbent assay (ELISA) as described previously [20]. Active VWF was determined by ELISA using a nanobody (AU/VWFa-11) that specifically recognizes the GPIb binding conformation of VWF, as described previously [6]. The multimeric pattern of VWF was analyzed using 2% agarose gel electrophoresis, followed by immunoblotting according to Raines and others [21]. ADAMTS13 activity was determined using the fluorescence resonance energy transfer (FRETS) assay for ADAMTS13 activity (Peptides International, Lexington, KY) [22], whereby the ADAMTS13 activity of normal pool plasma was set at 100%, and the values obtained in study participant samples were expressed as percentage of normal pool plasma. ADAMTS13 activity was also determined by a VWF proteolysis assay as described previously [23]. Briefly, proteolysis of recombinant type 2a VWF by study participant plasma samples was compared with normal pool plasma in the absence and presence of EDTA, which is known to inactivate ADAMTS13. In this assay, ADAMTS13 activity is taken to be absent in case ultralarge VWF multimers remain visible after proteolysis. ADAMTS13 antigen levels were measured by a commercially available ELISA according to the instructions of the manufacturer (American Diagnostica, Stamford, CA).

ADAMTS13 inhibitors assays. Presence of ADAMTS13 inhibitors was determined by measuring the residual ADAMTS13 activity of normal pool plasma after incubation with plasma of study participants (volume ratio 1:1) at 37°C for up to 3 hours. Presence of anti-ADAMTS13 IgG antibodies in plasma was measured using the Technozym inhibitor ELISA (Technoclone, Vienna, Austria), according to the manufacturer's instructions. In our laboratory, the upper limit of normal is a titer of 25 U/mL. A modification of this assay was used to detect the presence of IgM or IgA anti-ADAMTS autoantibodies. Anti-IgM and anti-IgA were added, instead of anti-IgG, as secondary detection antibody, and their OD values were compared with healthy hospital staff controls.

Mutations in the ADAMTS13 gene. DNA was extracted from whole blood using the Chelex-100 ion exchanger (BioRad Laboratories, Hercules, CA). Presence of the *P475S* genotype was determined using fluorescence-based genotyping (Assay on Demand; Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), as described in detail previously [24]. From five subjects, the exons of the *ADAMTS13* gene, including part of the intronic boundaries, were amplified by PCR on an ICycler PCR machine (Biorad Laboratories, Veenendaal, The Netherlands). Primers and PCR conditions are available on request. PCR products were purified using the High Pure PCR product purification kit (Roche Applied Science, Mannheim, Germany) and sequenced in both directions using the BigDye Terminator kit version 3 (Applied Biosystems) and a 3730 or 3100 DNA Analyzer (Applied Biosystems).

Measurement of sICAM-1 and IL-6. Concentrations of soluble intercellular adhesion molecule-1 (sICAM-1) and IL-6 were determined by sandwich ELISA technique using anti-human sICAM-1 (R&D DuoSet ELISA Development Systems) and antihuman IL-6 antibodies (ImmunoTools, Freiburg, Germany).

Statistical analysis

Data are presented as median followed by interquartile range in parentheses unless otherwise stated. Differences between more than two groups were assessed by Kruskal-Wallis test for quantitative variables with the Dunn procedure for pairwise comparisons. Mann-Whitney *U* test was used for comparisons between two groups. Relationships between laboratory parameters were assessed using the Pearson or Spearman correlation coefficient, depending on whether parameters were normally distributed. All analyses were performed with SPSS version 15.0 for Windows.

Table 1. Demographic and laboratory characteristics of study participants

	***P. falciparum* malaria**	***P. vivax* malaria**	**Hospital staff control**	***P***
Number of subjects (n)	25	15	11	
Sex (% female)	50.0	50.0	90.9	
Age (years)	20.0 (3.8–37.3)	11.0 (2.1–25.3)	25.5 (21.2–30.8)	0.066*
Parasitemia (parasites/μL)	3,240 (2,420–8,200)	880 (800–1,240)	0	< 0.001†
Hemoglobin level (g/dL)	9.3 (7.9–12.2)	11.6 (9.9–13.2)	13.2 (11.3–13.4)	0.009*‡
Platelet count ($\times 10^9$/L)	122 (70–154)	117 (77–220)	237 (172–258)	< 0.002*‡§
Thrombocytopenia (%) ¶	69.2	50.0	9.1	
White blood cell count ($\times 10^9$/L)	6.3 (5.0–8.0)	7.6 (6.2–8.6)	7.7 (6.6–9.4)	0.114*
Lymphocytes ($\times 10^9$/L)	0.9 (0.6–1.7)	1.8 (1.4–3.7)	2.1 (1.9–2.7)	0.001*‡
Granulocytes ($\times 10^9$/L)	4.4 (3.4–6.3)	4.3 (3.4–5.8)	4.0 (3.6–5.6)	0.985*
Interleukin-6 (pg/mL)	28.1 (2.0–87.5)	9.2 (2.0–45.6)	NA**	0.659†
sICAM-1 (pg/mL)	245.7 (213.7–283.4)	190.5 (141.8–402.5)	123.3 (94.6–140.4)	< 0.001*‡§

Data are median (interquartile range) unless otherwise specified. NA, not applicable; sICAM-1, soluble intercellular adhesion molecule-1.

* Differences between three groups by Kruskal-Wallis test, with post hoc testing by the Dunn procedure.

† Mann-Whitney *U* test.

‡ $P < 0.05$ between *P. falciparum* and control group.

§ $P < 0.05$ between *P. vivax* and control group.

¶ Thrombocytopenia defined as platelet number $< 150 \times 10^9$/L.

** Below assay's detection limit in all.

RESULTS

Demographic and laboratory characteristics

Characteristics of the patients presenting to hospital with *P. falciparum* malaria or *P. vivax* malaria and the healthy hospital staff controls are shown in Table 1. Two patients had severe *P. falciparum* malaria according to World Health Organization criteria (hyperparasitemia). All hospital staff controls were adults, whereas 52% of the malaria patients were < 18 years of age. Thrombocytopenia was a common finding in both *P. falciparum* and *P. vivax* malaria patients, and platelet numbers were significantly lower in the malaria patients than in hospital staff controls. Levels of the proinflammatory cytokine IL-6 and the endothelial cell activation marker sICAM-1 were significantly higher in both malaria groups than in hospital staff controls. In the malaria groups, no significant differences were present between children and adults in platelet number or in concentrations of measured mediators (data not shown).

VWF antigen concentrations and VWF activation factors

VWF concentrations were highest in patients with *P. falciparum* malaria (Figure 1A). However, compared with hospital staff controls, VWF concentrations in the *P. vivax* group were also significantly elevated. The mean VWF concentration in hospital staff controls was comparable to the concentration present in normal pool plasma obtained from healthy Dutch volunteers (10.1 versus 12.4 µg/mL, respectively). VWF activation factors were also significantly higher in P*. falciparum* malaria patients than in hospital staff controls (Figure 1B), indicating that their circulating VWF was in a more active conformation (i.e. in a conformation enabling spontaneous platelet binding).

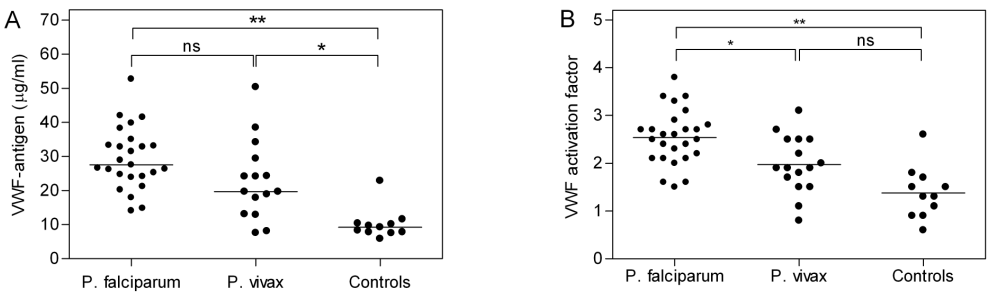

Figure 1. *Scatter dot plots with line at median showing von Willebrand factor (VWF) antigen concentrations (A) and VWF activation factors (B) in patients with symptomatic P. falciparum malaria (n = 25), P. vivax malaria (n = 15), and healthy Sumbanese hospital staff controls (n = 11). Group comparisons were done using Kruskal-Wallis test, whereas pairwise comparisons were done using the Dunn procedure (* P < 0.05; ** P < 0.001; ns P > 0.05).*

ADAMTS13 activity and antigen levels

ADAMTS13 activity levels were reduced in all patients with symptomatic *P. falciparum* or *P. vivax* malaria with median (IQR) levels of 13.5% (9.2 – 23.2%) and 13.3% (9.2 – 19.5%), respectively (Figure 2A). Levels in healthy Sumbanese hospital staff were comparable to levels found in healthy Dutch volunteers, whereas Dutch TTP patients typically had no demonstrable ADAMTS13 activity. ADAMTS13 antigen concentrations were also low in nearly all malaria patients (Figure 2B), and concentrations correlated well with ADAMTS13 activity levels (Pearson $r = 0.78$; $P < 0.001$). Additionally, the findings of low plasmatic ADAMTS13 activity by the FRETS-VWF73 assay were confirmed in a proteolytic VWF multimer assay. Figure 3A shows the results of this assay for three patients with a moderate decrease in ADAMTS13 activity and one with severely reduced activity (4%). Especially in the latter, large VWF multimers remained visible after incubation of synthetic VWF with patient plasma, confirming a strong reduction of plasmatic ADAMTS13 activity. Further confirmation was obtained by analysis of the VWF multimer pattern in plasma of malaria patients. As shown for two patients with *P. falciparum* in Figure 3B, UL-VWF could be detected in the plasma of these patients. Finally, we determined ADAMTS13 activity and antigen levels in Sumbanese with a negative malaria slide, living in remote villages, and found very low ADAMTS13 activity and antigen levels. Compared with the hospital staff controls, these villagers had significantly lower platelet counts (222 versus 331 × 10^9/L; $P = 0.012$) and borderline significantly higher VWF concentrations (15.3 versus 10.1 µg/mL; $P = 0.07$). However, although the differences in platelet number and VWF concentration might indicate that the low ADAMTS13 levels were a true finding, we decided to exclude these samples from further analysis because heat inactivation of ADAMTS13 during sample collection or transport to hospital could not be excluded with certainty.

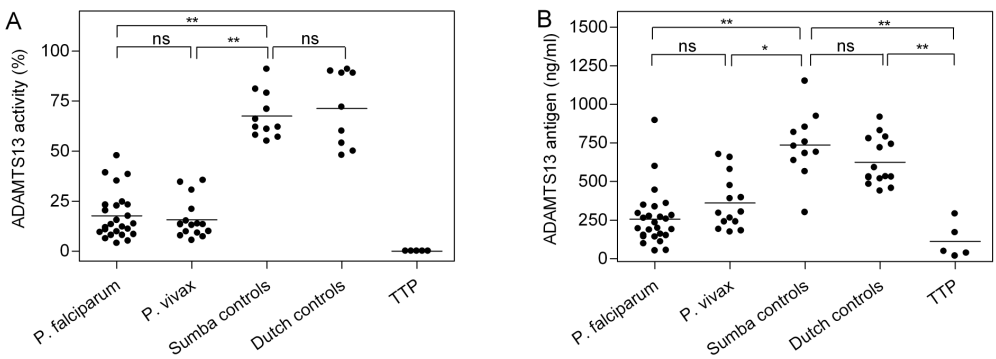

Figure 2. *Scatter dot plots with line at median showing ADAMTS13 activity (A) and ADAMTS13 antigen concentrations (B) in patients with symptomatic P. falciparum malaria (n= 25), P. vivax malaria (n= 15), healthy Sumbanese hospital staff controls (n = 11), healthy Dutch controls (n = 9), and thrombotic thrombocytopenic purpura (TTP) patients (n = 5). ADAMTS13 activity is expressed as percentage of normal pool plasma. Group comparisons were done using Kruskal-Wallis test, whereas pairwise comparisons were done using the Dunn procedure (* P < 0.05; ** P < 0.001; ns P > 0.05).*

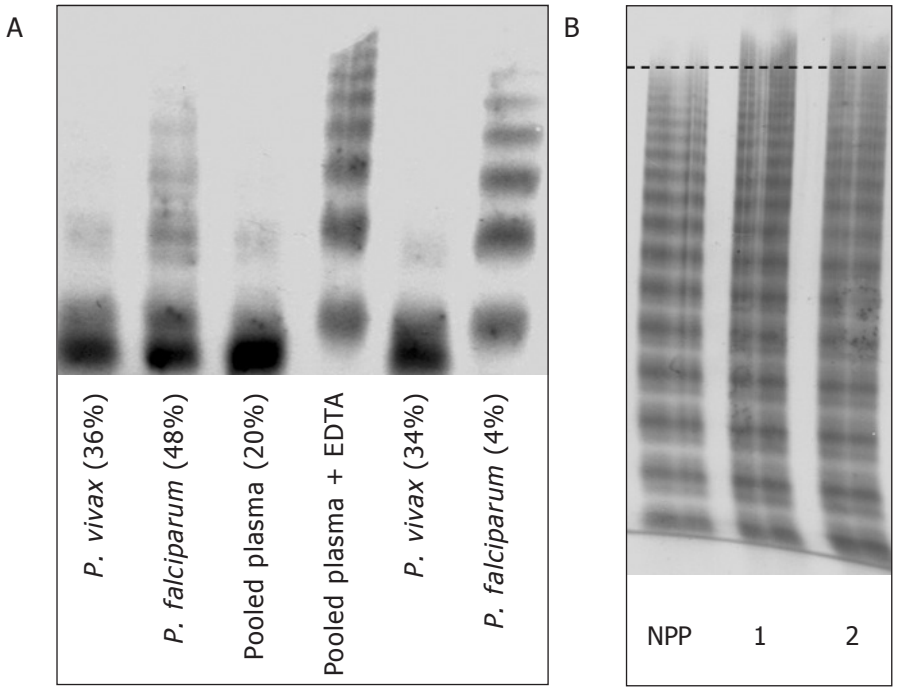

Figure 3. *(A) von Willebrand factor (VWF) proteolysis assay showing multimeric pattern of recombinant type 2a VWF after incubation with plasma from individual P. vivax and P. falciparum patients with moderate or severely reduced ADAMTS13 activity in FRETS-VWF73 assay and with normal pool plasma in absence and presence of EDTA (inactivator of ADAMTS13 activity). Percentages in parentheses indicate the ADAMTS13 activity in the plasma of the individual patients using FRETS-VWF73 assay. ADAMTS13 deficiency is shown by the presence of large VWF multimers. (B) VWF multimer pattern in the plasma of two patients with P. falciparum malaria with reduced ADAMTS13 activity (14% and 15%, respectively), showing presence of circulating ultra-large VWF multimers (multimers visible above the dashed line). NPP depicts normal pooled plasma of healthy Dutch subjects.*

Table 2. Mutations in the *ADAMTS13* gene identified in five Sumbanese individuals with the prevalence in 66 other Sumbanese individuals.

Subject	Exon	Nucleotide	Amino acid	Prevalence in 66 Sumbanese individuals		
				WT	Het	Ho
1 + 2 + 4 + 5	5	420 T > C	Silent	6%	31%	63%
1 + 2 + 3 + 4 + 5	15	1716 G > A	Silent	7%	36%	58%
1 + 2 + 4 + 5	19	2280 C > T	Silent	25%	23%	51%
4	29	4221 C > A	Silent	ND		
3	22	2814 G > T	K938N	100%	0%	0%

WT = wildtype; Het = heterozygous; Ho = homozygous; ND = not determined.

ADAMTS13 inhibitors and mutations in the *ADAMTS13* gene

In six malaria patients with strongly reduced ADAMTS13 activity levels, we determined the presence of ADAMTS13 inhibitors and ADAMTS13 autoantibodies. There was no difference in decline in residual ADAMTS13 activity between a mixture of normal pool plasma incubated with patient plasma and normal pool plasma alone (data not shown). In addition, no increased titers of ADAMTS13 autoantibodies of the IgG, IgA, or IgM class were found (data not shown). Because mutations in the *ADAMTS13* gene may also influence ADAMTS13 activity and antigen levels in the Sumbanese population, we screened a group of 71 Sumbanese for the presence of the *P475S* polymorphism, which is common in Japan [12]. This mutation was however not found in any of these subjects. Subsequent sequencing of the *ADAMTS13* gene of five Sumbanese to screen for the occurrence of other common polymorphisms yielded multiple silent exonic mutations in all five Sumbanese and one missense mutation in one subject (Table 2). The silent mutations have all been previously reported as single nucleotide polymorphisms (SNPs), 25 whereas the missense mutation has not been described before. However, although the silent mutations were highly prevalent in the remaining 66 Sumbanese, including our hospital staff controls with normal ADAMTS13 levels, the missense mutation was not found in others. Unfortunately, no ADAMTS13 level was available for the subject with the missense mutation.

Correlations between laboratory parameters

Figure 4 depicts correlations between VWF activation factor, platelet count, and ADAMTS13 activity and antigen concentrations in patients with *P. falciparum* or *P. vivax* infection. Higher VWF activation factors were associated with lower platelet counts (Figure 4A). Furthermore, ADAMTS13 antigen concentrations, but not ADAMTS13 activ-

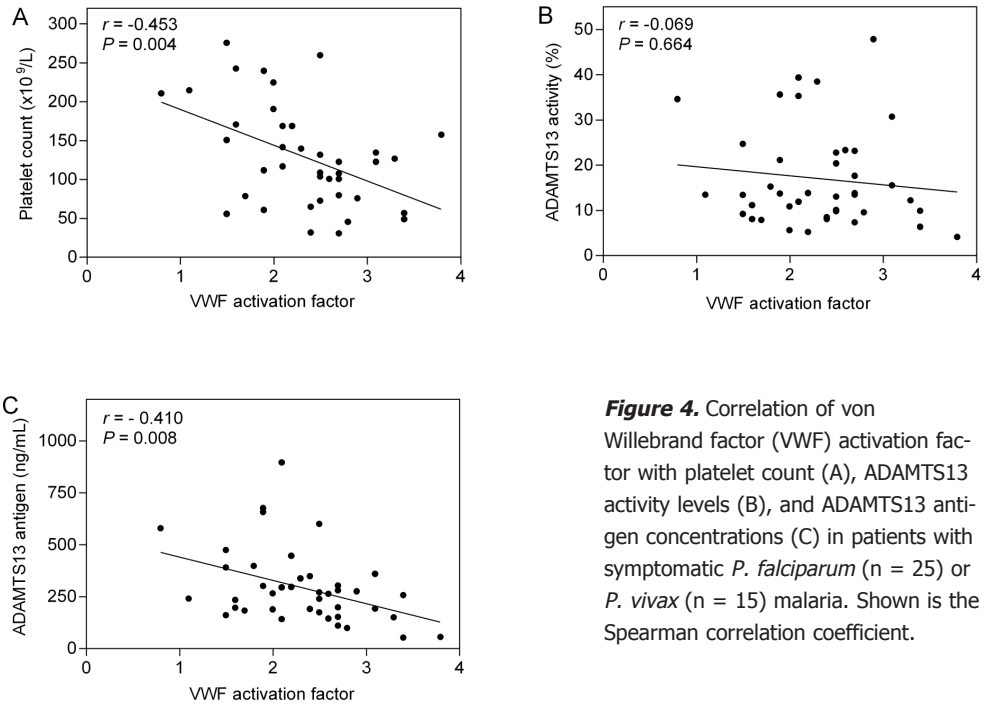

Figure 4. Correlation of von Willebrand factor (VWF) activation factor with platelet count (A), ADAMTS13 activity levels (B), and ADAMTS13 antigen concentrations (C) in patients with symptomatic *P. falciparum* (n = 25) or *P. vivax* (n = 15) malaria. Shown is the Spearman correlation coefficient.

ity levels, were inversely correlated with VWF activation factors (Figure 4B,C). ADAMTS13 antigen and VWF antigen concentrations were also inversely associated (Spearman r = -0.413; P = 0.007). Finally, there was an inverse correlation of IL-6 concentrations with ADAMTS13 activity levels (Spearman r = -0.417; P = 0.007) and with platelet count (Spearman r = -0.442; P = 0.006). No significant correlation was present between platelet numbers and either ADAMTS13 activity levels (Spearman r = 0.081; P = 0.626) or ADAMTS13 antigen concentrations (Spearman r = 0.273; P = 0.097).

DISCUSSION

In this study, we reported that symptomatic *P. falciparum* or *P. vivax* infections are associated with a significant increase in VWF and active VWF levels and a decrease in ADAMTS13 activity and antigen levels, resulting in the presence of circulating UL-VWF multimers.

The high VWF activation factors with circulating UL-VWF multimers indicated that an increased amount of the circulating VWF was in an active platelet binding conformation, which was supported by our findings of an inverse correlation between platelet numbers and VWF activation factors. We have previously found a similar association in our experimental human *P. falciparum* malaria model [5] and our current study expands these observations to naturally acquired *P. falciparum* and *P. vivax* infections. Both

sICAM-1 and VWF are known markers of endothelial cell perturbation, and these findings therefore suggest that endothelial cell perturbation is not a phenomenon restricted to *P. falciparum* but also occurs in *P. vivax* malaria, an infection in which thrombocytopenia is also a common observation [15]. This is in line with a previous study, which also found an almost similar increase in sICAM-1 concentrations in patients with either P. vivax or uncomplicated *P. falciparum* malaria [26]. We speculate that, in addition to providing a mechanistic explanation for the thrombocytopenia in *P. vivax* malaria, relapsing *P. vivax* blood infections, which arise from dormant liver stages, may induce repeated episodes of endothelial cell activation with excessive VWF release. In high-income countries, elevated VWF levels have been associated with adverse clinical consequences, such as increased risk for cardiovascular diseases [27]. However, the possible clinical consequences of high VWF levels and decreased ADAMTS13 activity need to be determined for low-income countries where malaria is endemic.

Recent evidence suggests that severe disturbances in the interplay of endothelial cells, platelets, VWF, and ADAMTS13 may result in secondary microangiopathy and thrombocytopenia-associated multi-organ failure in severely ill patients [28–30]. Our study included patients with symptomatic, but uncomplicated *P. falciparum* or *P. vivax* malaria. Although the occurrence of microvascular dysfunction was not routinely classified, the alterations in the balance between VWF secretion and ADAMTS13 activity in our patients probably did not seem to result in clinically relevant complications. However, at this moment, we cannot exclude with certainty that more severe disturbances in the VWF/ADAMTS13 system may contribute to the development of complications in severe malaria, as reported for severe sepsis. Future studies are needed in patients with severe malaria to test this hypothesis, as well as studies determining the functional threshold levels of ADAMTS13 activity below which complications may ensue. In addition, recent data have proposed a role for angiopoietin-2 in the pathogenesis of severe falciparum malaria [31]. Both angiopoietin-2 and VWF are constituents of endothelial cell Weibel-Palade bodies, highlighting the possible importance of Weibel-Palade body exocytosis in malaria pathogenesis.

At this time, the exact pathogenic mechanism behind the low ADAMTS13 activity levels in our malaria patients remains elusive. Induction of ADAMTS13 autoantibodies is the usual underlying pathogenic mechanism in TTP. Malaria may also induce various autoantibodies, as suggested by their high prevalence in malaria-endemic regions [32]. However, inhibitor assays could not show the presence of inhibitors or ADAMTS13 autoantibodies in our study population, although the used assays may not have been sufficiently sensitive to detect weak inhibitors or low ADAMTS13 autoantibody titers.

Alternatively, various non-immune mechanisms may be involved. First, consumption of ADAMTS13 is observed in situations with release of large amounts of VWF, as previously shown by desmopressin or endotoxin administration [33,34] whereas the proinflammatory cytokine IL-6 at high concentrations can inhibit, at least partially, ADAMTS13 activity [35]. In our study, VWF and IL-6 concentrations indeed correlated inversely with ADAMTS13 antigen concentrations. Regrettably, the limited sample size of our study did not allow a multivariate analysis. Second, parasites express various proteases and these might theoretically inactivate ADAMTS13, as has recently been shown for the bacterium *Bacillus anthracis* [36]. ADAMTS13 activity levels were, however, equally low in patients with *P. falciparum* and *P. vivax* malaria, despite the parasite density being much lower

in vivax malaria. Third, high concentrations of free haemoglobin [37] as can be found in massive intravascular hemolysis or *in vitro* hemolysis, and of the coagulation proteins plasmin and thrombin have been reported to reduce ADAMTS13 activity [38]. However, none of the plasma samples in our study were macroscopically hemolytic and a marked procoagulant state with diffuse intravascular coagulation is rare in malaria [39]. Fourth, reduced ADAMTS13 synthesis by liver stellate cells and/or endothelial cells may also result in ADAMTS13 deficiency. Recently, inflammatory cytokines were shown to reduce ADAMTS13 synthesis *in vitro* [40]. Finally, genetic mutations may influence ADAMTS13 activity and antigen levels, and malaria may have resulted in selection of certain polymorphisms in malaria-endemic regions. However, the *P475S* polymorphism, which is common in Japan, was not found in 71 Sumbanese individuals. In addition, sequencing of the *ADAMTS13* gene in a small number of Sumbanese subjects did show three highly prevalent SNPs, but these were also present in hospital staff controls with normal ADAMTS13 activity and antigen levels, suggesting that these SNPs are not associated with altered ADAMTS13 expression.

In conclusion, we showed that symptomatic *P. falciparum* and *P. vivax* infections are associated with endothelial cell perturbation, ADAMTS13 deficiency, and increased concentrations of active and UL-VWF. These combined mechanisms may contribute to malaria-induced thrombocytopenia. Future studies are needed to determine whether the disturbances in the interplay between endothelial cells, VWF, ADAMTS13, and platelets may also play a role in the complications observed in severe malaria.

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Chapter 8

General discussion

Introduction

Von Willebrand factor (VWF) plays an essential role in the adhesion of platelets to the injured vessel wall under conditions of blood flow. The platelet-binding site of plasma VWF, which is located in the A1 domain, is encrypted to prevent unwanted interaction with platelets. In order to arrest bleeding, VWF is converted from its latent conformation into an active conformation, a conformation in which the platelet-binding site in the A1 domain is exposed. Persistence of active VWF in the circulation would cause undesired VWF-platelet aggregate formation, resulting in thrombosis and/or bleeding from thrombocytopenia. Therefore, tight regulation of VWF activation and inactivation mechanisms is essential to prevent the occurrence of both bleeding and thrombotic events.

Undesired activation of VWF may occur under certain pathological conditions, among which thrombotic thrombocytopenic purpura (TTP), malaria and von Willebrand disease (VWD) type 2B. Although these conditions are of distinct origin, they share several clinical symptoms. TTP is caused by a genetic or auto-immune based deficiency of ADAMTS13. Deficiency of this VWF-inactivation machinery results in increased levels of active VWF in the circulation. Consequently, undesired VWF-platelet aggregates are being formed that occlude the microvasculature. Thrombocytopenia, microvascular thrombosis, anemia and organ failure are well-known complications of TTP. Malaria is a poverty-related disease caused by protozoan parasites from the genus *Plasmodium*. Its clinical symptoms closely resemble those of TTP. Thrombocytopenia is one of the hallmarks of malaria, although its pathology had not been unravelled so far. Finally, in VWD type 2B, a gain-of-function mutation in the VWF A1 domain causes conformational changes in the A1 domain that lead to exposure of the platelet-binding site. VWD type 2B patients are characterized by thrombocytopenia and a bleeding tendency.

This thesis aimed to contribute to a better understanding of the origin of active VWF (described in chapter 3) and focused on the relation between ADAMTS13, active VWF and consumptive thrombocytopenia in TTP (chapters 4 and 5) and malaria (chapters 6 and 7).

ADAMTS13

ADAMTS13 is a metalloprotease that cleaves VWF between tyrosine 1605 and methionine 1606 in the VWF A2 domain. The ADAMTS13 cleavage site is buried and not accessible in globular plasma VWF, although interaction between ADAMTS13 and the C-terminal domains on the surface does occur [1]. Proteolysis of VWF *in vitro* is typically studied in the presence of denaturants such as urea and guanidine hydrochloride. More physiologically, proteolysis of VWF is accelerated by shear stress and interactions with GpIbα [2,3] or coagulation factor VIII [4]. Alterations in ADAMTS13 proteolysis affect the multimeric size of VWF. A genetic or auto-immune based deficiency or dysfunction of ADAMTS13, resulting in TTP, is associated with the presence of ultralarge VWF multimers in the circulation. In contrast, increased proteolysis due to mutations in the VWF A2 domain causes a bleeding tendency in von Willebrand disease (VWD) type 2A patients. The bleeding tendency in VWD type 2B patients is paradoxical and poorly understood, as the gain-of-function mutation in the VWF A1 domain is suggestive for thrombosis. It is conceivable, however, that the conformational changes in VWD type 2B are not restricted to the GpIb-binding site in the VWF A1 domain, but that they also affect accessibility of the cleavage site in the VWF A2 domain. Indeed, it has been reported that proteolysis is increased in VWD type 2B patients [5,6]. Exposure of the cleavage site combined with the strong interaction with GpIba (being a co-factor for ADAMTS13 activity) could enhance ADAMTS13 cleavage, thereby preventing thrombus formation in VWD type 2B patients. Indeed, ADAMTS13 has been described to be able to regulate thrombus size *in vivo* [7].

Since the discovery of ADAMTS13 deficiency as the cause of TTP, much effort was put in the development of a rapid assay to determine ADAMTS13 activity in plasma of patients suspected for TTP. Initial attempts resulted in very labour intensive assays that were mostly based on the potential of plasma ADAMTS13 to cleave urea-denatured recombinant VWF. With the introduction of the FRETS-VWF73 assay, a more rapid assay became available that enabled quantitative measurement of ADAMTS13 activity within one hour [8]. This assay is based on a fluorescent substrate comprising 73 amino acids of the VWF A2 domain. We found a very good correlation between the results of the FRETS-VWF73 assay and the classical proteolytic multimer assay (chapter 5). The easy-use, rapidity and good reproducibility of the FRETS-VWF73 assay made it a valuable tool that is nowadays commonly used in TTP-diagnosis in the Netherlands. Quantification of the amount of ADAMTS13 activity has contributed to a better understanding of the pathogenesis of several diseases, though undetectable ADAMTS13 activity remains specific for primary TTP (TTP without demonstrable causes such as bone marrow transplantation) and pregnancy-related TTP. Based on clinical symptoms, it is often hard to discriminate pregnancy-related TTP from another pregnancy-related disease, namely the HELLP (hemolysis, elevated liver enzymes and low platelets)-syndrome. Results of ADAMTS13 activity analysis appears to be conclusive for differential diagnosis as ADAMTS13 deficiency is specific for pregnancy-related TTP, while ADAMTS13 activity was only mildly decreased in HELLP patients [9]. Limitations of the FRETS-VWF73 assay concern the small size of the peptide, the absence of endothelial cells and the fact that the assay is performed under static conditions. The influence of binding of ADAMTS13 to VWF and the effect of co-factors can therefore not be investigated.

Active VWF

The first illustrative evidence of conformational changes within the VWF molecule was described by Siedlecki and colleagues [10]. In the absence of shear stress, VWF has a globular conformation that stretches into an extended chain conformation by high shear stress. This stretching is relevant for platelet-binding as it causes exposure of the platelet-binding site within the VWF A1 domain, thereby allowing interaction with GpIba [11]. The interaction between active VWF and GpIbα has been further elucidated at atomic level. Crystal structure of the GpIbα–VWF A1 complex revealed two distinct areas of tight interaction [12]. The largest contact site mainly depends on conformational changes in the flexible loop in the C-terminus of GpIbα. The second contact site involves interaction between the N-terminus of GpIbα and the bottom face of the VWF A1 domain. Under normal conditions, terminal flanking peptides of the VWF molecule shield this bottom face binding site. It is thought that high shear stress, VWD-type 2B mutations and addition of ristocetin cause displacement of these flanking peptides, leading to exposure of the second binding site for GpIbα [13]. The llama derived antibody fragment AU/VWF-a11, which has been used throughout the manuscript to detect and quantify active VWF, displays high affinity for VWF upon introduction of a type 2B mutation in the VWF A1 domain and upon addition of ristocetin. Even though AU/VWF-a11 recognizes the platelet-binding conformation of VWF, it does not interfere with the interaction between VWF and GpIbα [14]. In that same paper by Hulstein and colleagues, a control nanobody was used that did inhibit platelet adherence to VWF. In contrast to AU/VWF-a11, this control nanobody does not display differential binding to wildtype or type 2B VWF. Possibly, AU/VWF-a11 binds an epitope in the VWF A1 domain that becomes exposed after displacement of the flanking peptides, thereby specifically recognizing the active conformation of the A1 domain. The binding epitope of the control nanobody might be at the larger contact site, thereby inhibiting the interaction between VWF and the C-terminus of GpIbα. Resolving the crystal structures of the nanobody-VWF A1 complexes will contribute to further understanding of the mechanism of VWF activation.

Endothelial activation and release of active VWF

VWF secreted from endothelial cells displays spontaneous binding to platelets. To prevent the formation of large VWF-platelet thrombi, ADAMTS13 rapidly cleaves the VWF multimers upon secretion from the endothelium. This process is thought to reduce the platelet-binding properties of VWF. The current dogma ascribes the platelet-binding properties of freshly secreted VWF to the UL-multimers. However, in chapter 4 it was shown that the active conformation of VWF is not restricted to UL-VWF, but rather covers the full spectrum of multimers. If ADAMTS13 is responsible for inactivation of VWF, it is likely that multimers of all sizes can hold their active conformation in the absence of ADAMTS13, even the smaller multimers. This is further supported by the fact that the recombinant VWF A1 domain, a small-sized fragment that cannot be cleaved by ADAMTS13, is able to bind GpIbα in the absence of modulators [15]. Of note, introduction of a VWD type 2B mutation or addition of ristocetin caused an increased activation state. Under conditions of flow, full-length endothelial cell-derived VWF can also be further activated by both means (chapter 3). A difference in activation state between wildtype and VWD type 2B endothelial cell-derived starts already in the Weibel-Palade

bodies of the endothelial cells. In wildtype endothelial cells, VWF is stored in a latent conformation, while stored in an active conformation in VWD type 2B cells. Upon secretion, VWF switches to its active conformation. The exact mechanism behind this transition has to be elucidated.

Perfusion experiments showed that platelets adhere randomly over the long VWF-strings that are secreted from the endothelial cells. Activation by ristocetin or perfusion over VWD type 2B endothelial cells remarkably increased the number of platelets adhering to a VWF-string, seemingly leaving no platelet-binding site unoccupied. Possibly, wildtype VWF under these conditions is not fully activated, even though AU/VWF-a11 staining indicated that the VWF-string is uniformly active. This might point at a two-step mechanism in VWF activation. In the first step, the flanking peptides that normally shield the bottom face binding site of the VWF A1 domain are displaced, which allows interaction with AU/VWF-a11 but not yet with platelets. In the second step, VWF becomes further activated so that platelets can bind. This second step in VWF activation could be the consequence of shear stress or displacement of VWF-bound proteins that were functioning as a shield in preventing the binding of platelets. So far, only ß2-glycoprotein I (ß2-GPI), the main antigenic target for autoantibodies in the antiphospholipid syndrome, has been to shown function as a physiological inhibitor of the VWF-GpIbα interaction [16]. However, ß2-GPI is not present in endothelial cells and could thus not be responsible for inhibition of platelet adhesion to VWF strings secreted from endothelial cells. Osteoprotegerin (OPG) was considered as a likely candidate as it was shown to (i) co-localize with VWF in the Weibel-Palade bodies (ii) remain in complex with VWF upon secretion and (iii) interact with VWF through the VWF A1 domain [17,18]. Platelet adhesion to a VWF-coated surface was reduced by 40% in the presence of OPG, indicating that OPG is a potent inhibitor of platelet adhesion to VWF (chapter 3). Further research is necessary to determine the clinical relevance of OPG in relation to VWF function.

Upon perfusion with plasma, the platelet-decorated VWF-strings are rapidly cleaved by ADAMTS13. Perfusion with ADAMTS13-deficient plasma, however, does not result in cleavage of the strings. This *ex vivo* model might therefore be helpful to study VWF proteolysis in patients of whom the ADAMTS13-related pathogenesis is not fully understood. As described in chapter 5, some acute TTP patients present with classical symptoms of TTP and respond well to plasma exchange. Surprisingly, these patients do display some residual ADAMTS13 activity in the FRETS-VWF73 and the proteolytic multimer assay. Moreover, as described in the next paragraph, several TTP patients in remission persistently lack ADAMTS13 activity without developing a relapse. The HUVEC-perfusion system could serve as a nice tool to test plasmas from these patients for their potential to cleave the VWF-strings. This will likely contribute to a better understanding of the pathogenesis of their disorders.

Nishio and colleagues have used static assays to demonstrate the stimulatory effect of recombinant GpIbα on ADAMTS13 proteolysis of truncated VWF-forms [2]. The HUVEC-perfusion system can now be used to study the effect of whole platelets on cleavage of full-length endothelial cell-derived VWF under conditions of flow, thereby more closely

resembling the *in vivo* situation. The perfusion experiments are generally performed using light microscopy. The VWF-strings are visualized indirectly by the adherence of platelets as they are too thin themselves. Cleavage of the VWF-strings by ADAMTS13 is subsequently visualized by disappearance of the platelets. In chapter 5 it has been shown that immunofluorescence techniques can be used to visualize the VWF-strings themselves, without the requirement of platelets (chapter 5). This approach will be valuable for future studies on the effect of platelets on proteolysis of VWF by ADAMTS13.

ADAMTS13 and active VWF in TTP in remission

Acquired TTP is caused by the development of auto-antibodies against ADAMTS13. During an episode, patients are treated with plasma exchange to remove the auto-antibodies and to supply the patient with fresh ADAMTS13 protein. Remission is achieved when patients do not longer require plasma to prevent thrombosis. Surprisingly, ADAMTS13 activity does not always return to normal values upon remission. Monitoring a cohort of TTP patients in remission revealed that ADAMTS13 activity remains undetectable in almost 25% of the patients without directly causing a new episode (chapter 4). Although our cohort was relatively small, it appeared that patients with low or absent ADAMTS13 activity upon remission are at highest risk for development of a new episode. These findings were in line with the results of a larger study in which it was shown that ADAMTS13 deficiency in remission increases the risk for relapsing 3-fold compared to patients that display normal ADAMTS13 levels [19]. Although acquired TTP is related to the presence of anti-ADAMTS13 antibodies, these antibodies could not be detected in all patients with low or undetectable ADAMTS13 activity. This phenomenon is also regularly seen in plasma of TTP patients in the acute phase. Either the commercially available immunosorbent assays are not sensitive and reliable enough to detect the patients antibodies, or the low affinity of these antibodies prevents detectable binding to the plate-immobilized ADAMTS13. Alternatively, ADAMTS13 antigen-antibody complexes are rapidly cleared from the circulation which prevents the detection of antibodies in plasma [20]. The latter is likely to occur as ADAMTS13 antigen levels are also undetectable in TTP patients with undetectable ADAMTS13 activity levels [21].

ADAMTS13 was found to have a strong influence on active VWF levels in TTP patients in remission. Although ADAMTS13 deficiency seems to increase the risk for developing a new episode, these strong pro-thrombotic conditions do not directly lead to platelet aggregation and microthrombus formation. Alternatively, if these thrombi are being formed, they remain undetectable as patients do not present any clinical signs of a microangiopathy. Apparently, ADAMTS13 deficiency is not enough to induce a new episode. This favours the idea of a second-hit model. Likewise, ADAMTS13-knockout mice do not present any clinical symptoms of TTP [22]. Introduction of a genetic background that is associated with increased VWF levels led to the development of spontaneous thrombocytopenia in a subset of the mice. Subsequent challenge with a bacterial toxin resulted in a phenotype closely resembling human TTP. Infections might not only play a role in the development of TTP by activating the endothelium, it might also induce the development of auto-antibody. Molecular mimicry, defined as similar structures shared by products of dissimilar genes, has been suggested to be associated with sev-

eral autoimmune diseases like rheumatoid arthritis [23] and the Varicella-autoantibody syndrome [24]. Assuming that molecular mimicry is involved in the pathogenesis of TTP, microbial structures resembling the spacer-domain of ADAMTS13 are of particular interest as epitope mapping revealed that the majority of TTP patients develops antibodies directed against these spacer-domains [25,26].

ADAMTS13 and active VWF in malaria

Malaria is a poverty-related disease that is caused by protozoan parasites from the genus *Plasmodium*. Thrombocytopenia is found in up to 80% of malaria patients and correlates with both parasite density and disease severity. The pathological mechanism behind thrombocytopenia in malaria had not been unravelled so far. Research has focussed on complement activation, platelet activation and activation of the coagulation system but none of these mechanisms could explain the thrombocytopenia. Meanwhile it was well established that endothelial activation plays an important role in the pathogenesis of malaria. In this thesis, the role of active VWF in the development of thrombocytopenia was investigated in an experimental human malaria model (chapter 6) and in a field study on the Indonesian island Sumba (chapter 7). In the experimental model, healthy volunteers were infected with *P. falciparum* and checked daily for the presence of parasites using a thick blood smear. Anti-malarial treatment was initiated upon a positive blood smear, when no clinical symptoms of infection were observed yet. Platelet numbers started to decrease very rapidly after infection. In this early stage of infection, the presence of parasites was confirmed by PCR rather than by a positive blood film as parasite densities were still at sub-microscopic detection level. The early drop in platelet count was associated with increased active VWF levels, which suggests causality.

The malaria patients from Sumba were characterized by a severe thrombocytopenia. Like in the experimental model, platelet counts showed an inverse correlation with active VWF levels. The increase in active VWF levels in the experimentally infected volunteers could not be explained by ADAMTS13 as activity levels did not significantly change throughout the infection. In contrast, malaria patients from Sumba showed a strong decrease in ADAMTS13 activity. This discrepancy might reflect the severity of the infection. In the experimental model, infection is abrogated before presentation of any clinical symptoms, while samples from Sumba were drawn from severely ill patients. As described for TTP, development of auto-antibodies against ADAMTS13 may result in clearance of ADAMTS13 from the circulation. Anti-ADAMTS13 antibodies were not detected in the Sumba-patients, although detection of these antibodies can be as difficult as discussed above for TTP. Inflammatory cytokines have been reported to inhibit ADAMTS13 activity or synthesis in the liver [27,28]. Alternatively, variations in the *ADAMTS13* gene might also affect functionality and/or clearance rate. Increased VWF antigen levels and reduced ADAMTS13 levels in malaria were confirmed by Larkin and colleagues [29]. Whereas we could not yet explain the reduction in ADAMTS13 activity, Larkin et al suggested the presence of an unidentified inhibitor. A time-dependent reduction in ADAMTS13 activity was shown in plasma from malaria patients, when mixed with normal pooled plasma. Similar experiments were performed in our lab, but this time-dependent reduction in ADAMTS13 activity was already found in normal pooled plasma. Therefore we cannot exclude the contribution of an experimental artefact in the

results presented by Larkin. Further research is necessary to clarify the exact mechanism behind ADAMTS13 activity reduction in malaria.

From both the experimental model and the Sumba study it can be concluded that vascular endothelial cell activation and the subsequent release of active VWF plays an important role in the development of thrombocytopenia in malaria. In the experimental infection model, increase in active VWF levels was already observed at the end of the liver stage, before the parasites entered the circulation. In this early infection, the liver may secrete (yet unidentified) factors that cause activation of the endothelium. Endothelial activation and the subsequent release of active VWF may support sequestration of the parasitized red blood cells, a process that is generally assumed to play an important role in the pathophysiology of malaria. It has been demonstrated that platelets facilitate cytoadherence by acting as bridges between parasitized red blood cells and endothelial cells [30]. In turn, secreted active VWF may function as a bridge between platelets and the endothelium. In this scenario, activation of the endothelium is advantageous for the parasite. Alternatively, activation of the endothelium might be a reaction of the host to trigger an immune reaction against the malaria parasites. Release of active VWF coincides with the release of IL-8, an important mediator of the innate immune system, which is also stored in the Weibel-Palade bodies of endothelial cells.

Results of both the experimental model and the field study indicate that active VWF may provide a mechanistic explanation for the development of thrombocytopenia in malaria, as it does in TTP. Prevalence of VWD, caused by qualitative or quantitative defects of VWF, has been estimated to be 1%. Combining future VWF- and ADAMTS13-studies in malaria endemic regions with screening for VWD will be very helpful to address the relevance of VWF in malaria. In case VWF deficiency or dysfunction is protective against malaria, intervention of the VWF-GpIbα axis could be a new target for anti-malarial treatment. However, this therapy will probably only reduce symptoms and as malaria is a disease of poverty, it might be too expensive for poor countries. Administration of an anti-malarial vaccine might thus be cheaper in relation to its effectiveness, although development of an effective vaccine has not been successful so far due to the high mutation rate in the genome of the parasite.

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Chapter 9

Nederlandse samenvatting

Inleiding

Von Willebrand factor is het grootste eiwit dat we in ons bloed hebben. In geval van bloedvatschade fungeert het als 'lijm' tussen de vaatwand en de bloedplaatjes om zo een korstje te vormen en de bloeding te stelpen. Mensen die geen of slecht functionerend von Willebrand factor hebben, hebben dan ook een bloedingsneiging. Mensen met verhoogde von Willebrand factor spiegels in hun bloed hebben daarentegen een verhoogde kans op trombose. Bekende voorbeelden van trombose zijn het trombosebeen, de longembolie en een hartinfarct. De hoeveelheid von Willebrand factor wordt gereguleerd door een enzym genaamd ADAMTS13. Verstoring van de balans tussen ADAMTS13 en von Willebrand factor leidt tot trombose klachten. Deze verstoring is het meest duidelijk in de ziekte trombotische trombocytopenische purpura (afgekort TTP), die veroorzaakt wordt door een tekort aan goed functionerend ADAMTS13. In dit hoofdstuk wordt uitleg gegeven over von Willebrand factor, ADAMTS13 en TTP. Daarnaast wordt ook ingegaan op tropische malaria. Ondanks het feit dat TTP en malaria twee ziektebeelden zijn met een totaal andere oorzaak, lijken ze qua klinische symptomen sterk op elkaar. Daarom hebben we in ons onderzoek ook gekeken of de symptomen van tropische malaria verklaard konden worden aan de hand van een verstoring in de ADAMTS13 / von Willebrand factor balans.

Von Willebrand factor en ADAMTS13

Von Willebrand factor is een eiwit dat normaal opgerold tot een bolletje door de bloedbaan stroomt. Als het een beschadiging in de vaatwand tegenkomt, bindt het aan de beschadigde plek en ontrolt vervolgens tot een sliert. In tegenstelling tot de bolvorm, kan de sliertvorm bloedplaatjes vangen. Von Willebrand factor fungeert dan als lijm tussen de beschadigde vaatwand en de bloedplaatjes om zo een korstje te vormen en de bloeding te stelpen. De bolvorm van von Willebrand factor, die dus niet de mogelijkheid heeft om bloedplaatjes te binden, noemen we rustend von Willebrand factor. De ontrolde sliert, die wel bloedplaatjes aan zich kan binden, noemen we actief von Willebrand factor. De oorsprong van rustend en actief von Willebrand factor wordt beschreven in hoofdstuk 3. Om aan de ene kant bloedingen, en aan de andere kant trombose te voorkomen, moet de balans tussen rustend en actief von Willebrand factor goed gereguleerd worden. Een aantal ziektebeelden is beschreven waarin teveel actief von Willebrand factor door de bloedbaan stroomt terwijl er geen vaatwandschade is. Dit actieve von Willebrand factor vangt bloedplaatjes terwijl dat niet nodig is. Hierdoor ontstaan ongewenste bloedpropjes die de bloedvaten kunnen blokkeren (trombose) met alle gevolgen van dien. Deze ziektebeelden worden veroorzaakt door een tekort of slecht functionerend ADAMTS13. ADAMTS13 is in staat om actief von Willebrand factor in kleinere stukjes te knippen waardoor het op kan rollen tot een bolletje; de rustende vorm van von Willebrand factor. Problemen aan de kant van ADAMTS13 kunnen dus via von Willebrand factor leiden tot trombose.

In hoofdstuk 3 is een nieuw eiwit beschreven dat de von Willebrand factor activiteit remt. Dit eiwit heet osteoprotegerine en is bekend vanwege zijn belangrijke rol in het voorkomen van osteoporose, oftewel botontkalking. Toekomstig onderzoek zal de exacte rol van osteoprotegerine in de regulering van von Willebrand factor activiteit moeten bevestigen.

Trombotische trombocytopenische purpura (TTP)

TTP is een zeer zeldzaam ziektebeeld met slechts 50-100 patiënten in Nederland. Patiënten melden zich bij hun arts met aanvankelijk algemene klachten als koorts, vermoeidheid, hoofdpijn en misselijkheid. Als er dan blauwe plekken bijkomen of de patiënt soms zelfs in coma raakt, is een snelle diagnose en de juiste behandeling van levensbelang.

TTP wordt veroorzaakt door een tekort aan goed functionerend ADAMTS13. In de meeste gevallen wordt dit veroorzaakt doordat het lichaam een auto-immuun reactie ontwikkelt tegen ADAMTS13. Een bekend voorbeeld van een auto-immuun ziekte is reuma. In het geval van TTP zorgen de auto-immuun antistoffen tegen ADAMTS13 ervoor dat ADAMTS13 niet meer kan functioneren. Hierdoor kunnen TTP patiënten actief von Willebrand factor niet meer omzetten naar rustend von Willebrand factor. Als gevolg hiervan stijgt de hoeveelheid actief von Willebrand factor in de bloedbaan en zal deze actieve vorm bloedplaatjes gaan binden. Er ontstaan bloedpropjes die trombose kunnen veroorzaken, wat bij sommige patiënten neurologische verschijnselen geeft of zelfs tot coma kan leiden. Doordat alle bloedplaatjes opgehoopt zitten in de bloedpropjes, daalt de concentratie bloedplaatjes in de bloedbaan. Dit zit verwerkt in het woord 'trombocytopenie', oftewel lage bloedplaatjes waarden. Patiënten die te weinig bloedplaatjes hebben, zullen gaan bloeden bij bloedvatschade. TTP patiënten hebben dan ook vaak blauwe plekken, wat terug te vinden is in het woord 'purpura', wat paars betekent in het Latijn.

Onbehandeld zal 90% van de patiënten overlijden. Er is slechts één effectieve manier om TTP te behandelen, namelijk plasmaferese. Dit is een soort dialyse waarbij de patiënt wordt gespoeld en zijn of haar bloedplasma wordt vervangen door donorplasma van de bloedbank. Dit is een kostenintensieve behandeling. Eén liter donorplasma kost 1000 euro. Gedurende een behandelcyclus van drie weken krijgt een TTP patiënt ongeveer 150 liter donorplasma toegediend. De gedachte achter plasmaferese is dat de patiënten worden voorzien van nieuw ADAMTS13. Donor-ADAMTS13 knipt dan de actieve von Willebrand factor moleculen van de patiënt in kleinere rustende stukjes waardoor ze niet meer aan bloedplaatjes zal binden. De arts verklaart een TTP patiënt genezen als zijn of haar aantal bloedplaatjes hoog blijft zonder dat de patiënt daar plasmaferese voor nodig heeft.

Als een arts vermoedt dat een patiënt TTP heeft, wordt de hoeveelheid ADAMTS13 in het bloed gemeten. Jarenlang werd dit gedaan met behulp van een moeilijke, arbeidsintensieve test die twee dagen duurde. Een Japanse groep heeft echter in 2005 een sneltest ontwikkeld waarmee de resultaten binnen een uur bekend zijn. Wij hebben in dit proefschrift de uitslagen van deze 1-uurs test vergeleken met de ouderwetse 2-daagse test (hoofdstuk 5). De testen gaven dezelfde uitslagen bij 79 TTP patiënten of patiënten die verdacht werden van TTP. Daarmee is de nieuwe sneltest van grote waarde geworden voor de TTP diagnostiek. Vandaag de dag wordt het dan ook in bijna alle ziekenhuizen in Nederland gebruikt.

Na ontslag uit het ziekenhuis zal de patiënt in het begin regelmatig op controle moeten komen om de bloedplaatjes te laten meten. Als die weer beginnen te zakken, ligt er mogelijk een nieuwe TTP aanval op de loer. In dit proefschrift hebben we een groep Nederlandse ex-TTP patiënten gevolgd na zo'n aanval (hoofdstuk 4). Bij deze patiënten hebben we gekeken naar actief von Willebrand factor en ADAMTS13 tijdens herstel en

bijgehouden wanneer ze weer een aanval kregen. Opvallend genoeg zagen we dat de ADAMTS13 waarden in 25% van de patiënten niet normaliseren, terwijl deze patiënten geen klinische symptomen van TTP lieten zien. In veel andere patiënten kruipt de waarde iets omhoog, maar blijft ze veel lager dan in gezonde mensen. Zoals verwacht, bleek er een sterk verband te zijn tussen de ADAMTS13 activiteit en de hoeveelheid actief von Willebrand factor. In de patiënten die lage ADAMTS13 waarden behielden na een aanval, was de hoeveelheid gemeten actief von Willebrand factor dan ook beduidend hoger dan in de patiënten met genormaliseerde ADAMTS13 waarden. Het blijkt dan ook dat patiënten met afwezige of lage ADAMTS13 waarden de grootste kans hebben om opnieuw een TTP-aanval te ontwikkelen.

Tropische malaria

Malaria is een ziekte die voornamelijk voorkomt in de tropen. Met 1-3 miljoen sterfgevallen per jaar is malaria een van de ernstigste dodelijke infectieziekten ter wereld. Malaria wordt veroorzaakt door een infectie met Plasmodium falciparum, een parasiet die door de malariamug wordt overgedragen. Ondanks dat malaria veroorzaakt wordt door een parasiet en TTP een auto-immuun ziekte is, zijn er qua ziektebeeld sterke overeenkomsten. Koorts en neurologische verschijnselen (zelfs coma) zijn de duidelijkste overeenkomsten. Na bloedprikken blijkt dat ook malaria patiënten een diepe trombocytopenie (lage bloedplaatjes waarden) hebben, net als TTP patiënten. Daarom hebben we gekeken of de symptomen van tropische malaria ook verklaard zouden kunnen worden aan de hand van actief von Willebrand factor en ADAMTS13.

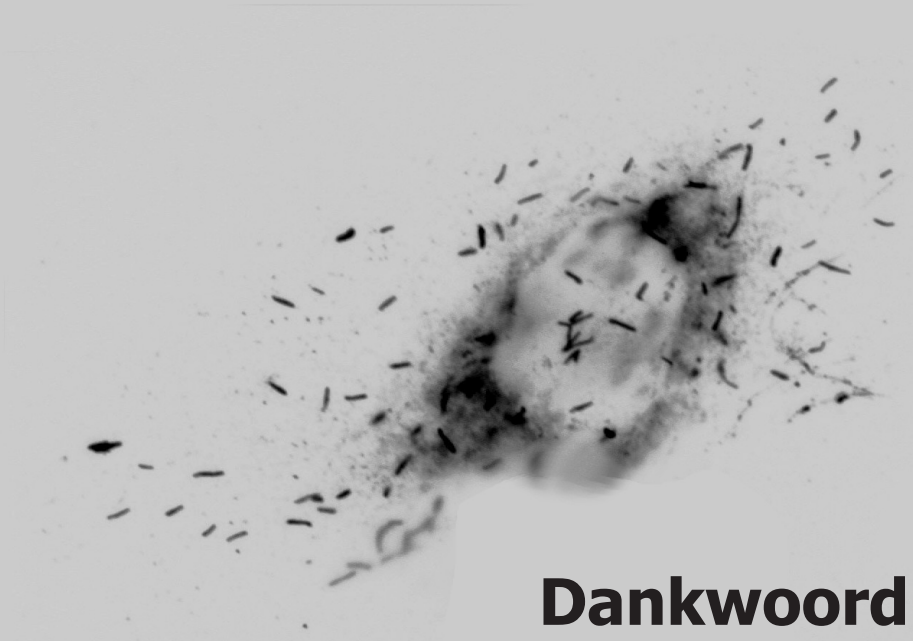
In eerste instantie hebben we een model gebruikt waarin gezonde vrijwilligers onder gecontroleerde omstandigheden geïnfecteerd werden met malaria (hoofdstuk 6). De vrijwilligers kregen niet tot nauwelijks klachten, sommigen hooguit wat koorts. Na ongeveer een week werden de parasieten in een uitstrijkje van het bloed zichtbaar. Dit was het moment dat de vrijwilligers direct anti-malaria medicatie kregen toegediend en volledig herstelden van de infectie. Zoals bekend was van malaria, daalde het aantal bloedplaatjes in het bloed van de vrijwilligers na infectie. Vanaf het moment dat de bloedplaatjes daalden, begon de von Willebrand factor spiegel in het bloed te stijgen. Het von Willebrand factor bleek in de actieve vorm te zijn. Kennelijk leidt een malaria infectie ook tot een verhoging in actief von Willebrand factor. Net als in TTP kunnen deze actieve von Willebrand factor moleculen aan bloedplaatjes binden waardoor kleine bloedpropjes kunnen ontstaan en het aantal bloedplaatjes in de bloedbaan daalt. De vrijwilligers vertoonden echter geen symptomen van trombose, wat waarschijnlijk komt omdat gezonde vrijwilligers genoeg ADAMTS13 hebben om de eventueel kleine bloedpropjes snel op te ruimen.

Vervolgens hebben we onze hypothese getest op bloed van malaria patiënten van het Indonesische eiland Sumba (hoofdstuk 7). Deze patiënten verkeerden in een veel ernstiger stadium van infectie, sommigen zelfs tot aan coma toe. Analyse van het bloed van deze patiënten liet zien dat deze patiënten, net als TTP patiënten en de gezonde vrijwilligers, een sterke daling in bloedplaatjes aantallen hadden. Dit bleek samen te gaan met een toename in von Willebrand factor spiegels. Ook hier bleek het te gaan om actief von Willebrand factor. In deze patiënten bleek verder dat de ADAMTS13 waarden fors lager waren in vergelijking met gezonde inwoners van Sumba. Weliswaar waren de ADAMTS13 waarden nog niet zo laag als bij TTP patiënten, maar in een aantal gevallen

toch zorgwekkend laag. De oorzaak hiervan is helaas nog onbekend. Toekomstig onderzoek zal zich richten op het effect van een malaria infectie op de synthese van ADAMTS13 en op afwijkingen in het DNA die kunnen bijdragen aan de daling in waarden.

Conclusie

TTP is een zeldzame bloedziekte die veroorzaakt wordt door een tekort aan ADAMTS13. Hierdoor stijgt de actief von Willebrand factor spiegel in het bloed, waardoor het risico op trombose toeneemt. Onbehandeld is de kans groot dat een patiënt komt te overlijden. Snelle diagnose is daarom van levensbelang. Met de introductie van een 1-uurs test voor de ADAMTS13-bepaling is dit veel makkelijker geworden. Het is gebleken dat TTP patiënten, die tijdens hun periode van herstel weinig ADAMTS13 in hun bloed hebben, de grootste kans hebben om weer ziek te worden. Zorgvuldige monitoring van deze patiënten is daarom van groot belang. Met de kennis van het zeldzame TTP hebben we veel geleerd over het grote malaria. De klachten bij malaria patiënten blijken net als bij TTP patiënten gedeeltelijk verklaard te kunnen worden aan de hand van een verstoorde ADAMTS13 – von Willebrand factor balans. Toekomstige therapie, die erop gericht is om de interactie tussen actief von Willebrand factor en bloedplaatjes te verhinderen, zal mogelijk een waardevolle bijdrage kunnen leveren aan het herstel van TTP en malaria patiënten.



Dankwoord

Promoveren doe je niet alleen en het tot stand komen van dit boekje is dan ook mede te danken aan mijn lieve collega's, familie en vrienden.

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De AIO-kamer heeft de hoogste bezettingsgraad van de Hematologie-gang. Het biedt plaats aan 7 AIO's die allemaal in hetzelfde schuitje zitten: artikelen schrijven, deadlines voor een abstract, presentaties maken, 's avonds eten in De Brink. En regelmatig met z'n allen op ~~vakantie~~ congres! Dank jullie wel voor alle gezelligheid en steun! Dianne, je bent een supercollega. We hebben samen heel wat afgelachen als komisch duo. Promoveren krijgen we niet kado, maar ik heb alle vertrouwen in jou! Anja, vier jaar geleden we zijn samen begonnen met ons onderzoek. Je wilt verder in het diabetesonderzoek, of toch niet…Maar zoals ze in Frisco zeggen: 'You go girl!!!' Erik, voor jou heb ik grote waardering. Hoe heb je het al die tijd met zoveel vrouwen op een kamer uit kunnen houden? Je kennis van dameslaarzen is in die tijd bewonderenswaardig gegroeid! Gwen, altijd vol wilde ß2-plannen. ß2 plakt aan VWF, dus succes gegarandeerd voor jouw onderzoek. Esther, enige hemofilie AIO van onze afdeling, maak er wat moois van. Mirjam, opvallend genoeg worden jouw dagen in Utrecht ter gelegenheid van een borrel gekenmerkt door zeer hoge odds-ratios…

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Lab III is altijd het drukste lab van onze afdeling geweest. Sandra, de lab-bitch met een heerlijke dosis humor. Het komt vast niet stoer over als ik zeg dat ik je een schatje vind…maar toch is het zo! Arnold, man van principes en grootmoeders tegeltjes. Succes met je nieuwe functie, ik heb het volste vertrouwen in je! Suzanne, altijd enthousiast om iets te organiseren of om rond te gaan met een kaartje. Wat is het stil nu je weg bent! Suzanne, jij bent al wat langer weg, maar voor mij hoor je er nog helemaal bij.

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TTP is een zeldzame ziekte met maar weinig patiënten in Nederland. Door de grote interesse en welwillendheid van de patiënten hebben we toch heel veel kunnen doen. Ik wil alle patiënten dan ook hartelijk bedanken voor hun bijdrage aan ons onderzoek. Lia, als voortrekker van de TTP-club wil ik vooral jou bedanken. Ik kon altijd op je rekenen. Had ik weer wat gegevens nodig? Eén mail naar jou en binnen een week had ik alles binnen. Kristel, je bent een geval apart. Dacht ik als onderzoeker nieuwe inzichten in TTP te hebben behaald? Bij jou was alles natuurlijk nét weer even anders. Je hebt me veel meegegeven over hoe het is om met TTP te leven. Kop op meid, samen met jouw kleine muis sla jij je overal doorheen!

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Evelyn

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