HAEMOSTATIC AND THROMBOTIC DISORDERS: A JOURNEY FROM BENCH TO BEDSIDE

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Sarah Muriel Meiring
December 2019

DECLARATION

Hereby I, Sarah Muriel Meiring, declare that the compilation in respect of the D Med Sc degree that I herewith submit at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education. I declare that in all applicable publications, I had been involved in writing up the process of literature research, conceptualisation, and refining techniques on the bench; I had been deeply involved in the study design, data management, analysis and interpretation of data; condensing drafts, figure selection and approving the final versions of the manuscripts.

I am aware that the copyright vests in the University of the Free State and that all royalties with regard to intellectual property that had been developed during the course of and/or in connection with the study at the University of the Free State, will accrue to the university.

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SYNOPSIS

This compilation focuses on thrombotic and haemostatic disorders, illustrating my journey from basic research on thrombotic and haemostatic disorders to the differential diagnoses of these disorders. For purposes of clarity I shalll divide it in three parts.

The first part includes the testing of antithrombotic agents. My scientific career started with the testing of antithrombotic drugs in a baboon model of arterial thrombosis. These antithrombotic drugs were mostly targeted at platelets and, to a lesser extent, coagulation. For my PhD, I clarified the catabolism, pharmacokinetics and exctretion of recombinant hirudin, an anti-thrombin drug.

The second part includes the development of cost-effective diagnostic tests, mostly for von Willebrand disease (VWD), the most common congenital bleeding disorder. This comprises the largest part of the thesis. I developed four anti-thrombotic peptides by using Phage Display technology that I mastered during my post-doctoral study at the University of Leuven in Belgium. I was also part of the group of researchers that developed many new thrombosis models in baboons. For my M.Med Sc study, I developed a flow chamber model to study *in vitro* endothelial function, which was subsequently used to test the thrombogenicity of tissue-engineered small vessels. This study was the first where endothelial cells were successfully seeded onto decellularised baboon arteries. This study was undertaken in collaboration with the Department of Cardiothoracic Surgery at the University of the Free State, Bloemfontein.

The third part is a spin-off from my research on VWD. I established the only Special Haemostasis laboratory of the NHLS, situated at the University of the Free State in Bloemfontein, South Africa, by developing, validating and implementing four diagnostic assays. As a result the laboratory now functions as a reference centre for von Willebrand disease; the most prevalent, but underdiagnosed bleeding disorder in South Africa. The developed diagnostic

assays resulted in nineteen peer-reviewed publications on the diagnosis of haemostatic and thrombotic disorders. As reference centre for VWD in South Africa, we published eight articles on its diagnosis, together with international leaders in the field. Additionally, we also published the first South African recommendations regarding the differential diagnoses of VWD. We published on other bleeding disorders and on a fatal thrombotic disorder, thrombotic thrombocytopenic purpura (TTP). Lastly, is it important to note that the research on VWD, haemophilia and TTP is ongoing.

1. INTRODUCTION

Thrombosis is considered as a major cause of death worldwide, being responsible for heart attacks and strokes (Heidenreich *et al*., 2011). In addition, venous thromboembolism (VTE) is the major preventable cause of death in hospitals (Tsai *et al*., 2015). Strong evidence indicates that prophylaxis is still under-prescribed (Spyropoulos, 2010). Although thrombosis is the main cause of death in industrialized countries, thrombosis complications in patients infected with the human immuun difficiency virus (HIV) are on the rise in developing countries. HIV infection is now a known pro-thrombotic condition, because cases where HIV infection was linked to venous thrombo-embolisem (VTE) had been reported since the late 1980's (Tsongo Vululi *et al*., 2018).

A survey conducted by the Medical Research Council (MRC) in South Africa stated that, every day, 195 people died from some type of cardiovascular disease during the time period 1997 to 2004. Of these, 33 people died due to heart attack, 60 due to stroke and 37 people due to heart failure. Furthermore, the MRC projected that chronic disease (including heart disease) would increase significantly by 2010 and that this increase would continue thereafter. Before the age of 65, most people die due to chronic disease. These premature deaths have a huge effect on the workforce and a major economical impact and were expected to increase by 41% by 2030. It was also estimated that the cardiovascular disease (CVD) burden in South Africa would increase among all age groups to become the prime contributor to overall morbidity and mortality (Bradshaw *et al*., 2003). The American Heart Association further estimated that by 2030, the CVD death rate would be more than 23 million people world-wide (Heidenreich *et al*., 2011). Thus, the main challenge in cardiovascular research is to develop safe antithrombotics to prevent thrombosis, but cause less bleeding than the existing, commonly used clinical drugs (coumarin, heparin, direct thrombin inhibitors and factor Xa inhibitors). All these anticoagulants have limited success to maintain the haemostatic balance between thrombosis and bleeding (Hirsh & Weitz, 1999, Wolberg *et al*., 2012).

Bleeding disorders, that are on the other side of the haemostatic balance, largely influence the quality of life of patients and are also a burden on the economy. Haemophilia has a prevalence of 1:5000 boys in South Africa. Unfortunately, many patients with haemophilia or other bleeding disorders are either not diagnosed or diagnosed at a very late stage of the disease. Even more concerning, the South African Haemophilia Foundation (SAHF) estimates that many of those that are diagnosed remain without adequate care (Mahlangu, 2009). Von Willebrand disease (VWD) is the bleeding disorder with the highest prevalence of 1% in the general population. Despite this high prevalence, the diagnosis and classification remain a challenge (Sadler, 2005). The severity of a bleeding disorder usually depends on the actual amount of clotting factor that is missing or not functioning (Jacobson, 2013; Eyal & Veller, 2009). Our Specialised Haemostasis Laboratory is the reference centre for VWD in the country and is thus the only centre that diagnoses the sub-classes of VWD in sub-Saharan Africa. Figure 1 indicates the coagulation factors in haemostasis that I targeted in my research.

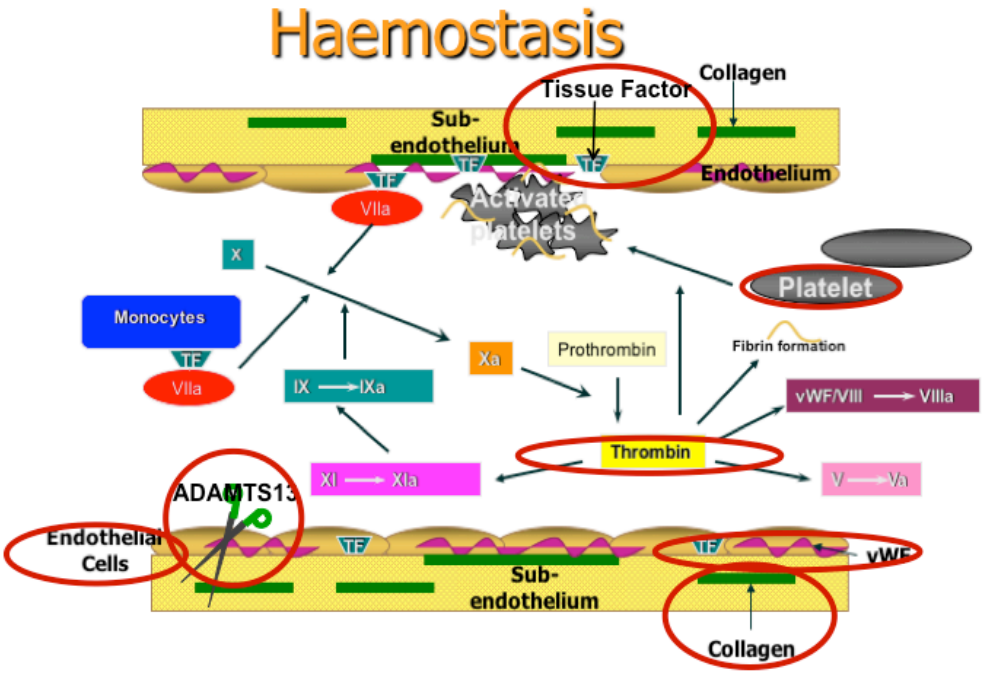

Figure 1: *The targeted thrombotic factors (red circles) that I researched in this compilation*

I investigated endothelial cells and their function for my M.Med.Sc. Although I did not include my M.Med.Sc study in this compilation, my main research theme thereafter was concerned with the function of endothelial cells which include VWF and its cleaving protease ADAMTS13, and the important role that tissue factor (TF) plays in thrombosis and bleeding. I also developed and tested drugs that inhibit platelets and thrombin, both main players in thrombosis. These studies will be explained further in the compilation.

For my PhD, I determined the sites of excretion and the pharmacokinetics of the recombinant thrombin inhibitor, recombinant (r)-hirudin, kindly supplied by Prof Fritz Markwardt from the Medical Academy in Erfurt, Germany **(S-1)**. The sites of excretion and/or uptake of r-hirudin was previously unknown and I was the first to show that the half-life to r-hirudin, administrated as an intravenous bolus or 30 minute infusion, did not differ significantly. For this purpose, the r-hirudin was labelled with Iodine-131 and its *in vivo* distribution and sites of elimination were monitored with a scintillation camera, coupled with a computer-assisted image analysis program. The half-life of Iodine-131-r-hirudin was 21 ± 3 minutes. I also found that only 50% to 60% of lepirudin was excreted by the kidney. This was in contrast to the general assumption that r-hirudin is mainly excreted by the kidneys. I also showed that a sizeable portion of r-hirudin was found in the bile that indicated that it was eliminated by the liver. It is important to note that the method of delivery (bolus or infusion) did not influence the plasma half-life and clearance of r-hirudin.

This compilation explains the journey through the different parts of my research. I shall discuss the contribution of my publications to the international body of knowledge in three distinct parts. For purposes of inclusiveness, the publications to which I refer is attached at the end of the document.

The three parts of this compilation are outlined in Figure 2.

S-1 Meiring, S.M., Lötter, M.G., Badenhorst, P.N., Bucha, E., Nowak, G.& Kotzé, H.F. (1999). Sites of elimination and pharmacokinetics of recombinant ^{131}I-lepirudin in baboons. *Journal of Pharmaceutical Sciences* 88(5):523-529. (Cited by 2)

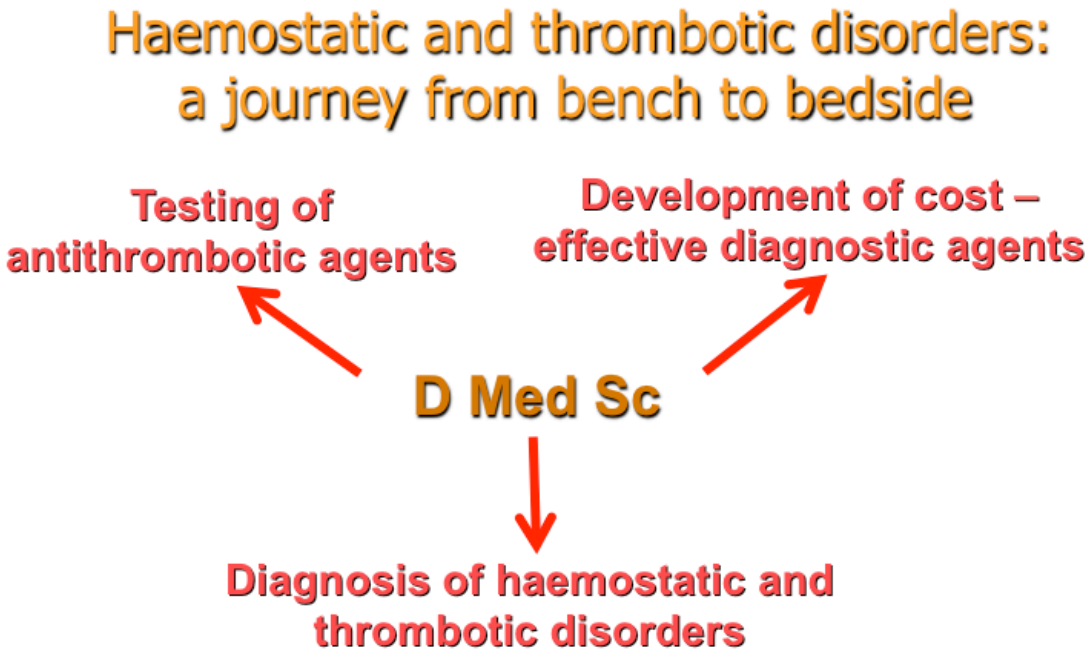

Figure 2: *The three parts of this compilation include testing of antithrombotics, development of cost-effective diagnostic methods and diagnosis of haemostatic and thrombotic disorders.*

2. A JOURNEY FROM BENCH TO BEDSIDE

My journey from bench to bedside may accurately be described as "Haemostatic disorders: towards a better understanding of mechanisms and treatment. It is important to note that unraveling the molecular mechanisms underlying haemostatic diseases does not only lead to a better understanding thereof, but also to the potential development of novel drugs for treatment and of tests to diagnose them (Bradshaw et al., 2003).

2.1 Part 1: Testing of antithrombotics

Following my research for the M.Med.Sc and PhD (See section 1: Introduction), I started my research by testing anti-thrombotic agents in baboon thrombosis models. In collaboration with Prof. Hans Deckmyn from the Laboratory for Thrombosis Research of the Kortrijk Campus of the University of Leuven in Belgium, we tested the efficacy of platelet membrane receptor human monoclonal antibodies that inhibit arterial thrombosis.

My colleagues developed, in collaboration with Dr Stephen R. Hanson from the Scripps Clinic and Research Foundation in La jolla, California, USA, a baboon model of platelet dependent arterial-type thrombus formation (Hanson *et al*., 1985). This model consists of an arterial-venous shunt that is interposed between the femoral artery and vein and that contains a Dacron vascular graft insertion acting as an arterial-dependent thrombus generator.

The first monoclonal antibody, MA-16N7C2 that we tested in our baboon model contains an echistatin-like RGD sequence that binds to the platelet receptor glycoprotein IIb/IIIa and prevents fibrinogen binding and so prevents platelet-platelet interaction (**S-2**). A bolus injection of 1mg/kg MA-16N7C2 occupied significantly more GPIIb/IIIa receptors for a longer period than a lower dose of 0.4 mg/kg, suggesting that the receptors might be internalised by the platelets. In the baboon, bolus injections of 1.0 and 0.4 mg/kg significantly inhibited *ex vivo* platelet-dependent deposition on the vascular graft material. It also prolonged the bleeding time and inhibited *ex vivo* platelet aggregation. These effects showed a dose-dependent response and lasted for several days. Therefore, MA-16N7C2 is regarded as a potent and long-acting GPIIb/IIIa inhibitor. Unfortunately, its effects were so substantial that it increased the risk of uncontrolled *in vivo* bleeding. In addition, because they are large proteins, monoclonal antibodies trigger the immune system to develop antibodies against them. It is therefore conceivable that MA-16N7C2 can only be used once in a patient. This study nevertheless showed that inhibition of the GPIIb/IIIa receptor effectively inhibits thrombosis.

The platelet receptor glycoprotein Ibα interacts with VWF and mainly support platelet adhesion in arterial flow conditions at the site of injury to prevent platelet binding to subendothelial collagen. Prof. Hans Deckmyn developed a F(ab) fragment of the monoclonal antibody 6B4. This antibody inhibits the binding of platelet glycoprotein Ib (GpIb) to VWF. In addition, this F(ab)

S-2 Kotzé, H.F., Badenhorst, P.N., Lamprecht, S., Meiring, M., Van Wyk, V., Nuyts, K., Stassen, J.M., Vermylen, J. & Deckmyn, H. (1995). Prolonged inhibition of acute arterial thrombosis by high dosing of a monoclonal anti-platelet glycoprotein IIb/IIIa antibody in a baboon model. *Thrombosis and Haemostasis* 74:751-757.

fragment does not trigger the immune response. We investigated the antithrombotic effect of 6B4 and its F(ab) and F(ab')$_2$ fragments in our baboon model (**S-3**). In this study we substituted the Dacron vascular graft material insertion (**S-2**) with fixed bovine pericardium to provide a collagen-rich surface. When we injected this antibody in baboons, both the intact IgG and its F(ab)$_2$ fragments immediately caused thrombocytopenia and could not be investigated further. In contrast, the F(ab) fragments did not cause thrombocytopenia. Baboons were treated with bolus injections of 80 μg/kg, 160 μg/kg, 320 μg/kg and 640 μg/kg 6B4 F(ab) fragments. These doses remarkably reduced *ex vivo* platelet deposition on a collagen-rich surface by 43%, 53%, 56% and 65% respectively. The highest dose of 640 μg/kg significantly prolonged the bleeding time. With this study we were the first to show that an anti-human GpIb antibody is able to successfully prevent platelet adhesion and thrombus formation *in vivo*. This finding confirms the predominant role of GpIb in *in vivo* platelet adhesion.

Another baboon model that we used to study the antithrombotic effect of the F(ab) fragment of 6b4 was the modified Folts model where we induced thrombus formation at a mechanically injured and stenosed site of the femoral artery. In this case, cyclic flow reductions (CFRs) measured on an extracorporeal femoral arterial-venous shunt was used as measure of the antithrombotic effect (**S-4**). We also measured receptor binding and the *in vitro* and *ex vivo* platelet aggregation response due to the F(ab) fragment. Increasing doses of 6B4 F(ab) decreased the platelet deposition onto the injured femoral artery devices in a dose-dependent manner. The F(ab) fragments prevented ex vivo ristocetin and botrocetin-induced platelet agglutination dose-dependency. The IC_{50} (dose that inhibit agglutination by 50%) was 1.8 ug/ml at a plasma F(ab) concentration of 36nmol/L and 2.5 ug/ml at 40 nmol/L. The F(ab) fragments bind to baboon platelets in a dose-

S-3 Cauwenberghs, N., Meiring, S.M., Vautarin, S., Van Wyk, V., Lamprecht, S., Roodt, J.P., Novak, L., Harsfalvi, J., Deckmyn, H. & Kotzé, H.F. (2000). Antithrombotic effect of platelet glycoprotein Ib-blocking monoclonal antibody Fab fragments in nonhuman primates. *Arteriosclerosis, Thrombosis and Vascular Biology* 20:1347-1353. (Cited by 141)

S-4 Fontayne, A., Meiring, M., Lamprecht, S., Roodt, J., Demarsin, E., Barbeaux, P. & Deckmyn, H. (2008). The humanized anti-glycoprotein 1b monoclonal antibody h6B4-Fab is a potent and safe antithrombotic in a high shear arterial thrombosis model in baboons. *Thrombosis and Haemostasis* 100:670-677. (Cited by 58)

dependent manner and reached a saturation level of 52%. It is important to note that this treatment had a small and insignificant influence on bleeding time. In addition, no blood loss, spontaneous bleeding or thrombocytopenia was observed. These results clearly indicated that the F(ab) fragments of monoclonal antibody 6B4 successfully prevent platelet adhesion and subsequent thrombus formation *in vivo*. These findings emphasized the important role that GPIbα plays in *in vivo* platelet adhesion and thrombosis.

Based on the abovementioned results, we also predicted that the F(ab) fragments of the anti-GPIbα MoAb 6B4 may be used to constitute a useful approach to prevent arterial thrombosis in patients after balloon catherisation, vascular engraftment or endarterectomy.

The modified Folts model was also used to compare, separately and in combination, the antithrombotic effect of the GPIb/IX MoAb fragment of 6B4 and the GPIIb/IIIa blocking MoAb 16N7C2 (**S-5**). A dose as high as 2 mg/kg 6B4-F(ab) completely prevented the CFRs without prolonging the bleeding time. MoAb 16N7C2 also abolished the CFRs at a high dose of 0.3 mg/kg, but with significant prolonging of the bleeding time. By combining a low dose of 0.6 mg/kg 6B4-F(ab) with a low dose of 0.1mg/kg MA-16N7C2, CFRs was inhibited again, but without prolonging the bleeding time. This study showed that partial inhibition of both GPIb and GPIIb/IIIa blocking is more effective and safe than inhibiting only one platelet receptor, since it abolished the CFRs in our model without any bleeding risks.

We also evaluated the inhibitory effect on platelet adhesion by blocking the binding of VWF to collagen in the modified Folts model using murine antihuman VWF mAb 82D6A3 which had been developed in Belgium (**S-6**). This antibody binds to the A3 domain of VWF and interacts with collagen fibres I and III, but not to collagen fibre VI. This was the first study that

S-5 Wu, D., Meiring, M., Kotzé, H.F., Deckmyn, H. & Cauwenberghs, N. (2002). Inhibition of platelet glycoprotein Ib, glycoprotein IIb/IIIa, or both by monoclonal antibodies prevents arterial thrombosis in baboons. *Arteriosclerosis, Thrombosis and Vascular Biology* 22:323-328. (Cited by 84)

S-6 Wu, D., Vanhoorelbeke, K., Cauwenberghs, N., Meiring, M., Depraetere, H., Kotzé, H.F. & Deckmyn, H. (2002). Inhibition of the von Willebrand-collagen interaction by an antihuman VWF monoclonal antibody results in abolition of *in vivo* arterial platelet thrombus formation in baboons. *Blood* 99(10):3623-3628. (Cited by 139)

showed conclusively that the monoclonal antibody *in vivo,* even at a high dose of 600 μg/kg 82D6A3 abolished CFRs without significant prolonging the bleeding time. This clearly indicates a safe approach to target platelet adhesion inbibition of arterial thrombosis.

As a follow-up to the study described in **S-6**, we decided to test the antithrombotic efficacy of this antibody in a baboon model of in-stent stenosis where we used standard treatment with heparin, aspirin and clopidogrel (**S-7**). This was the first reported study to investigate the possible antistenotic effect of an inhibitor of the VWF-collagen interaction following stent deployment. The studies were carried out 28 days after deployment. Our results did not supply sufficient evidence that additional inhibition of platelet adhesion reduces neointimal hyperplasia compared to the current routine clinical setting. This may indicate an overestimation of the role of vascular smooth muscle cells' mitogenic and attractant factors that are released upon platelet activation following percutaneous transluminal coronary angioplasty and stenting. This study therefore underscored the importance of evaluating new antistenotic therapies in a clinically relevant model. This model of neointimal hyperplasia may become important in future studies assessing the prevention of restenosis.

With the last study of this section, we tested the antithrombotic activity and pharmacodynamics of an anti-factor VIII human monoclonal antibody LE2E9Q in our baboon model of *ex vivo* thrombosis described in S1 (**S-8**). Mab-LE2E9Q was administered as a single intravenous dose of 1.25 and 5 mg/kg and thrombosis development was recorded in an expansion chamber (coagulation-dependent venous thrombosis) and on Dacron vascular material (platelet-dependent arterial thrombosis) in an extracorporal arteriovenous shunt that was implanted between the femoral artery and femoral vein

S-7 De Meyer, S.F., Staelens, S., Badenhorst, P.N., Pieters, H., Lamprecht, S., Roodt, J., Janssens, S., Meiring, M., Vanhoorelbeke, K., Bruwer, A., Brown, S, & Deckmyn, H. (2007). Coronary artery in-stent stenosis persists despite inhibition of the von Willebrand factor – collagen interaction in baboons. *Thrombosis and Haemostasis* 98: 1343-1349. (Cited by 18)

S-8 Jaquemin, M., Stassen, J-M., Saint-Remy, J-M., Verhamme, P., Lavend'Homme, R., VanderElst, L., Meiring, M., Pieters, H., Lamprecht, S., Roodt, J. & Badenhorst, P. (2009). A human monoclonal antibody inhibiting partially factor VIII activity reduces thrombus growth in baboons. *Journal of Thrombosis and Haemostasis* 7:429-437.

(Cadroy et al., 1989). The effect on thrombosis was measured at different time points (1 hour, 24 hours and 7 days) after administration of Mab-LE2E9Q. We found that a significantly lower number of platelets was deposited in both the arterial and venous chambers with both concentrations of Mab-LE2E9Q after 1 hour and 24 hours after administration, but not after 7 days. The antibody also did not lengthen the bleeding time. The results thus suggested that this antibody may be used as a novel type of long-acting antithrombotic agent and that it displayed an optimal safety/efficacy profile.

2.2 Part 2: The use of phage display technology to develop antithrombotic peptides and antibodies and to develop cost-effective diagnostic tests

The successful testing of anti-platelet drugs in our baboon models prompted me to develop new anti-thrombotic drugs myself. These peptides and proteins were eventually used to develop diagnostic assays. I therefore applied to the South African National Research Foundation and received funding to become a post-doctoral fellow at the laboratory of our collaborator, Prof. Hans Deckmyn, head of the Laboratory for Thrombosis Research at the University of Leuven (Kortrijk Campus), Belgium. While there as a post-doctoral fellow, I mastered the technology of phage display. It is a powerful method to select proteins with a specific function where a protein or peptide is fused to a coat protein on the surface of a filamentous phage. These proteins or peptides then act as ligands or enzymes because they can bind to specific antigens of choice.

During my post-doctoral study in Belgium, we identified a collagen-binding protein from the hematophagous human parasite, *Necator americanus* (**S-9**). We developed a cDNA-expressing phage display library from *Necator americanus* and cloned it as fusions into phagemids with the C-terminal part of the phage bound to coat protein pVI. Phages that bind to human collagen type I and III were enriched through four rounds of panning. All collagen-

S-9 Viaene, A., Crab, A., Meiring, M., Pritchard, D. & Deckmyn, H. (2001). Identification of a collagen-binding protein from Necator americanus by using a cDNA-expression phage library. *Journal of Parasitology* 87(3):619-625.

binding phages were sequenced and one common 135-amino acid peptide sequence was identified that binds in a concentration-dependent manner to human type I and III collagen and rat type I collagen. This was my first experience of using phage display technology to select proteins based on their binding properties.

On my return to South Africa, I published an invited review article on the application of phage display technology in thrombosis and haemostasis (**S-10**). In this review I suggested that phage display technology was ideally suited to develop more effective anti-thrombotic agents.

The first anti-thrombotic peptide that I identified by phage display technology was a thrombin inhibition peptide (TIP) (**S-11**). To achieve this, I used a cyclic heptapeptide phage display library to select phages that bind to human alpha-thrombin. One of the phage clones that bound the strongest to thrombin also competed with Phe-Pro-Arg chloromethyl ketone (PPACK) for binding to thrombin. The clone was selected and the aminoacid sequence determined. A peptide with the exact cyclic aminoacid sequence Cys-Asn-Arg-Pro-Phe-Ile-Pro-Thr-Cys was then synthesised. TIP competitively inhibited the function of thrombin with an inhibition constant (*Ki*) of 0.4974 mM. It prolonged the thrombin time and inhibited platelet activation by thrombin dose-dependently. It also prevented platelets from adhering onto a human micro-vascular endothelial matrix in a parallel plate flow chamber. This effect was seen under both high shear (arterial) and low shear (venous) conditions. The flow chamber studies were done in the laboratory of Prof Jolan Harsfalvi from the University of Debrecen, Hungary as described previously (Harsfalvi *et al*., 1995).

S-10 Meiring, S.M., Kotzé, H.F., Pretorius, G.H.J. & Badenhorst, P.N. (1999). Die toepassing van peptiedblootlegging op fage in trombose en hemostase (The application of phage display technology in thrombosis and haemostasis). *Suid-Afrikaanse Tydskrif vir Natuurwetenskap en Tegnologie (South African Journal of Science and Technology)* 18 (3):76-81.

S-11 Meiring, S.M., Littauer, D., Harsfalvi, J., Van Wyk, V., Badenhorst,P.N., Kotzé, H.F. (2002). *In vitro* effect of a thrombin inhibition peptide selected by phage display technology. *Thrombosis Research* 107:365-371. (Cited by 16)

By using the same cyclic heptapeptide library, I also selected a peptide with sequence Cys-Ala-Trp-Pro-His-Thr-Pro-Asp-Cys. This peptide competes with tissue factor for binding to coagulation factor VII (**S-12**). This peptide also lengthened the prothrombin time in a dose-dependent manner. It reduced platelet adhesion to both tissue factor and human endothelial cell matrices in a parallel plate flow chamber under high shear arterial flow conditions. Similarly, it also competes with tissue factor for binding factor VIIa with *Ki* 123.2 μM.

In the early 2000's, the development of antibodies and fragments thereof represented the fastest growing development in the biopharmaceutical market (Nicolaides *et al*., 2006). As a result, I decided to use a human single chain antibody fragment library to select single chain antibody fragments that inhibit tissue factor, the principal protein trigger of coagulation. These antibody fragments were only 26kD in size and are unlikely to be immunogenic in humans. I selected and purified a single chain variable antibody fragment that inhibited the action of human tissue factor. It prolonged the prothrombin time dose-dependently with an IC_{50} of 0.5 μM. Interestingly, this antibody fragment inhibited thrombogenesis more effectively in thrombophilic plasma from patients with thrombophilia than in normal plasma. Thus, TF inhibitors might achieve anti-coagulant activity in thrombophilic plasma without significantly interfering with haemostasis in normal plasma. This antibody fragment showed the strongest TF inhibitory activity currently described in the literature (**S-13**).

Although I applied for an international patent cooperation treaty (PCT) , it was not granted because the results were published in *Drug Development Research* a few days before the patent application date. This intervention related to novel antibody fragments that inhibit the action of human tissue

S-12 Meiring, S.M., Roets, C.E. & Badenhorst, P.N. (2006). Funksionele beskrywing van 'n faktor VIIa inhiberende peptied, IP-7, geselekteer deur faagblootleggingstegnologie. (Functional characterisation of a factor VIIa inhibiting peptide, IP-7, selected by phage display technology) *Die Suid Afrikaanse Tydskrif vir Wetenskap en Tegnologie (South African Journal of Science and Technology)* 25(4):209-220.

S-13 Meiring, S.M., Vermeulen, J. & Badenhorst, P.N. (2009). Development of an inhibitory antibody fragment to human tissue factor using phage display technology. *Drug Development Research* 70:199-205. (Cited by 2)

factor. Unfortunately the current single chain variable antibody fragments (scFv) phage display technology is limited by the inability to produce sufficient scFv fragments for extensive testing *in vivo*. A larger production system with higher yield is therefore required to allow further characterisation and *in vivo* studies.

Therefore, in a follow-up study, we manipulated expression of the low-yielding scFv in an attempt to enhance production (**S-14**). We improved the expression of the scFv in the cytoplasm of *E-coli* BL21 (DE3) by modifying the expression systems and optimizing the codons of the gene. We also evaluated two commercially available methods of protein recovery: *in vitro* refolding and the use of cold shock expression systems together with *E.coli SHuffle*®. This approach increased cytoplasic expression of the scFv expression in *E. coli* up to 5 times and delivered high functionality. Further improvements and/or upscaling the expression volume needs to be investigated in order to produce sufficient scFv to test for efficacy in animal models and, eventually, in humans.

We used an antibody fragment library to select antibodies that bind to the VWF-propeptide to develop cost-effective assays to test for the differential diagnoses of VWF disease (**S-15**). In South Africa, commercially available assays are very expensive because the antibodies that are used in these assays are produced in animals and are also influenced by the unfavourable Rand/US$ exchange rate. We were the first to use human antibody proteins that were produced by phage display technology in our assays. Initially we displayed the VWF propeptide protein on yeast. We then selected antibody fragments (scFv) against the displayed VWF propeptide by using phage display technology. We selected two antibody fragments that bound with high specificity to the VWF propeptide. After purification of the antibody fragments,

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S-14 Vermeulen, J., Burt, F., Van Heerden, E., Cason, E. & Meiring, M. (2018). Evaluation of *in vitro* refolding vs cold shock expression: production of a low yielding single chain variable fragment. *Protein Expression and Purification* 151:62-71. (Cited by 1)

S-15 Meiring, S.M., Setlai, P., Theron, C. & Bragg, R. (2018). The use of phage display and yeast based expression system for the development of a von Willebrand factor propeptide assay: Development of a von Willebrand factor propeptide assay. *BioMed Research International* 2018:632091:1-7 (https://doi.org/10.1155/2018/6232091).

we developed a cost-effective VWF propeptide assay that functions to detect VWF propeptide in normal plasma. We compared our assay's performance to that of commercial VWF propeptide kits with antibodies (CLB-Pro 35 and CLB-pro 14.3, Cell Sciences, USA). Our selected antibodies showed a higher binding affinity for VWF propeptide than the commercially available antibodies. The limit of detection of our assay was 1.56%, where the commercial assay only detected VWF propeptide from 6.35%. Finally, we successfully secured a SA patent (2011/05426).

In 2007, I established my laboratory as the reference laboratory for von Willebrand disease in South Africa and also developed it as a centre of international excellence to confirm diagnosis of von Willebrand disease. In order to achieve this, I developed and evaluated an assay that monitors VWF activity, the Collagen-Binding Assay (VWF:CB), using type III collagen. This assay is ten times more cost-effective than the commercially available methods and is also very sensitive to detect the collagen-binding activity in patients with VWD (**S-16**). The validation of our assay showed that the intra- and inter-assay coefficients of variation were <8% and <9% for normal values. The normal reference range varies between 51% and 143%. We could also demonstrate that this assay is sensitive to the presence of large VWF multimers. Type 2A VWD patients have a collagen-binding activity/antigen (CB/Ag) ratio of 0.18 and the CB/Ag ratio of type 2B patients is 0.4. Both these values indicate functional discordance. Furthermore, the mean CB/Ag ratios of type 2M and type 1 VWD, were 1.1 and 1.0, respectively. Although we did not compare our assay to commercial assays, our results were similar to literature findings on VWF:CB assays using type III collagen from Sigma, USA (Favaloro, 2000).

I also developed a rapid and cost-effective test to visualise VWF multimer patterns to further differentially diagnose VWD (**S-17**). This test is used to identify VWD subclasses. Krizek & Rick (2000) described a highly sensitive

S-16 Meiring, M., Badenhorst, P.N. & Kelderman, M. (2007). Performance and utility of a cost-effective collagen-binding assay for the laboratory diagnosis of von Willebrand disease. *Clinical Chemistry and Laboratory Medicine.* 45(8):1068-1072. (Cited by 19)

S-17 Meiring, M., Badenhorst, P. & Kelderman, M. (2005). A rapid and cost-effective method to visualize von Willebrand factor multimers in plasma. *Medical Technology SA* 19(2):16-18.

and rapid method to visualise the multimeric structure of VWF using the Western blot technique. This test includes agarose gel electrophoresis followed by the transfer of proteins onto a polyvinylinine fluoride membrane. The multimeric pattern of VWF is visualised by immunolocalisation on the membrane and luminographic detection on an X-ray film. An advantage of this method is that no radioactivity is used. I modified this method comprehensively to increase its sensitivity and to reduce the cost whilst producing results quickly. I used one instead of two localisation antibodies and so reduced the immunolocalisation time by more than two hours. In the case of the Krizek test, results are available only after 3 additional hours. I further reduced the cost by using two carbon plates instead of the blotter instrument. This approach reduced the cost of the assay by at least 40%. In addition, degassing the agarose before casting and by using a 0.65% agarose gel instead of a 0.7% agarose, improved sensitivity to visualise type 1 VWD plasmas with low VWF levels sufficiently. My method also distinguishes between the multimer patterns of type 2M VWD and normal plasma, since plasma of type 2M patients shows a higher density of small multimers and a lower density of larger multimers than normal plasma.

Since 2010, my reference laboratory for the diagnosis of VWD gained international recognition because we formed part of an international quality assurance programme (RCPA) where we blindly analyse test samples sent by other laboratories to assess the accuracy of their methods. In this regard I published, in collaboration with Prof. Emmanuel Favaloro from the Royal College of Pathologists of Australasia (RCPA), a definitive communiqué on the value and accuracy of the diagnostic tests for VWD. We conducted a cross-laboratory study where we compared how sensitive the three VWF activity assays are to recognise the loss of the high molecular weight multimers, represented by type 2A and type 2B VWD (**S-18**). We sent out a set of eight test samples, six of which had a stepwise reduction in the high

S-18 Favaloro, E.J., Bonar, R., Chapman, K., Meiring, M. & Funk, D. (2012). Differential sensitivity of von Willebrand Factor (VWF) 'activity' assays to large and small molecular weight forms: a cross-laboratory study comparing ristocetin cofactor, collagen-binding and mAb-based assays. *Journal of Thrombosis and Haemostasis* 10(6):1043-1054. (Cited by 47)

molecular weight multimers, to be tested by 51 different laboratories enrolled in the RCPA quality assurance programme, using a variety of assays. The collagen-binding assay was the most sensitive measure of the loss of high molecular weight VWF-multimers.

The survey was recently repeated but included all the latest VWF activity assays; a total of five (**S-19**). In this study we sent out a set of four samples with a stepwise reduction in high molecular weight (HMW) VWF to be tested by more than 400 laboratories around the world, using all available assays. We also sent out a set of two samples representing type 1 vs 2A VWD-like plasma to be tested by a second set of 251 laboratories. Both the collagen-binding assay and the VWF-activity assay, based on spontaneous binding of VWF to a gain-of-function mutant GPIb fragment (VWF:GPIbM), showed the highest sensitivity to assess reduced HMW-multimers. The sensitivity of the ristocetin-cofactor assay and the VWF-activity assay that are based on ristocetin-induced binding of VWF to a recombinant wild type GPIb fragment (VWF:GPIbR) had intermediate sensitivity to assess reduced HMW-multimers. The VWF activity assays based on the binding of a monoclonal antibody to a VWF A1 domain epitope had the lowest sensitivity to assess reduced HMW-multimers.

It is important to take these different assays into account, since they have significant clinical implications for both the diagnosis and monitoring of therapy for VWD. It follows that the least sensitive assays, and to a certain extent the intermediate sensitivities, can fail to diagnose patients with VWD, and that success of treatment may be misinterpreted.

Von Willebrand factor multimers compete for clearance and for proteolysis through its cleavage protein in plasma, ADAMTS13. Exaggerated proteolytic cleavage by ADAMTS13 can cause von Willebrand disease. Conversely,

S-19 Favaloro, E.J., Bonar, R., Hollestelle, M.J., Jennings, I., Mohammed, S., Meijer, P., Wood, T. & Meiring, M. (2018). Differential sensitivity of von Willebrand factor activity assays to reduced VWF molecular weight forms: A large international cross-laboratory study. *Thrombosis Research* 166:96-105. (Cited by 7)

defective proteolysis can cause thrombotic thrombocytopenic purpura (TTP). TTP used to be a rare disorder but its incidence has increased dramatically concurrent with the HIV/AIDS epidemic (Brecher *et al*., 2008). The underlying cause of the disease may be the result of low ADAMTS13 levels, functional abnormalities of ADAMTS13, endothelial dysfunction or the development of autoantibodies to ADAMTS13. Although ADAMTS13 antigen kits are available, the tests remain expensive, especially for small laboratories and laboratories in developing and lower-income countries. I developed a cost-effective ELISA test, using commercial antibodies to ADAMTS13 (**S-20**). We compared the result, given as a percentage of ADAMTS13 antigen levels of our in-house assay using two commercial antibodies. A murine anti-human ADAMTS13 antibody from R&D systems, USA was used to coat the ELISA plate and a rabbit anti-human detection antibody obtained from Santa Cruz Biotechnology, USA was used as a detection antibody. We compared our assay with a commercial ADAMTS13 antigen kit by using plasma of 40 HIV-associated TTP patients and 40 healthy individuals. The intra- and inter-assay coefficients of variation for our ADAMTS13 antigen assay were 8% and 7% respectively. The assay's linearity ranged from 0.78% to 12.5% ADAMTS13. The limit of detection was 0.2% ADAMTS13 and the limit of quantification 0.8%. Our assay compared favorably with the commercial test kit from Sekisui Diagnostics (USA) with an R^2-value of 0.9. The cost of our in-house assay was at least 90% less than the commercial kit. As a result, we now use this assay routinely to diagnose HIV-associated TTP.

In a ground-breaking study published in 2012 as an educational report in *European Oncology and Haematology* we did not only show low levels of ADAMTS13 in HIV-associated TTP patients, but also extremely high levels of VWF and tissue factor (TF) (**S-33**). We had no ready explanation for the high levels of VWF. We then examined the effects that inflammatory cytokines and clotting factors such as tissue factor and thrombin and especially combinations of clotting factors and cytokines have on the synthesis and release of VWF, as well as the cleavage of ultra large von Willebrand factor

S-20 Meiring, M. & Myneni, S. (2017). Evaluation of a cost-effective ADAMTS13 antigen assay. *Medical Technology SA* 31(1):1-4.

(ULVWF) by cultured human umbilical cord endothelial cells (HUVECs). We also measured endothelial micro-particle formation and the VWF content in these microparticles (**S-21**). We found that tissue factor alone and in combination with inflammatory cytokines substantially increased VWF release from cultured HUVECs. These studies resulted in two M.Med.Sc degrees titled: “The effect of inflammatory cytokines and coagulation factors on the von Willebrand factor synthesis and cleavage“ and “Microparticles derived from stimulation of human umbilical endothelium“. The results also mimic the findings where extremely high VWF levels are present in patients with thrombosis and inflammation (Lopez, 2006). Furthermore, less ADAMTS13 was secreted with all treatments. These results proved that the inflammatory cytokines IL-8 and TNF-alpha, clotting factors thrombin and tissue factor and combinations of cytokines and clotting factors stimulate human endothelial cells to release ULVWF and downregulate the release of ADAMTS13. We hypothesized that this resulted in an overload of ULVWF in plasma ready to bind platelets that lead to platelet aggregation and might lead to the almost always fatal HIV-associated TTP.

The endothelial layer is fortunately responsible for many of the anti-thrombotic functions of blood vessels. However, endothelialisation of vascular grafts has been limited due to the cost and availability of reagents, and because it is difficult to get seeded endothelial cells to attach to the de-endothelialised blood vessels. This pertains to de-endothelialisation by balloon catheterisation or through air injury to the endothelium (Kakisis *et al*., 2005). In collaboration with Prof. Francis Smit from the Robert W.M. Frater Research Institute of the Department of Cardiothoracic Surgery at the University of the Free State, we prepared tissue-engineered decellularised small-vessel conduits using baboon arteries (**S-22**). We seeded the decellularised baboon arteries with cultured human umbilical endothelial cells (HUVECs) and compared its

S-21 Meiring, M., Allers, W. & Le Roux, E. (2016). Tissue Factor: a potent stimulator of von Willebrand factor synthesis by human umbilical vein endothelial cells. *International Journal of Medical Science* 13(10):759-764. (Cited by 4)

S-22 Meiring, M., Khemisi, M., Laker, L., Dohmen, P.M. & Smit, F.E. (2017). Tissue engineered small vessel conduits – the anti-thrombotic effect of re-endothelialization of decellularized baboon arteries: a preliminary experimental study. *Medical Science Monitor Basic Research* 23:344-351. (Cited by 6)

thrombogenicity to that of decellularised arteries. In both instances, the blood vessels were circulated for one hour with native blood in the flow chamber that I designed to study endothelial function. The decellularised arteries had no endothelial cell lining with an intact basement membrane as confirmed by scanning and transmission electron microscopy. The seeding process resulted in a complete endothelial layer on the surfaces of the arteries. Perfusion with native blood did not dislodge the seeded cells on the decellularised surface. After perfusion, no thrombi formed in intact control arteries and in the re-endothelialised vessels. In contrast, there was widespread platelet adhesion and activation in the decellularised vessels despite a relatively intact basal membrane. To our knowledge, this was the first study that achieved successful re-endothelialisation in small-diameter arteries (carotis, radial and femoral). Ultimately, the success of this approach needs to be assessed *in vivo* in baboons where decellularised recellularised vessels are implanted.

2.3 Part 3: Diagnosis of thrombotic and haemostatic disorders

All the prior research work led to a substantial number of publications on the diagnosis of von Willebrand disease. The first was in 2005 where we used an algorithm of Prof. Federici from Italy (Federici *et al*., 2002) as a guideline for the diagnosis and treatment of von Willebrand disease type 1, 2A, 2B and 2M VWD (**S-23**). Importantly, we also studied four patients with VWD where the types and subtypes were not clear. In this study we also outlined some pitfalls to look out for when diagnosing VWD. These pitfalls are mainly due to the limited sensitivity, reproducibility and the huge interlaboratory variability of the ristocetin-cofactor assay and the ristocetin-induced platelet agglutination assay (Favaloro, 2000). We unexpectedly found that the low collagen-binding activity in type 2A and type 2B patients was more consistent with the reduction in high molecular weight VWF multimers than the ristocetin cofactor activity. As a result we strongly recommend that the collagen-binding assay

S-23 Meiring M., Badenhorst, P.N. & Kelderman, M. (2005). The use of an algorithm for the laboratory diagnosis of von WIllebrand disease. *Medical Technology SA* 19(1):15-18.

should form part of the diagnosis of VWD.

In 2009, as a result of my extensive experience in the field, I was invited by the editors of *European Oncology and Haematology* to write a review paper on the laboratory diagnosis of von Willebrand disease. In this paper I proposed a modified algorithm that must include all the diagnostic tests needed to diagnose VWD (**S-24**). These tests include the VWF antigen assay (VWF:Ag), the ristocetin cofactor assay (VWF:RCo), the collagen-binding assay (VWF:CB), the VWF propeptide assay (VWF:pp), the factor VIII-binding assay (VWF:FVIIIB), the ristocetin-induced platelet agglutination assay (RIPA) and RIPA mixing studies. We included an algorithm to diagnose type 1 VWD with increased clearance and also suggested how to distinguish between platelet-type VWD and type 2B VWD. We also included an approach to diagnose type 2N VWD. We ultimately proposed that it is vitally important to follow a systematic approach to diagnose VWD. As I mentioned in S-24, it is very important to keep in mind that each laboratory test only forms one part of the diagnostic puzzle. This makes it necessary to put all the puzzle pieces together to build the bigger diagnostic picture.

In 2011, *Seminars in Thrombosis and Haemostasis* published an edition to include all the laboratories world-wide that diagnose VWD. I was asked to write a review paper on the laboratory diagnosis and management of VWD in South Africa. I listed the 17 Haemophilia Treatment Centres in South Africa where patients with VWD are cared for (**S-25**). I indicated that we do not know the prevalence of VWD in South Arica. I also included the genotypic data that we determined in five type 2 VWD patients in our laboratory. Two of these presented with type 2B VWD, two with type 2M VWD and one with type 2A VWD. We sequenced exon 28 and identified single nucleotide polymorphisms (SNPs). The 4641T/C SNP was found in all five patients. A 4141A/G SNP was found in three patients while a silent SNP 2923G/A was found in one patient and a new silent SNP 4923G/A in another patient. We also found that forty-

S-24 Meiring, M., Kelderman, M. & Badenhorst, P.N. (2009). Laboratory diagnosis of von Willebrand Disease. *European Oncology and Haematology* 3(1):33-36. (Cited by 1)

S-25 Meiring, M., Coetzee, M., Kelderman, M., & Badenhorst, P. (2011). Laboratory diagnosis and management of von Willebrand disease in South Africa. *Seminars in Thrombosis and Haemostasis* 37(5):576-580. (Cited by 9)

five percent of our type 1 VWD patients present with VWF:pp/VWF:Ag ratio of 1.9 ± 0.3. This indicates an increased VWF clearance phenotype. Our reference range in normal subjects for the VWF:pp/VWF:Ag ratio is 1.3 ± 0.2. We rejected approximately 15% of the samples we received mainly due to poor storage conditions. In order to scientifically show, and to stress the importance of storage conditions on the quality and accuracy of the diagnostic tests, we exposed normal plasma samples to diferent strorage conditions and measured the effect on the VWF:Ag, VWF:RCo, VWF:CB ratios and multimeric analysis. Storage at -20°C broke down the large VWF multimers. The functional assays confirmed this. Storage at -70°C had no measurable effect on the multimer pattern, even when samples were thawed and frozen up to five times. In this study we also point out that the VWF:Ag concentration in the FVIII/VWF concentrate that is used to treat patients in South Africa is approximately double that of the FVIII concentrate. This fact must be consided when this product is used to treat patients for VWD and is thus important for clinicians to be aware of.

In 2017, I published a paper detailing the challenges we face to diagnose VWD in the laboratory and to manage these patients in our country (**S-26**). We reported on the distribution of VWD subtypes and determined the percentage misdiagnoses that occurred if the only two tests, the VWF:Ag and VWF:RCo assays were used. It must be noted that these two assays are mostly used in the other laboratories in South Africa. Retrospective analysis of data from 250 VWD patients indicated that 6% of type 1 VWD patients would have been misdiagnosed as type 2 VWD, 13% of type 2A would have been misdiagnosed as type 1 and 77% of them as type 2B, 8% of type 2B would have been misdiagnosed as type 1 and 55% of them as type 2A; 28% of type 2M patients would have been misdiagnosed as type 1 and 28% of them as type 2A or type 2B and 1% of type 3 VWD patients would have been misdiagnosed as type 1VWD. When the multimeric analysis was included together with the VWF:Ag and VWF:RCo tests, 20% of patients would still

S-26 Meiring, M., Haupt, L., Conradie, C. & Joubert, J. (2017). Challenges in the laboratory diagnosis and management of von Willebrand disease in South Africa. *Annals of Blood* 2:19-25. (Cited by 2)

have been misdiagnosed.

My research and publications on VWD led to continued collaboration with Prof Emmanuel Favaloro, head of the RCPA quality assurance programme in Australia. Our first collaboration on the diagnosis of VWD was a study in 2006 evaluating the diagnosis of type 2B VWD (**S-27**). Six different type 2B plasma samples were sent out for testing by 52 laboratories. Those laboratories that used only the VWF:Ag and VWF:RCo assays misdiagnosed 26% of the samples either as "normal" or as "type 1 VWD". When the VWF:CB assay was added to the test panel, 11% misdiagnosed the samples. In addition, VWF sub-assays influenced the diagnosis of type 2 VWD. Of concern was that the use of automated platelet agglutination to assay VWF:RCo resulted in a more consistently functional discordance to identify VWF function as compared to the classic platelet aggregometry. Our in-house VWF:CB assays performed better than the commercial kits. The automated LIA-based VWF-activity assays also performed better than the ELISA-based assays. The majority of laboratories were proficient to test for VWD, but unfortunately incorrectly interpreted the results. We concluded that the correct diagnosis was more likely when more diagnostic tests were used and when the VWF:CB assay was included in the diagnosis. We finally provided a series of recommendations in the form of an algorithm to be used to properly identify type 2B VWD in the laboratory. I am happy to report that the majority of laboratories now follow our recommendations by referring VWD samples to expert VWD testing centres for investigation.

Despite these helpful approaches, the laboratory diagnosis of VWD remains problematic for many laboratories world-wide. Together with Prof Emmanuel Favaloro's quality assurance programme, in 2014 we evaluated the laboratory errors in the diagnosis of VWD (**S-28**). In this evaluation we used 29 plasma samples of both quantitative VWD deficiencies (type 1 and 3 VWD) and

S-27 Favaloro, E.J., Bonar, R., Meiring, M., Street, A. & Marsden, K. (2007). 2B or not 2B? Disparate discrimination of functional VWF discordance using different assay panels or methodologies may lead to success or failure in the early identification of type 2B VWD. *Thrombosis and Haemostasis* 98:346-358. (Cited by 41)

S-28 Favaloro, E.J., Bonar, R., Meiring, S.M., Duncan, E., Mohammed, S., Sioufi, J. & Marsden, K. (2014). Evaluating errors in the laboratory identification of von Willebrand disease in the real world. *Thrombosis Research* 134:393-403. (Cited by 49)

qualitative defects (type 2 VWD) that were tested by 55 participating laboratories. We found considerable variation between laboratories and between the different methods that led to errors in the identification of VWD. Samples with a moderate quantitative VWF deficiency were misdiagnosed as qualitative defects in 30 of 334 occasions (9% error rate). Qualitative VWF defects were also misdiagnosed as quantitative deficiencies at a 9% error rate. Of concern was that most laboratories misdiagnosed their own data. In addition, in most instances the misdiagnoses were due to limited or insufficient test panels. As a result we again stressed that laboratories should use at least the VWF:Ag and two other activity (functional) tests, e.g. the VWF:RCo and the VWF:CB to diagnose VWD. Adding multimeric analysis will possibly help to distinguish between qualitative and quantitative defects. It is important to note that only a small percentage (3.5%) of laboratories used this.

In another collaborative study with Prof. Emmanuel Favaloro we assessed how many times qualitative type 2M VWD that is not associated with the loss of high molecular weight multimers, is misdiagnosed (**S-29**). We sent four type 2M VWD samples; two type 2B VWD and 2 type 2A-like samples to 60 laboratories world-wide. The results were alarming. Only 29% of laboratories identified the type 2M samples correctly; the error rate was 71%. These misdiagnoses were the result of insufficient test panels (42%), wrong interpretation of results (10%) and other analytical errors (13%). The findings clearly indicated that type 2M VWD was diagnosed more often incorrectly than correct. This clearly indicates that type incidence of 2M VWD is under-reported in literature.

In an attempt to improve on the limited value or insufficient test panels that are used, we assessed if using a supplementary PFA-100 test together with VWD testing will minimize the misdiagnosis of VWD. FPA-100 measures

S-29 Favaloro, E.J., Bonar, R.A., Mohammed, S., Arbelaez, A., Niemann, J., Freney, R., Meiring, M., Sioufi, J. & Marsden, K. (2016). Type 2M von Willebrand disease – more often misidentified than correctly identified. *Haemophilia* 22:e145-e55.

platelet adhesion to a collagen surface (**S-30**). We analysed retrospective data obtained from patients with type1, type 2A or type 2M VWD and also from patients with haemophilia and carriers of haemophilia. Laboratory analyses included factor VIII, VWF:Ag and VWF:RCo assays and the PFA-100 closure times pre- and post-desmopressin treatment or pre- and post-FVIII-concentrate treatment. The normalisation of the PFA-100 closure times after desmopressin (DDAVP) treatment correlated well with the normalisation of the VWF:CB post-treatment. Based on this we strongly suggest that the supplementary use of the PFA-100 closure time and VWF:CB together with the VWF:Ag and VWF:RCo will reduce misdiagnosis of VWD and also improve the treatment management of patients with VWD.

I also collaborated in studies with researchers at other universities e.g. the North-West University, University of Cape Town and the University of the Witwatersrand in South Africa by measuring VWF levels in several studies. In collaboration with Prof Leoné Malan from the subject group Physiology, Faculty of Health Sciences, Potchefstroom Campus, North-West University, we investigated possible associations between salivary testosterone levels with fibrinogen, di-dimer and VWF levels in plasma in 102 African and 165 Caucasian male teachers in the Northwest province of South Africa (**S-31**). Cardiovascular competency was assessed by blood pressure monitoring. The Stroop Colour-Word Conflict test was used to evoke acute mental stress responses (Stroop, 1935). The African sub-group showed elevated blood pressure with a slight inflammation response, hyperglycaemia and low testosterone levels. This might lead to a higher cardiovascular risk. The Caucasian group displayed only higher fibrinogen levels. Multiple regression analysis found an association of higher fibrinogen and VWF levels with a low testosterone environment.

S-30 Favaloro, E.J., Thom, J., Patterson, D., Just, S., Baccala, M., Dixon, T., Meiring, S.M., Koutts, J., Rowell, J., Baker, R. (2009). Potential supplementary utility of combined PFA-100 a functional von Willebrand factor testing for the laboratory assessment of desmopressin and factor concentrate therapy in von Willebrand disease. *Blood Coagulation and Fibrinolysis* 20(6):475-483. (Cited by 22)

S-31 Malan, N.T., Von Kanel, R., Schutte, A.E., Huisman, H.W., Smith, W., Schutte, R., Smith, W., Mels, C.M., Kruger, R., Meiring, M., Van Rooyen, J.M. & Malan, L. (2013). Testosterone and acute stress are associated with fibrinogen and von Willebrand factor in African men: The SABPA study. *International Journal of Cardiology* 168 (5):4638-4642. (Cited by 11)

In another study we compared in the same group as in **S-31** the coagulation response to acute mental stress. We also studied the relationship of these coagualtion responses to physical well-being (**S-32**). We measured circulating levels of VWF:Ag, fibrinogen and D-dimer before and after the Stroop Colour-Word Conflict test. All participants completed health questionnaires to establish symptoms of depression. Africans showed lower VWF and fibrinogen reactivity in response to laboratory mental stress than Caucasians. The symptoms of depression in Africans were associated with a higher increase in VWF levels and decrease in fibrinogen plasma levels than in Caucasians. Thus we could postulate that depressive mood might be associated with an increased risk of hypercoagulability, as reported by Frasure-Smith & Lespérance (2010).

The composition of the von Willebrand factor multimers in the circulation is a result of the competition between the liver clearance of VWF and proteolysis through its cleavage protein in plasma, ADAMTS13. Defective proteolysis of VWF by ADAMTS13 can result in thrombotic thrombocytopenic purpura (TTP), a fatal thrombotic disorder (Moake *et al*., 1982). TTP used to be a rare disorder but its incidence has increased dramatically concurrent with the HIV/AIDS epidemic (Gunther *et al*., 2006). The underlying cause is likely to be the result of low ADAMTS13 levels, fundamental abnormalities of ADAMTS-13, endothelial dysfunction, or autoantibody production against ADAMTS13.

In 2012, we established low levels of ADAMTS13 in HIV-associated TTP patients in conjunction with extremely high plasma VWF and tissue factor (TF) levels (**S-33**). Interestingly, we found that only 50% of our patients with HIV-associated TTP presented with autoantibodies to ADAMTS-13. Based on our results, we propose that the trigger for HIV-associated TTP is HIV infection that increases cytokine release from endothelium, resulting in increased VWF

S-32 Von Kanel, R., Hamer, M., Malan, N.T., Scheepers, K., Meiring, M. & Malan, L. (2013). Procoagulant reactivity to laboratory acute mental stress in Africans and Caucasionas, and its relation to depressive symptoms: The SABPA Study. *Thrombosis and Haemostasis* 110(5):977-986. (Cited by 7)

S-33 Meiring, M., Webb, M., Goedhals, D. & Louw, V. (2012). HIV associated Thrombotic Thrombocytopenic Purpura – What we know so far. *European Oncology and Haematology*, 8(2):89-91. (Cited by 10)

synthesis. VWF that is released by the endothelium consequently leads to microvascular thrombus formation. In addition, micronutrient deficiencies in HIV-infected patients also cause decreased ADAMTS13 synthesis that leads to microvascular thrombus formation (Kaiser *et al.*, 2006). We also found that HIV infection increases the production of autoantibodies to ADAMTS13. It is plausible that some of these autoantibodies might inhibit the function of ADAMTS13 which can also lead to microvascular thrombus formation. Ultimately, haemolytic anemia and thrombocytopenia result in HIV-associated TTP, independent of the cause of microvascular thrombus formation. To my knowledge, this is the first proposed mechanism that explains the onset of HIV-associated TTP which has changed the paradigm about TTP. The findings in this study provided me with a highly focused, ongoing research project that will lead to further publications and provide research projects for postgraduate students.

The treatment products for TTP in South Africa include fresh frozen plasma (FFP), cryosupernatant (CSP) and and Bioplasma FDP, a solvent/detergent-treated plasma (SDP). In another study on TTP (**S-34**), we compared the ADAMTS13 and VWF levels and activities, as well as the plasminogen levels in plasma products that are currently used to treat TTP. With this study, we explored possible differences in VWF and ADAMTS13 levels and activities between the products that may offer treatment advantages. All products had normal to high ADAMTS13 levels and activities, making them suitable to treat TTP. Bioplasma FDP had normal VWF levels and CSP had typically reduced VWF levels. FFP had a large inter-batch variation where some batches contained decreased VWF levels and some normal levels. Statistically higher ADAMTS13 activities were found on FFP samples from the O-blood group than the non-O blood groups. This finding might indicate that FFP of blood group O may be preferential to treat TTP patients. We concluded that one should consider the viral safety, the cost, the availability of the product and the impact of the within-product variations when choosing a product for

S-34 Van Marle, A.C., Joubert, J. & Meiring, S.M. (2019). Comparison of ADAMTS13 and von Willebrand Factor levels and activities, and plasminogen levels, in plasma products currently available for the treatment of thrombotic thrombocytopenic purpura in South Africa. *Transfusion and Apheresis Science* 58:72-78.

treatment.

The inclusion of ADAMTS13 testing in our routine diagnostic setup opened the way for collaboration on projects that require these results. Together with the group of Prof. Marc Combrinck from the Department of Neurology, Faculty of Health Sciences at the University of Cape Town, we investigated the role of VWF and ADAMTS13 in HIV-related strokes in young patients of 35 years of age (mean age) in a case-control study with three participant groups: HIV-positive antiretroviral therapy-naïve young stroke patients, HIV-negative young stroke patients and HIV-positive antiretroviral therapy-naïve non-stroke control patients (**S-35**). In the case of the stroke patients, plasma VWF and ADAMTS13 levels were measured five and seven days post-stroke. The HIV-positive non-antiretroviral stroke patients had significantly higher VWF levels and lower ADAMTS13 levels than the HIV-positive antiretroviral treated non-stroke controls. In addition, VWF levels in all HIV-positive participants correlated negatively with CD4 counts, i.e. the higher the VWF levels, the lower the CD4 counts. Thus, stroke in HIV infection was associated with a pro-thrombotic state with increased VWF and decreased ADAMTS13 levels. The pro-thrombotic state of HIV is not yet fully elucidated and require ongoing research. We have several studies planned, as well as studies in collaboration with the University of the Witwatersrand.

A case study describes that a patient, treated with valproic acid (a commonly used anticonvulsant), developed serious bleeding during surgery (**S-36**). Platelet aggregometry in response to arachidonic acid, adenosine diphosphate, epinephrine, collagen and ristocetin was tested. The platelet aggregation in response to arachidonic acid was abolished and reduced in response to adenosine diphosphate, adrenaline, collagen and ristocetin. Valproic acid treatment was halted for two weeks, with close monitoring for epilepsy. All platelet functions returned to normal and no bleeding developed

S-35 Allie, S., Stanley, A., Bryer, A., Meiring, M. & Combrinck, M.I. (2015). High levels of von Willebrand factor and low levels of its cleaving protease, ADAMTS13, are associated with stroke in young HIV-infected patients. *International Journal of Stroke* 10(8):1294-1296. (Cited by 13)

S-36 Jafta, A.D., Meiring, M. & Conradie, C. (2010). Valproic acid associated platelet dysfunction: Case Report. *African Journal of Haematology and Oncology* 1(2):54-56.

during follow-up surgery. It is very likely that free fractions of valproic acid in plasma may reduce platelet aggregation, in spite of the fact that most patients on valproic acid do not experience platelet dysfunction. We are currently testing this hypothesis in collaboration with local neurologists from the Department of Neurology and pharmacologists from the Department of Pharmacology at the UFS.

3. GENERAL SUMMARY

My main contribution to the knowledge of haemostatic disorders was to develop and test new and existing antithrombotic drugs, to develop cost-effective tests to diagnose these disorders, and to unravel the mechanisms underlying the diagnoses of these disorders.

We investigated several monoclonal antibodies for their effect in platelet-dependent thrombogenesis. It is important to note that some caused almost immediate thrombocytopenia when injected into baboons. Others had no effect on the circulating platelet count. Antibody 6B4 and the $F(ab)_2$ fraction thereof caused an immediate drop in platelet count (profound thrombocytopenia) **(S3)** when injected into baboons. Thehe F(ab)-fragment did not decrease the platelet count. The fact that monoclonal antibodies, as large proteins and foreign to the body, trigger the immune system should also be kept in mind. As a result second treatments will not be successful because they will be recognised by the immune system as foreign and immediately removed from the circulation. It is therefore advisable to use the smaller, non-immunogenic F(ab) fragments.

In my group we reported on the first echistatin-like RGD-sequence containing monoclonal antibody MA-16N7C2 that significantly inhibited platelet glycoprotein IIb/IIIa function. In addition, it appeared that the antibody was internalised to prolong the inhibitory effect (**S-2**). We also described the first anti-human GpIb/IX monoclonal antibody MA-6B4 (**S-3**) that successfully prevented platelet adhesion to the subendothelium and subsequent thrombus formation. This finding confirmed the important role of GpIb/IX in platelet adhesion *in vivo*. An important finding was that the bleeding time was not markedly affected, while no blood loss, spontaneous bleeding or thrombocytopenia was observed. We also conclusively showed that inhibition of both GPIb/IX with antibody 6B4 and GPIIb/IIIa with antibody 16N7C2 was more effective and safer than using them separately (**S-5**). It is also important to note that the superior inhibition of platelet-dependent thrombosis was achieved at much lower individual doses when the antibodies were used in

combination. We also described, for the first time, the antithrombotic effect of the monoclonal antibody 82D6A3 (**S-6**) that effectively inhibits the VWF binding to fibrillar collagens type I and III in baboons without significantly lengthening the bleeding time, even at high doses. This antibody is therefore a safe target to prevent arterial thrombosis. We also showed that the factor VIII inhibitory monoclonal antibody LE2E9 (**S-8**) prevented platelet-dependent thrombosis without prolonging the bleeding time and so decreases the tendency of *in vivo* bleeding. This may present a novel approach to a long-acting antithrombotic agent with an optimal safety/efficacy profile. We also found that inhibiting VWF with antibody 82D6A3 (**S-7**) prevented stenosis in a clinically relevant model. We also developed a model to use to prevent restenosis following stent deployment.

In my endeavours to develop cost-effective anti-thrombotic drugs, we identified a collagen-binding protein from the hookworm *Necator americanus* by using a cDNA-expressing phage display library (**S-9**). This was the first study using phage display technology to select proteins based on their binding properties in which I was involved. Through phage display technology we developed a competitive thrombin inhibition peptide (TIP), that inhibited thrombin-induced platelet activation in a dose-dependent manner (**S-11**). It also reduced platelet adhesion onto a human microvascular endothelial matrix in a parallel plate flow chamber under both arterial and venous shear conditions. Using the same cyclic heptapeptide library, we also selected a peptide that binds to factor VII and competes with tissue factor for binding to factor VII (**S-12**). This peptide prolonged the prothrombin time and reduced platelet adhesion to both human endothelial cells and tissue factor matrices in a flow chamber under arterial flow conditions. We also selected single chain antibody fragments that inhibit tissue factor (**S-13**), the initiator protein of blood coagulation. We expressed this single chain variable fragment (scFv) by increasing the levels in the cytoplasm of *E. coli* than what we had previously achieved in the periplasm (**S-14**). Both *in vitro* refolding and cold shock strategies were able to produce functional tissue factor inhibitory scFv, although with varying degrees of success. This approach successfully

improved the production of low-yielding single chain variable fragments. This will ultimately lead to the production of large amounts of product.

We used an antibody fragment library to select antibodies that bind to the VWF-propeptide in order to develop cost-effective assays for diagnosis of haemostatic disorders (**S-15**). Our assay compared favourably with that of the available commercial kit. Of particular importance is that our antibodies had a higher binding affinity for VWF propeptide than the commercial antibodies. We also filed an South Africa patent 2011/05426. In order to widen our scope of diagnoses of VWD, I developed a cost-effective assay that monitors VWF activity by measuring its binding to type III collagen, the collagen-binding assay (**S-16**). This assay is ten times cheaper than other commercially available assays. It is also very sensitive to measure the collagen-binding activity in VWD patients. I also developed the VWF multimer analysis as an additional instrument to diagnose VWD (**S-17**); this is 10 times cheaper than the commercially available assay. We developed a cost-effective ELISA test, using commercial antibodies to ADAMTS13 (**S-20**). Our in-house assay was 90% cheaper than the commercial kit and we therefore recommend that this assay be used to diagnose HIV-associated TTP. Therefore, by developing these essays, we were able to significantly decrease the high cost of diagnoses as a result of purchasing these tests from commercial sources.

In summary, I developed cost-effective assays to increase the scope of tests in order to improve the ability of my reference laboratory to diagnose VWD. In order to provide an extensive and accurate diagnostic service to the South African public, our battery of tests for the diagnosis of von Willebrand disease includes the VWF antigen test, the collagen-binding assay, the ristocetin cofactor assay, the multimeric analysis, the factor VIII-binding assay and the VWF propeptide assay. By developing cost-effective diagnostic tests, we can now diagnose VWD at a fraction of the cost that it would have been if we had used imported commercial assays. As I mentioned before, it is crucial to use a systematic approach to diagnose VWD. Each test only forms one piece of the diagnostic puzzle. It is therefore necessary to put all the puzzle pieces together for the whole diagnostic picture to emerge.

Building this puzzle, we found that 45% of our type 1 VWD patients had an increased VWF clearance phenotype (**S-25**). We also determined the inaccuracy of diagnosis if only the VWF:Ag and VWF:RCo assays are used (**S-26**). We did this because these tests are often the only ones used by the majority of laboratories in South Africa. Retrospective analyses of data of 250 VWD patients in our database indicated that 6% of type 1 VWD patients would have been misdiagnosed as type 2 VWD, 13% of type 2A would have been misdiagnosed as type 1 and 77% as type 2B; 8% of type 2B would have been misdiagnosed as type 1 and 55% as type 2A;, 28% of type 2M patients would been misdiagnosed as type 1 and 28% as type 2A or type 2B, and 1% of type 3 VWD patients would have been misdiagnosed as type 1 VWD. Even if multimeric analyses were added to the VWF:Ag and VWF:RCo tests, 20% of patients would still have been misdiagnosed. Importantly, storage at -20°C broke down the large VWF multimers; a finding confirmed by the functional assay. Storage at -70°C caused no breakdown of multimers (**S-25**). This will further exacerbate misdiagnosis. We also found a new silent SNP 4923G/A in one of our type 2 VWD patients (**S-25**). I emphasize the errors in diagnosis because it directly affects the success of treatment of patients with VWD. Errors in diagnosis lead to wrong treatment approaches that is mostly ineffective and expensive and may increase the risk to patients.

In collaboration with Prof. Emmanuel Favaloro from the Royal College of Pathologists of Australasia (RCPA), we conclusively showed that the collagen-binding assay and the VWF activity assay based on spontaneous binding of VWF to a gain-of-function mutant GPIb fragment (VWF:GPIbM) had the highest sensitivity to assess the loss of the high molecular weight multimers. This confirms my finding on the sensitivity of the collagen-binding assay to assess VWF-multimer decrease (**S-18**). The ristocetin-cofactor assay and the VWF activity assay based on ristocetin-induced binding of VWF to a recombinant wild type GPIb fragment (VWF:GPIbR) displayed intermediate sensitivity to assess decreased HMW multimers (**S-19**). These results ultimately hold significant clinical implications to diagnose and to monitor the

outcome of therapy in patients with VWD. In a further study using a larger sample size (**S-28**), we discovered that most international laboratories were proficient in testing to diagnose VWD, but that their interpretation of the assays was incorrect when they used only the VWF:Ag and VWF:RCo assays. It became clear that inclusion of the VWF:CB assay improved the accuracy of diagnosis. Based on these results, we made a series of recommendations in the form of an algorithm on the tests to be used to enable a laboratory to correctly identify VWD. In most cases the misdiagnoses by laboratories were due to limited or insufficient test panels (**S-27**). We again stressed that laboratories should use an extensive test panel to diagnose VWD. If it is not possible, the VWF:Ag and two other functional tests, e.g. the VWF:RCo and the VWF:CB, may be used as the minimum tests to ensure accurate diagnosis. Similarly, the use of the multimeric VWF analysis will be important to distinguish between qualitative and quantitative defects, since the treatment depends on the correct classification of VWD. Unfortunately only a small percentage of laboratories perform this test. In the survey we also found that type 2M VWD is misdiagnosed more regularly than it is diagnosed correctly (**S-29**). This may explain the relative under-reported incidence of type 2M VWD in the literature. Because of this, we strongly recommended that supplementary combination of the PFA-100 assay be done together with measuring VWF:CB. This, together with the VWF:Ag and VWF:RCo tests, may possibly improve diagnosis and treatment efficacy in patients with VWD.

Based on our findings on patients with TTP, we were the first to propose a mechanism to explain the initial onset of the disorder (**S-33**). We proposed that the trigger for HIV-associated TTP is HIV infection that increases cytokine release from endothelium, resulting in increased VWF synthesis and release by the endothelium leading to microvascular thrombus formation. This changed the paradigm of thought pertaining to TTP. We furthermore proved that human umbilical vein endothelial cells decrease VWF in reaction to tissue factor and cytokines interleukin 6 and tumor necrosis factor. This explained the extremely high VWF level in HIV and HIV-associated TTP patients.

The proposed mechanism of onset of TTP was based on our findings that

inflammatory cytokines such as IL-8 and TNF-alpha, coagulation factors such as thrombin and tissue factor, as well as combinations thereof, stimulate the release of ULVWF and inhibit the release of ADAMTS13 in human umbilical endothelial cells (HUVECs, **S-21**). We also indicated that all products that are currently used to treat TTP have normal to high ADAMTS13 levels and activity. Of particular importance was the finding that the VWF varied considerably in the commercially available products that are used to treat these patients. Bioplasma contains normal VWF levels. Cryosupernatant has typically reduced VWF levels. Fresh frozen plasma had a large inter-batch variation where some batches contain decreased VWF levels and some normal levels. This may adversely impact on the success of treatment of HIV-positive patients presenting with TTP. Furthermore, statistical differences were found across products and ABO blood groups of donors. Fresh frozen plasma from blood group O donors contains more ADAMTS13 than those from non-O donors and would be more beneficial for the treatment of patients with HIV-associated TTP. In addition to the choice of product based on VWF and ADAMTS13 availability, we also emphasised that factors such as viral safety, costs and product availability must be taken into account.

In collaborative studies, we were the first to show that stroke in HIV-infected patients was associated with a pro-thrombotic state, characterised by elevated VWF and low ADAMTS13 levels (**S-35**). In addition, depression was associated with higher plasma concentration of VWF increase in Africans compared to Caucasians. It is possible that ethnic differences in the vasculatory energic stress response may partially explain this difference. Multiple regression analysis suggested an association of higher circulating levels of fibrinogen and VWF with low testosterone levels (**S-32**). We also described a case where valproic acid, a commonly used anticonvulsant, was associated with excessive bleeding during surgery (**S-36**). In this patient *in vitro* platelet aggregometry was decreased in response to aggregation induced by with arachidonic acid, adenosine diphosphate, epinephrine, collagen and ristocetin.

In a new avenue of research that I recently started in collaboration with Prof. Francis Smit from the Robert W.M Frater Institute for Cardiovascular Research, we developed re-endothelialised tissue-engineered small-vessel conduits with no thrombogenicity **(S-22)**. We studied re-endothelialisation of decellularised baboon arteries in the *in vitro* flow chamber described in S-22. We conclusively found that HUVECs irreversibly bind to the decellularised scaffold in an *in vitro* flow chamber. It is important to stress that, to our knowledge, this was the first study to show success with re-endothelialisation. Up to this point, attempted re-endothelialisation of arteries that were damaged by balloon catheterisation, or endothelial damage by air or magnetism, had been unsuccesfull. Although this research is in an infantile stage, we have high hopes of positive results following implantation of re-endothelialised decellularised arteries into baboons.

In conclusion, my research contributed significantly to the knowledge base of thrombosis inhibition and accurate differential diagnosis of VWD. The success of particularly the research on VWD resulted in my laboratory functioning as the reference laboratory for VWD in RSA. In addition, my laboratory is also part of the world-wide quality assurance programme for the diagnosis of VWD. In this regard, I work closely with Prof. Emmanuel Favaloro from the Royal College of Pathologists' quality assurance programme.

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Sites of Elimination and Pharmacokinetics of Recombinant [^{131}I]Lepirudin in Baboons

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Abstract □ Lepirudin has a short half-life, and only 50–60% of the intravenously administered dose is excreted by the kidneys. The fate of the remainder is unknown. We designed a study to determine the fate of this lepirudin. In each of six baboons, [^{131}I]lepirudin was given intravenously as a bolus or infused over 30 min, 24 h apart. The in vivo redistribution of [^{131}I]lepirudin was determined and quantified by scintillation camera imaging. In all studies, the half-life of [^{131}I]lepirudin, as determined from the disappearance of radioactivity, was 21 ± 3 min. The half-life determined from the disappearance of lepirudin, measured by the Ecarin Clotting Time (ECT) method, was similar at 23 ± 8 min. Results obtained with the labeled lepirudin are therefore comparable with those obtained using the plasma concentration of lepirudin. When lepirudin was administered as a bolus, the half-life was 18 ± 4 min, and lepirudin was cleared from the plasma at a rate of 42 ± 12 mL/min and by the kidneys at 23 ± 2 mL/min. Following infusion over 30 min, the half-life and total and renal clearances were not significantly different. In both studies, between 50 and 60% of the administered lepirudin was excreted by the kidney. Studies on sacrificed baboons showed that appreciable amounts of lepirudin were present in the bile, indicating the liver as a contributor to the elimination of lepirudin.

Introduction

Hirudin is regarded as the most potent direct inhibitor of thrombin and its recombinant form, lepirudin ([leu^1, Thr^2]-63-disulfatohirudin), has the same physiochemical characteristics and biochemical properties as the native protein.[1,2] Lepirudin is a 65 amino acid polypeptide (7 kDa) produced by transfected yeast cells.[1] The interaction between hirudin and thrombin results in the formation of a stable, noncovalent stoichiometric 1:1 complex that inhibits all functions of thrombin.[1,2] Apart from its inhibition of coagulation, it also inhibits platelet-dependent arterial-type thrombosis when given in high enough dosages.[3−5] Although the pharmacokinetics of lepirudin is well-known, there are still some unresolved issues. It is generally accepted that lepirudin is mainly excreted in an unchanged form by the kidneys.[1,6] However, approximately 40 to 50% of injected protein cannot be accounted for in the urine. The sites of metabolism and/or uptake of this lepirudin are unknown. We designed this study to investigate the metabolism and fate of lepirudin in baboons.

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Subjects and Methods

Experimental Animals—Eight male baboons (Papio ursinus) supporting permanent femoral arteriovenous shunts (A-V shunts) of silicone rubber tubing[7,8] were used. The animals were sedated with intramuscular ketamine hydrochloride (10 mg/kg body mass, Centaur Laboratories, South Africa) to enable handling. Anesthesia was maintained with subsequent administrations when necessary. One hundred milliliters of saline were given intravenously (iv) to each baboon 30 min before the start of a study to ensure that they were normovolaemic.

Experimental Protocol—Six baboons received [^{131}I]lepirudin as an iv bolus of 0.3 mg/kg as well as an infusion at a rate of 0.01 mg/kg/min for 30 min on separate days. The treatment requirements were such that an equal number of baboons received the bolus or infusion on each day. The lepirudin (HBW 023, Hoechst AG, Frankfurt and Behringwerke AG, Marburg, Germany) was labeled with 131-iodine (^{131}I; Radiochemical Centre, Amersham, UK) using the IODO-GEN method.[9] Labeling efficiency was 98 ± 1%. It is important to note that only labeled lepirudin was infused and that we determined the plasma concentration of the labeled lepirudin (see later). Therefore, when reference is made to lepirudin, it refers to the results obtained from the plasma concentration as determined using the ECT method.[10] Similarly, reference to labeled lepirudin refers to results obtained from radioactive count rates of [^{131}I]lepirudin.

Scintillation Camera Imaging—Imaging of the in vivo distribution of labeled lepirudin was done with a Large Field of View Scintillation Camera fitted with a high-energy collimator. Image analysis was done with an A^3-MDS data processing system that was interfaced with the camera. Imaging was done in two phases; they are, a dynamic image acquisition phase and a static acquisition phase.

Dynamic Image Acquisition—The baboons were positioned in front of the detector of the camera so as to include the kidneys, bladder, liver, and spleen in the field of view. Dynamic image acquisition, consisting of 3-min images (64 × 64 word mode) for 120 min (infusion study) and 90 min (bolus study), started simultaneously with the infusion of [^{131}I]lepirudin. For analysis, a region of interest was selected for the kidneys, the bladder, the spleen, and the liver, and the radioactivity in each region was expressed as a percentage of injected [^{131}I]labeled lepirudin, which was estimated by the geometrical mean method of quantification (see Static Image Acquisition). The radioactive count rates of the left and right kidneys were summed to obtain total kidney radioactivity.

Static Image Acquisition and Quantification of In Vivo Distribution of [^{131}I]Lepirudin—After the dynamic image acquisition, static anterior and posterior images of 3 min (64 × 64 word mode) were acquired of the head, thorax and abdomen, and legs. A background image was also acquired to correct whole body and region of interest (organ) radioactivity for background radioactivity. Before treatment on the second day, anterior and posterior images of the thorax and abdomen were acquired to determine and correct for residual ^{131}I radioactivity as a result of infusion of [^{131}I]lepirudin on the previous day.

To obtain anterior and posterior whole body radioactivity, the radioactivity in the head, thorax and abdomen, and legs was

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corrected for background radioactivity and/or residual radioactivity and summed. Regions of interest for the kidneys, bladder, spleen, and liver were selected to determine organ radioactivity. On the first day of the experiment, these radioactivities were corrected for background radioactivity. On the second day, organ radioactivity was corrected for background radioactivity and residual radioactivity, measured before the infusion of [^{131}I]lepirudin. The in vivo organ distribution of [^{131}I]lepirudin was quantified by the geometric mean method.[11]

Blood Collection and Analysis—Blood, 4.5 mL in 0.5 mL of a 3.2% sodiumcitrate solution, was collected before a study was started and then every 15 min thereafter. The radioactivity in 1 mL of plasma was determined in a gamma counter. Plasma levels of lepirudin were determined by the ECT method, which was specifically developed to measure hirudin levels in plasma and body fluids.[10] The activated partial thromboplastin time (aPTT) was measured with a fibrinometer (Clotex II, Hyland Division, Travenol Laboratories, Costa Mesa, CA), and reagents were supplied by the same company. The aPTT was measured to give an indication of the level of anticoagulation achieved and to show that the labeled lepirudin was functional.

To determine if the labeling procedure affected the function of [^{131}I]lepirudin, unlabeled and labeled lepirudin were added to plasma obtained from 4 baboons. Plasma concentrations were 2.5 and 5.0 μg/mL of lepirudin. In addition, a 50/50 mixture of labeled and unlabeled protein was also used. The aPTT was determined on all samples.

Urine Collection and Analysis—Urine was collected into a urine-collecting bag with a Teflon-coated latex Foley catheter (GRS Medicals, Kelvin, SA) for up to 24 h and analyzed for lepirudin by the ECT method.[10] The total amount of lepirudin was calculated from the total urine volume and the concentration in urine.

Urine was also analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples analyzed were [^{131}I]lepirudin, urine without [^{131}I]lepirudin (native urine), [^{131}I]-lepirudin added to native urine, and urine collected from the treated baboons. The results strongly suggest that [^{131}I]lepirudin was excreted in an unchanged form. The results are not given.

Calculations—*Plasma Half-Lives of Lepirudin and [^{131}I]-Lepirudin*—The elimination half-lives of lepirudin and [^{131}I]-lepirudin were calculated by adjusting a one-phase exponential function to the appropriate phases of the log−linear plasma concentration−time and radioactive count rate−time profiles by the method of least-squares analysis. The value k, in the function $C_{max} \times e^{-kt}$, was thus found, where C_{max} is the maximum lepirudin concentration or maximum radioactive count rate in plasma. The half-lives were calculated as $t_{1/2} = 0.693/k$.

Total Clearance of Lepirudin and [^{131}I]Lepirudin—For the constant *infusion*, total clearance (CL_{total}) was calculated by dividing the rate of infusion (mg/min or radioactive count rate/min) by the maximum concentration of lepirudin or radioactive count rate in the plasma. To estimate the maximum plasma concentration or plasma radioactive count rate, an exponential association function was fitted to the data points obtained during the infusion period and extrapolated to maximum (i.e., an estimate of steady-state levels).[12]

For the *bolus* injection, CL_{total} was calculated by dividing the total dose, or the total counts injected, by the area under the curve ($AUC_{0-\infty}$) of the plasma lepirudin concentration (μg/mL)−time or plasma radioactivity (radioactive count rate/ml)−time profiles. The area under the plasma concentration−time and plasma radioactive counts rate−time curves up to 90 min (AUC_{0-90}) were calculated using the trapezoid rule. The curves were extrapolated to ∞ using the formula $AUC_{0-\infty} = AUC_{0-90} + C_p^*/k_e$, where C_p^* is the plasma lepirudin concentration or plasma radioactive count rate at the last sampling time (90 min). The terminal rate constant (k_e) was determined from the slope of the terminal portion of the log-linear C_p versus time curve.

Renal Clearance of [^{131}I]Lepirudin—For the constant *infusion*, renal clearance (CL_{renal}) was calculated by dividing the rate of appearance of [^{131}I]lepirudin in the bladder (radioactive count rate/min) by the maximum counts in the plasma. The latter was estimated by fitting an exponential association function to the available radioactive count rate/mL−time profiles and extrapolated to estimate the maximum value where a steady state was reached.

For the *bolus* injection, CL_{renal} was calculated by dividing the maximum radioactive count rate in the bladder by $AUC_{0-\infty}$ of the plasma radioactivity (radioactive count rate/mL)−time profiles.

Extravascular Clearance—The plasma clearance of lepirudin by the extravascular compartment was estimated by calculating the difference between the total clearance and the renal clearance.

Determination of Sites of Lepirudin Accumulation in Sacrificed Baboons—Two baboons were used. The one baboon was sacrificed after 30 min of infusion of [^{131}I]lepirudin. The other one received no lepirudin and was sacrificed to obtain organs and body fluids that served as controls for immunohistochemistry and lepirudin determinations, respectively. The baboons were deeply anesthetized. The test baboon was exsanguinated through the arterial side of the A-V shunt. About 2 L of saline at 37 °C were simultaneously infused under pressure (140 mmHg) into the venous side of the shunt to replace the blood that was lost.

Ex Vivo Quantification of the Distribution of [^{131}I]Lepirudin—The liver, spleen, kidneys, and bladder of the sacrificed baboons were immediately excised by careful dissection. Residual radioactivity in the body and that present in the liver, spleen, kidneys, and bladder were determined by scintillation camera imaging.[11] Images of 3 min (64 × 64 word mode) were acquired. Organ radioactivity was then expressed as a percentage of total body radioactivity, obtained by summation of residual and organ radioactivities.

Organ Biopsies—Biopsies of the kidneys (cortex and medulla), spleen, and liver were collected to establish the location of the lepirudin by using immunohistochemistry techniques. A primary antibody, rabbit anti-hirudin antibody (diluted 1:100 in PBS; Celsus Laboratories, Cincinnati, OH) was added to thin processed sections of the tissues and incubated for 60 min. A secondary goat anti-rabbit antibody (Signet kit, Johnson & Johnson, Johannesburg, Republic of South Africa) was added to bind to the primary antibody and a third antibody complex, a peroxidase−antiperoxidase immune complex, was used as the marker complex. These antibody complexes become visible when the tissues are exposed to the substrate diaminobenzidine. By adding hydrogen peroxide to the diaminobenzidine solution, a brown precipitate forms where lepirudin is present. Light microscopy was used to interpret the stains. Lightmicrographs of all tissues were enlarged 200 times and examined for the presence of lepirudin in cells.

Biopsies of the kidneys, liver, and spleen were also homogenized in saline and centrifuged at 8000 g for 24 h. The ECT method[10] was used to test for the presence of lepirudin in the supernatant of the homogenates.

Collection and Analysis of Duodenal and Gastric Juices and Bile—Duodenal and gastric juices and bile were collected from the sacrificed baboons to test for the presence of lepirudin by the ECT method.[10]

Statistics—Statistical comparison was done using Student's t test for paired data. Values of $p < 0.05$ were considered significant. The results are expressed as mean ± 1 standard deviation (SD).

Results

Changes in aPTT and Pharmacokinetics of Lepirudin—In the in vitro studies, concentrations of 2.5 and 5.0 μg/mL lepirudin lengthened the aPTT to 87 ± 4 and 174 ± 5 s. [^{131}I]Lepirudin, at the same concentrations, lengthened the aPTT to 92 ± 4 and 180 ± 6 s. When labeled and unlabeled lepirudin were mixed, the aPTT lengthened to 90 ± 3 and 178 ± 4 s. The difference in measurements was not significant. The labeling process therefore does not affect the function of lepirudin.

The in vivo changes in aPTT are summarized in Table 1. The labeled hirudin was biologically active because it caused a 2−3-fold lengthening in the aPTT. When lepirudin infusion was stopped and following the bolus injection, the aPTT rapidly shortened to reach near pre-infusion values after 90 min.

The changes in circulating lepirudin and labeled lepirudin are summarized in Figure 1. Following infusion or the bolus injection, the plasma levels of lepirudin and [^{131}I]-lepirudin decreased rapidly. In all studies, the plasma half-life of lepirudin was 24 ± 9 min. When calculated from the

Table 1—Changes in APTT[a]

time (min)	infusion	bolus
–30	40 ± 4	–
–10	–	–
0	131± 20	42 ± 6
30	79 ± 10	98 ± 16
60	64 ± 9	74 ± 13
90	56 ± 8	64 ± 12

[a] Results are given in seconds and are expressed as mean ± 1 SD.

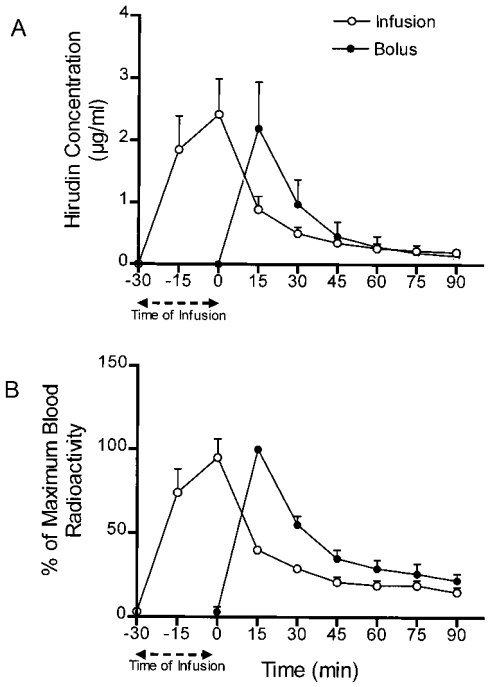

Figure 1—Dynamic changes of (A) plasma lepirudin and (B) [^{131}I]lepirudin following infusion (open circles) or bolus administration (black circles). Values are given as a mean ± 1 SD.

Table 2—Total, Renal, and Extravascular Clearance of Lepirudin and [^{131}I]Lepirudin

route	clearance	r-Lepirudin	[^{131}I]Lepirudin
infusion	total	49 ± 16	52 ± 10
	renal	–	32 ± 10
	extravascular	–	20 ± 8
bolus	total	42 ± 12	45 ± 12
	renal	–	23 ± 2[b]
	extravascular	–	22 ± 12

[a] Values are given in mL/min and are expressed as mean ± 1 SD. [b] $p <$ 0.05, infusion versus bolus (Student's *t* test for paired data).

disappearance of radioactivity from the blood, it was 21 ± 6 min. The difference was not statistically significant ($p >$ 0.05). After infusion of [^{131}I]lepirudin was stopped, lepirudin was eliminated from the plasma with a half-life of 23 ± 8 min ([^{131}I]lepirudin = 21 ± 3 min). When the same amount of [^{131}I]lepirudin was given as a bolus, the half-life was 18 ± 4 min ([^{131}I]lepirudin = 19 ± 8 min). The mean difference in the half-lives between infused and bolus injected lepirudin was 5 ± 10 min. The 95% confidence interval of between −5 and 16 min indicates that the difference was not statistically significant.

Total, renal, and extravascular clearance of lepirudin is summarized in Table 2. Total clearance was ≈45 mL/min, whether it was calculated from the data obtained from the concentration of lepirudin or from labeled lepirudin. Between 51 and 61% of this lepirudin was cleared by the kidneys.

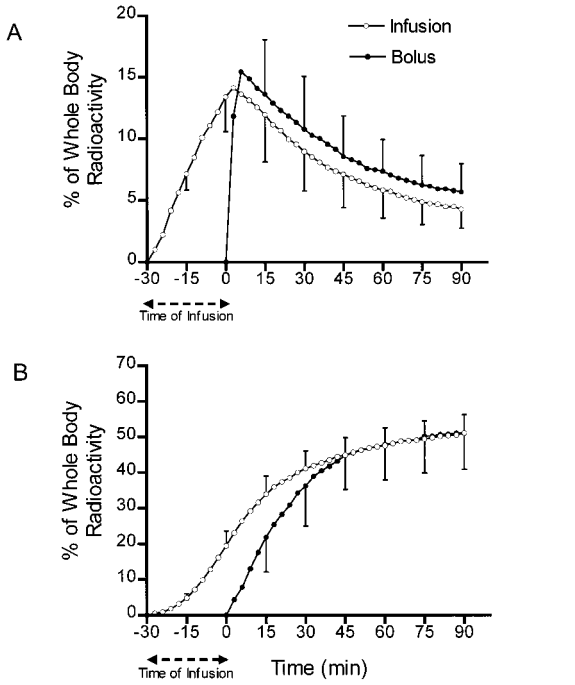

Figure 2—Dynamic changes of [^{131}I]lepirudin in the (A) kidneys and (B) bladder following infusion (open circles) and bolus administration (black circles). The radioactivity is expressed as a mean percentage ± 1 SD of whole body radioactivity at the end of each study.

Table 3—Organ Distribution of [^{131}I]Lepirudin at the End of Treatment

organ	infusion	bolus
kidneys	4.3 ± 1.5	5.7 ± 2.3
bladder	51.2 ± 5.2	51.2 ± 10.3
liver	1.7 ± 0.8	1.8 ± 0.9
spleen	3.6 ± 2.4	3.0 ± 2.7

[a] Percent of whole body radioactivity at the end of each study. Values are given as mean ± 1 SD.

Table 4—ECT of Bile and Duodenal and Gastric Juices, and of the Supernatant of Homogenized Kidney, Liver, and Spleen Tissue[a]

sample	ECT, s
bile	229
gastric juice	49
duodenal juice	39
kidney	77
liver	44
spleen	49

[a] Control values for the ECT method are between 38 and 40 s.

In Vivo Distribution of [^{131}I]Lepirudin—The changes in radioactivity in the kidneys and bladder are summarized in Figure 2, and the in vivo organ distribution at the end of the study in Table 3. Only ≈5% of total injected [^{131}I]-lepirudin was quantified in the liver and the spleen. The results obtained in the sacrificed baboon, 0.1% of the injected radioactivity in the spleen and 1.5% in the liver, also showed that the liver and spleen contained little [^{131}I]-lepirudin. Between 50 and 60% of the injected labeled lepirudin could be accounted for in the bladder. At the end of infusion or shortly after the bolus was injected, the kidneys contained ≈15% of the injected radioactivity (Figure 2A).

The presence of lepirudin in the bile and gastric and duodenal juices of the sacrificed baboon was measured by the ECT method, and the results are shown in Table 4. Of these, only the bile contained appreciable amounts as indicated by the markedly longer than normal ECT results.

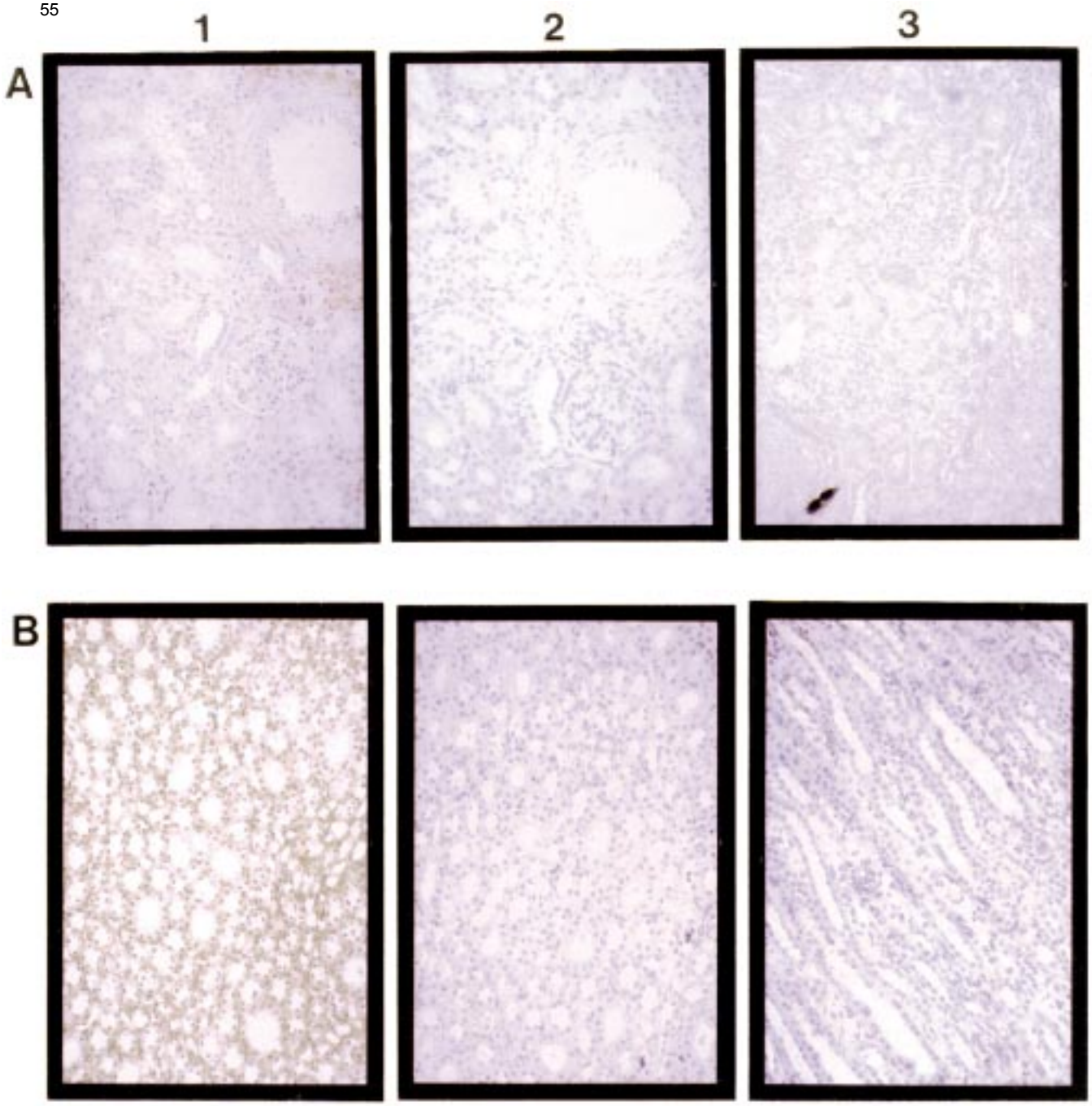

Figure 3—Light micrographs (enlarged 200X) of the (A) renal cortex and (B) medulla. A1 and B1 are from baboons that received lepirudin. A2 and B2 are negative controls where no anti-hirudin antibody was used. A3 and B3 show the renal cortex and medulla of the control baboon that did not receive lepirudin but was treated with anti-hirudin antibody.

The amount of lepirudin excreted by the kidneys was calculated from the total urine volume and the concentration of r-hirudin in urine. In two cases (1 × bolus study and 1 × infusion study), very little urine could be collected. The results are nevertheless given. After infusion of labeled lepirudin, 2.2 ± 1.9 mg of lepirudin was excreted, after the bolus 2.2 ± 1.5 mg was excreted. This amount was ≈54% of the total dose of lepirudin given.

Immunohistochemisty—The results are presented in Figures 3 and 4. The kidney, liver, and spleen of the baboon that was not treated with lepirudin stained negative when the tissues were incubated with the anti-hirudin antibody (Figure 3, A3 and B3, and Figure 4, A3 and B3). This result indicates that these tissues did not contain proteins that interact with the antibody. Kidney, spleen, and liver tissue from baboons treated with lepirudin also stained negative when the tissues were not incubated with the antibody (Figure 3, A2 and B2, and Figure 4, A2 and B2), indicating that the positive staining obtained in the test samples was not a result of the staining procedure.

In the renal cortex, the cytoplasm of the epithelial cells of the convoluted tubules, the cytoplasm of the endothelial cells of the blood vessels, and the connective tissue stained for lepirudin. The glomeruli stained negative (Figure 3, A1). The renal medulla stained intensely. It is likely that lepirudin was present in the epithelium of the thick and thin segments of the loop of Henly and the collecting tubes. The cytoplasm of the endothelial cells of the vas recta and the connective tissues between the cells stained strongly (Figure 3, B1).

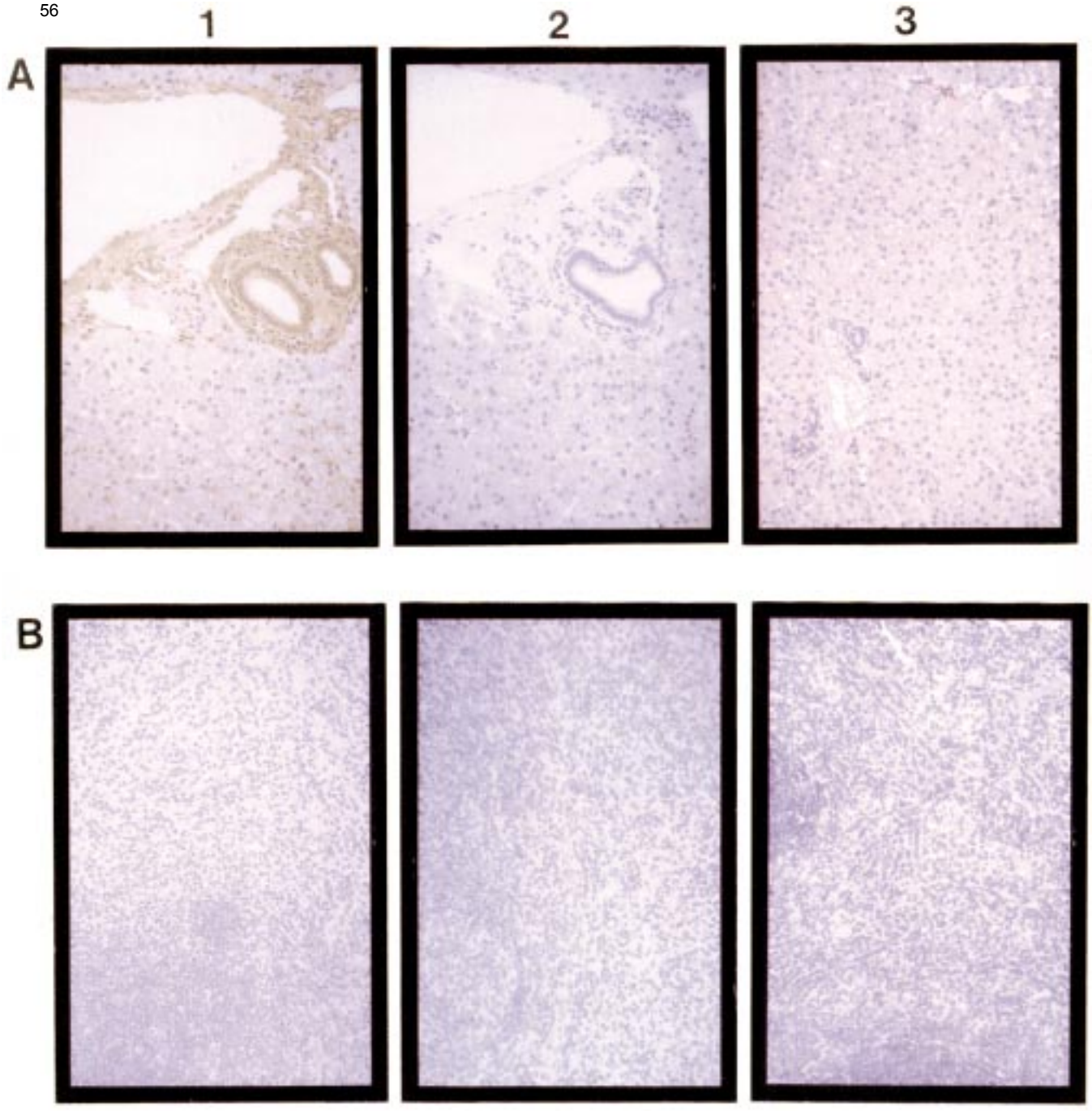

Figure 4—Light micrographs (enlarged 200X) of the (A) liver and (B) spleen. A1 and B1 are from baboons that received lepirudin. A2 and B2 are negative controls where no anti-hirudin antibody was used. A3 and B3 show the liver and spleen of the control baboon that did not receive lepirudin but was treated with anti-hirudin antibody.

The spleen did not stain for lepirudin (Figure 4, A1). On the other hand, staining of liver is ambiguous (Figure 4, B1). The cytoplasm of the hepatocytes did not stain strongly, but the cytoplasm of the endothelial cells of the hepatic artery and portal vein and that of the epithelial cells of the bile duct stained strongly.

Discussion

In a study of this nature, where the pharmacokinetics and mechanism of clearance of a compound labeled with a radioisotope is studied, it is vital that the labeled compound reacts in the same way as the native compound. There are several lines of evidence to show that the behavior of lepirudin, when labeled with ^{131}I, was not markedly affected by the labeling procedure. First, treatment with the labeled lepirudin lengthened the aPTT 3−4-fold (Table 1). In addition, when unlabeled and labeled hirudin, either alone or as a 50/50 mixture, were added to plasma in vitro, the lengthening in aPTT was equivalent. This result indicated that the labeled lepirudin was functional. Second, when the half-life was measured from the radioactive count rate in blood, it was 21 ± 6 min, which was not significantly different from that estimated for lepirudin (24 ± 9 min). These estimates were also not significantly different from that obtained in another study.[5] Third, after 90 min of treatment with labeled lepirudin, no accumulation of ^{131}I was imaged in the thyroid or spleen, both of which rapidly

remove free iodine in plasma.[13] It is therefore reasonable to conclude that labeling of lepirudin with ^{131}I did not adversely affect the function and plasma clearance of lepirudin and that the ^{131}I remained bound to the lepirudin. The results obtained with the labeled lepirudin can therefore be compared with those of lepirudin, and conclusions relating to the pharmacokinetics of lepirudin can be made from the results obtained with labeled lepirudin.

It is evident that lepirudin was mainly excreted by the kidneys (Figures 2A and B). Between 50 and 60% of the injected lepirudin was detected in the bladder 90 min after it was injected (Table 3), which agrees with results obtained in humans and rhesus monkeys.[6,14,15] Because the clearance of lepirudin approximates that of creatinine in humans, it was proposed that excretion be by glomerular filtration.[14,15] We used immunohistochemical techniques in an attempt to verify this proposal (Figure 3). The results were rather confusing and do not confirm glomerular filtration. Glomeruli in the renal cortex did not stain for lepirudin. Tissue surrounding the glomerulus and the blood vessel did stain. Even more surprising was the fact that the tubuli in the medulla, and especially the epithelial cells lining the tubules, stained intensely, which suggests tubular reabsorption and secretion. A well-planned study to investigate glomerular filtration and possible tubular reabsorption and secretion is called for. Such a study will provide definitive answers about the mechanisms by which the kidneys excrete lepirudin.

It is generally accepted that the fraction of lepirudin that is not excreted by the kidneys is distributed in the extravascular compartment.[14] The in vitro results in this study however showed that the liver plays a part in the catabolism of lepirudin and that lepirudin was excreted in the bile. Although we could not quantify appreciable quantities of [^{131}I]labeled lepirudin in the liver (Table 3), immunohistochemical studies showed that lepirudin was present in the hepatocytes and the tissues surrounding the blood vessels and bile ducts. The finding that lepirudin was present in the bile means that it has to be excreted into the digestive system. We explain the absence of lepirudin in the duodenal juices (Table 4) and the finding that lepirudin was not present in the feces of normal humans 24 h after it was administered[15] by digestion and absorption of lepirudin by the enzymes present in the duodenum. It is reasonable to assume that the 40% of administered lepirudin that was not excreted by the kidneys, could be excreted by the liver into the bile. However, a well-planned study is needed to quantify the contribution of the liver to the clearance of lepirudin from plasma. It is further evident that the spleen plays no part in the catabolism of lepirudin (Table 3, Figure 4).

The plasma elimination half-life of lepirudin was 18 ± 4 min when it was injected as a bolus. When the same quantity was infused over 30 min, the estimated half-life was not significantly different (23 ± 8 min). This result is similar to the estimated half-life that we determined in baboons where different dosages of lepirudin were infused over 30 min.[5] The half-lives estimated in the baboons are shorter than the 55−70 min measured in humans, rhesus monkeys, rats, rabbits, and dogs.[6,15−17] We have no easy explanation for this discrepancy.

The total clearance of lepirudin from plasma was ≈45 mL/min, whether the same amount was infused or given as a bolus. Similar results were obtained when clearance was calculated from the radioactive radioactive count rates (Table 2). This value is not very different from that determined in rhesus monkeys,[18] but differs greatly from the results reported for humans, rats, rabbits, and dogs.[6,15] Labeled lepirudin, infused over 30 min, was cleared by the kidneys at a significantly higher rate than that administered as a bolus (Table 2). However, when renal clearance was expressed as a percentage of plasma clearance, 62 ± 16% of infused lepirudin was cleared by the kidneys. The corresponding value for bolus administrated lepirudin, 55 ± 15%, was not significantly different ($p > 0.05$, Student's *t* test for paired data). Thus, although the rate of clearance was different, the relative contribution of the kidneys to plasma clearance was the same. It is important to note that in rhesus monkeys that received a bolus of lepirudin, renal clearance also contributed ≈50% to plasma clearance.[18] These results are similar to those obtained in this study. The method of administration of the labeled lepirudin did not affect the extravascular clearance (Table 2).

In summary, whether 0.3 mg/kg of labeled lepirudin was infused over 30 min or given as a bolus, it did not influence its plasma half-life and clearance. This result suggests that the study was performed with plasma concentrations below the maximum threshold levels of excretion of lepirudin by the kidneys. The mode of administration did not affect the clearance of lepirudin from plasma, nor that by the kidneys or the extravascular compartment. The kidneys were the main sites of excretion of lepirudin, where between 50 and 60% was excreted, probably by tubular secretion. It has been speculated that the remainder may be catabolized by the kidneys and that the methods used to determine lepirudin are unable to recognize the degradation products.[6,14,18,19] The quantification of the percentage labeled lepirudin found in the bladder argues against renal catabolisation. If the labeled lepirudin was catabolized, much more than 50 to 60% of administered labeled lepirudin would have been detected in the bladder at the end of the study. In addition, SDS-PAGE suggested excretion in an unchanged form. A surprising finding was that the bile contained appreciable amounts of lepirudin.

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Prolonged Inhibition of Acute Arterial Thrombosis by High Dosing of a Monoclonal Anti-platelet Glycoprotein IIb/IIIa Antibody in a Baboon Model

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Summary

The in vivo activity of MA-16N7C2, the first monoclonal antibody that contains an echistatin-like RGD-sequence and inhibits platelet glycoprotein (GP)IIb/IIIa function, was determined in baboons. A dose-finding study assessing haemostatic variables such as bleeding time and ex vivo platelet aggregation showed that doses of as low as 0.2-0.3 mg/kg resulted in a pronounced effect. The effects were dose-dependent and lasted for several days, implying that MA-16N7C2 is a potent and long-acting GPIIb/IIIa inhibitor. Following the initial studies, the antithrombotic effect of 0.1 and 0.3 mg/kg of the antibody, given as a bolus, was determined in a baboon model of platelet-dependent, arterial-type thrombus formation. In these studies, a thrombogenic device consisting of Dacron vascular graft material was inserted as extension segments into a permanent arteriovenous shunt. The results confirmed the potent and long-lasting antithrombotic effect of MA-16N7C2. Surprisingly, the antithrombotic effect was stronger 48 h after a dose of 0.3 mg/kg administration than on the day of treatment with 0.1 mg/kg, despite the fact that comparable numbers of GPIIb/IIIa receptors were occupied on resting platelets. We postulate that with the high dose of MA-16N7C2 and after an extended period, occupied GPIIb/IIIa may be internalised by the platelets. Upon platelet activation, these receptors become reexposed but are unable to participate in thrombus formation. This is in contrast to unoccupied internal GPIIb/IIIa receptors early after a low dose of MA-16N7C2.

Introduction

Blood platelets are the first line of host defence when normal blood vessels are injured. Platelet adhesion, aggregation and further platelet recruitment culminate in haemostatic plug formation to prevent excessive bleeding. This is accompanied by the consolidating effect of fibrin(ogen) binding to glycoprotein (GP) IIb/IIIa, the fibrinogen receptor on the platelet membrane. Endothelial cells in the proximity possess thromboregulators that limit the size of the haemostatic plug. However, induction of excessive platelet activation by biomaterials and structures such as an ulcer or fissure in the fibrous cap of atheroma overcomes the normal thromboregulatory mechanisms. The platelets then become a major prothrombotic offender predisposing to arterial vaso-occlusive disease (1-6). Inhibition of platelet function is therefore a major avenue of search for potent antithrombotic agents. The present mainstay of this approach is to develop monoclonal antibodies, peptides and peptidomimetics that can prevent the binding of fibrinogen, through the carboxy terminal dodecapeptide (7) or the Arg-Gly-Asp (RGD) recognition sequence of fibrinogen to GPIIb/IIIa (8-15). Such agents prevent in vitro and ex vivo platelet aggregation and in vivo thrombosis in experimental models and patients (13-17). The major differences amongst the agents are mainly in their affinity for GPIIb/IIIa and in their plasma half-life, i.e. the time they can act as inhibitors of platelet function in vivo (8-16). An intriguing further possibility is that platelets can become loaded with the inhibitor: indeed, evidence has been provided that GPIIb/IIIa is a cycling receptor (18, 19) and as such can internalize not only fibrinogen but also anti-GPIIb/IIIa products into the platelet α-granules (20, 21). Such process would be favored when products are used with a low off-rate, such as high affinity antibodies.

In this study we report on the antithrombotic effects of a murine monoclonal antibody, MA-16N7C2, in a baboon model of platelet-dependent arterial type thrombus formation. MA-16N7C2 inhibits GPIIb/IIIa function and is the first antibody described with an echistatin-like RGD-containing sequence in the CDR3-region of its heavy chain, which however is not fully responsible for the inhibitory effect (22). The antibody furthermore recognises the Ca^{2+}-dependent GPIIb/IIIa complex also on resting platelets, but binding is accelerated and with higher affinity upon platelet activation.

Materials and Methods

Antibody Preparation

Antibody MA-16N7C2 was purified from ascitic fluid from BALB/c mice by protein A Sepharose 4B affinity chromatography in 0.1 M Tris-HCl, pH 8.3. After elution at low pH (0.1 M glycine, pH 2.8), the antibody was dialyzed against PBS and purity was analyzed with SDS-PAGE. Protein content was determined using the Bio-Rad protein assay (BioRad Laboratories, Brussels, Belgium) with bovine gamma globulin as standard.

Species Specificity

Blood from dogs, cats, rabbits, pigs and baboons was anticoagulated with 0.12 vol 145 mM trisodium citrate, whereas for rat, hamster and guinea pig blood, an additional 1 U/ml heparin (Liquemine; Roche, Basel, Switzerland) was added.

Platelet-rich plasma was obtained after centrifugation of the blood for 10 min at 150 × g. Platelet aggregation was performed on PRP with ADP (Sigma Chemicals Co., St. Louis, MO) or collagen (Hormon Chemie, München, Germany) as inducers using the optical method. Gel-filtered platelets were prepared from PRP by chromatography on a 20 × 2.5 cm Sepharose 2B column (Pharmacia, Uppsala, Sweden) using 5 mM Hepes, 0.34 mM Na_2PO_4, 12 mM $NaHCO_3$, 134 mM NaCl, 2.9 mM KCl, 10% ACD, pH 5.5.

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Platelets were counted in a Platelet counter (Cell-Dyn 610; Sequoia-Turner Co., Mountain View, CA) and 200 µl of 150-200.10^3/µl gel-filtered platelets was put into the wells of microtiter plates which had been pretreated with 10 µg/ml poly-L-lysine (100 µl/well). The plates were centrifuged at 4° C for 15 min at 150 g. After removal of the supernatant, the platelets were fixed for 15 min at 25° C with 0.5% (v/v) formalin (50 µl/well). After washing with PBS containing 0.002% (w/v) Tween 80, plates were blocked with 1% bovine serum albumin and next incubated with MA-16N7C2 for 1 h at room temperature. After another washing step, the bound material was detected with rabbit anti-mouse IgG (H + L) coupled to horseradish peroxidase (Nordic Immunology, Tilburg, The Netherlands) diluted 1/3000 in PBS, 0.1% BSA, 0.002% Tween for 1h. Following a final wash, colour development was done using 160 µl of a solution containing O.4 mg/ml 1,2-orthophenylene-diamine (Fluka Chemie, Buchs, Switzerland) in 17 mM citric acid, 65 mM Na_2HPO_4 and 0.003% H_2O_2. The reaction was stopped with 50 µl of 4 M H_2SO_4 and absorption was measured at 442 nm in an ELISA-reader (EAR 40; AT, SLT-Labinstruments, Austria).

Animal Studies

1. Dose-finding studies. Baboons (Papio hamadryas) of either sex, weighing 7-12 kg, were anaesthetized with 10 mg/kg ketamine hydrochloride (Imalgene 500; Sanofi, Toulouse, France) and 0.06 mg/kg atropine i.m. During the first experimental day anaesthesia was maintained with 30 mg pentobarbitol i.v. (Nembutal; Abbott Laboratories, North Chicago, IL) at hourly intervals.

Antibody used for i.v. injection was in PBS, 1 mM $CaCl_2$ and appropriately diluted with saline in order to have an injection volume of 1 ml. The preparation contained less than 29 EU/ml endotoxin (KabiVitrum, Stockholm, Sweden). Samples were taken before and 1 h after injection until a dose was obtained giving a prolongation of the bleeding time of at least 30 min. Subsequent determination of bleeding times and blood collection of samples were carried out until the bleeding time was back into the normal range (≤10 min).

Bleeding times were performed on the shaved volar surface of the arm using an automated template device (Simplate II; General Diagnostics, Morris Plains, N. J.). No pressure cuff was applied. Blood was collected for determination of haematocrit, platelet count, ADP- and collagen induced platelet aggregation, determination of remaining binding sites for MA-16N7C2 and determination of plasma levels of murine monoclonal antibodies.

Determination of remaining binding sites. ^{125}I-labeling of antibody was done using Iodogen-reagent (Pierce Chemical Company, Rockford, IL) as described (22, 23). Remaining binding sites on platelets from baboons treated with antibody, were determined in duplicate by incubating PRP, with platelet counts adjusted to 200,000 pl/µl with autologous plasma, with a saturating concentration (2 µg/ml) of ^{125}I-MA-16N7C2 for 15 min at room temperature. 80 µl of this was then carefully layered over either 200 µl of an oil-layer consisting of 3/4 vol silicone oil (Jansen Chimica, Beerse, Belgium) and 1/4 vol paraffine oil (VEL, Haasrode, Belgium) or over 200 µl 15% sucrose in Eppendorf tubes and centrifuged at 12.000 g for 4 min. The tips, containing the platelet pellet, were cut and platelet-associated radioactivity was measured with a gamma-counter (Packard, Meriden, CT).

Plasma levels. Plasma levels of murine monoclonal antibodies were measured in an ELISA using plates coated with 4 µg/ml affinity-purified (AP)-goat anti-mouse IgG (H + L) antibodies (Jackson Immuno Research, West Grove, PA) and developed with AP-goat anti-mouse IgG coupled to horseradish peroxidase (Jackson Immuno Research, West Grove, PA). Standard curves were constructed by adding known amounts of MA-16N7C2 to baboon plasma.

2. Thrombosis studies. Thirteen normal male baboons (Papio ursinus) were used. The animals weighed between 10 and 15 kg and were disease-free for at least 6 weeks prior to use. All procedures were approved by the Institutional Ethics Committee for Animal Experimentation in accordance with Federal Guidelines (Guide for the Care and Use of Laboratory Animals, Public Health Service National Institute of Health publication number 85-23, 1985). The peripheral blood platelet counts, 282 ± 62 × 10^9/l, were normal. The baboons supported permanent Teflon-Silastic arteriovenous (AV) shunts implanted in the femoral vessels (24). These shunts do not detectably shorten platelet survival or produce measurable platelet activation (24-26).

Study protocol. Platelet dependent, arterial type thrombus formation was induced by Dacron vascular graft material (0.5 cm^2) that was built into the wall of silicone rubber tubing (inside diameter = 4 mm). The method of preparation of the thrombogenic device is described in detail (27).

In each experiment, a thrombogenic device, prefilled with saline to avoid a blood-air interface, was incorporated as an extension segment into the permanent AV-shunt by means of teflon connectors (24). The devices were kept in place for 2 h whereafter they were removed and blood flow through the permanent shunt was reestablished. On the day before the treatment studies (day 0), control studies were done in all baboons. On day 1, the baboons were treated with a bolus injection of 0.3 mg/kg (n = 7) or 0.1 mg/kg (n = 6) of MA-16N7C2 15 min before the devices were placed. Devices were again placed on day 2 and 3. Blood flow rate through the device was 100-140 ml/min, producing wall shear rates similar to that found in medium to large sized arteries (25). The antibody was diluted in saline for injection.

Graft imaging and quantification of platelet deposition. Autologous blood platelets were labeled with ^{111}In-chloride as previously described (28) and reinjected at least one hour before thrombogenic devices were placed on the day the control studies were done. Imaging and quantification of the deposition of ^{111}In-platelets were done as described in detail (24). Image acquisition of the grafts, including proximal and distal silastic segments, was done with a Searle Pho scintillation camera fitted with a high resolution collimator. The images were stored on and analyzed with a Medical Data Systems A^3 computer (Medtronic, Ann Arbor, MI) interfaced with the camera. Dynamic image acquisition, 5 min images (128 × 128 byte mode) for 2 h, was started simultaneous with the start of blood flow through the devices. A five minute image (128 × 128 byte mode) of a 3 ml autologous blood sample was also acquired each time that the grafts were imaged to determine circulating blood radioactivity (blood standard). A region of interest (1 × 1.5 cm) of the graft segment was selected to determine the deposited and circulating radioactivity in the proximal segment of the extension tubing. This activity was substracted from the radioactivity in the graft region to calculate deposited radioactivity. The ratio between deposited ^{111}In-platelet radioactivity and circulating radioactivity in one ml blood (from the blood standard) was calculated. The latter was appropriately corrected for plasma ^{111}In-radioactivity (24). The number of platelets deposited on the graft was calculated by multiplying the ratio with the number of platelets per ml of blood (27).

Laboratory measurements. In each experiment, blood samples were collected before and after exposure of the blood to the thrombogenic device. The platelet count and haematocrit were determined in blood collected in 2 mg/ml disodium EDTA using a Technicon H2 blood cell analyser (Bayer Diagnostics). This sample was also used to correct whole blood radioactivity for plasma radioactivity in order to calculate platelet radioactivity (24).

Blood from MA-16N7C2-treated baboons without inplants was furthermore collected in 3.8% sodium citrate (9 volumes blood to 1 volume citrate) to determine the activated partial thromboplastin time (APTT) and the plasma fibrinogen concentration. APTT (Dade) was measured on a fibrinometer (Clotex II, Hyland) and fibrinogen was indirectly determined with an Automated Coagulation Laboratory (Instrumentation Laboratories). The template bleeding time was measured as described (25).

The in vitro aggregation response to platelets before, 10 min and 24 and 48h after treatment with MA-16N7C2 was measured turbidimetrically in a Monitor IV Plus aggregometer (Helena Laboratories). The method has been fully described elsewhere (29). The aggregation response, measured at 4 min following addition of ADP (20 µM) or collagen (0.05 g/l), was related to the difference in light transmission between platelet-rich and platelet-poor plasma and expressed as a percentage.

The number of GPIIb/IIIa receptors occupied by MA-16N7C2 was calculated as described above. Platelet-rich plasma was incubated with near saturating levels of ^{125}I-MA-16N7C2 and the number of radiolabelled molecules bound per platelet determined.

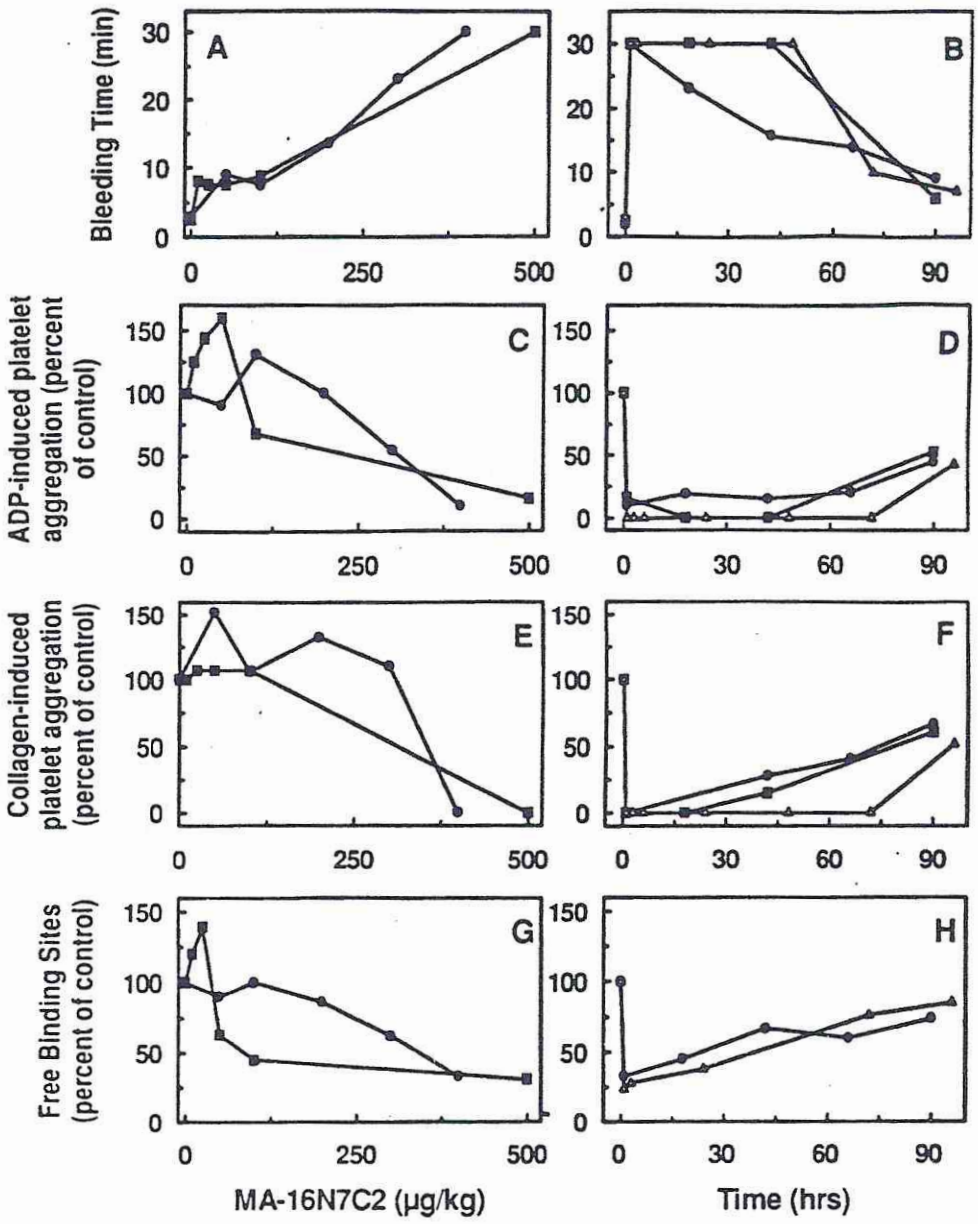

Fig. 1 Bleeding times (A, B), ADP- (C, D) and collagen- (E, F) induced platelet aggregation, and remaining binding sites for ^{125}I-MA-16N7C2 (G, H), as a function of cumulative doses of MA-16N7C2 (left) resulting in a final total dose of 0.4 (●) or 0.5 (■) mg/kg, and as a function of time after administration of a total dose of 1 (Δ), 0.5 (■) or 0.4 (●) mg/kg MA-16N7C2 (right) to baboons

The number of receptors occupied by the monoclonal antibody was finally obtained by subtracting the number of labelled molecules bound to the platelets after infusion of antibody from that obtained before infusion.

Statistical analysis. Student's test (two-tailed) was used to test for differences when the data were normally distributed. Data in the text are given as the mean ± SD.

Results

1. Dose-finding Studies

Preliminary studies indicated that MA-16N7C2 did not bind to platelets or inhibit platelet aggregation when platelets from dogs, cats, pigs, rabbits, rats, hamsters, mice or guinea pigs were used. Platelets from baboons on the other hand clearly did bind and were inhibited by the antibody. We therefore used this species to evaluate the in vivo potential of the antibody.

First a dose finding study was done, the results of which are represented in Fig. 1. In the initial experiment a bolus injection of 1 mg/kg MA-16N7C2 was given, in the second and third, consecutive doses at one hour intervals were administered. Following these injections, the studies on the baboons were continued until bleeding times returned into the normal range.

I.v. administration of MA-16N7C2 had profound, dose-dependent and long-lasting effects on bleeding times and platelet aggregability. Full recovery from the total dosage of 0.4 mg/kg, 0.5 mg/kg and 1 mg/kg required about 90 h, with some dose-dependent variation. Platelet counts never dropped below 150.000/µl. An overall significant correlation was found between the number of remaining binding sites and the extent of aggregation induced by ADP ($r = 0.85$; $p < 0.001$), collagen ($r = 0.74$; $p < 0.001$) and the bleeding times ($r = -0.83$; $p < 0.001$). However, normalization of bleeding times and collagen-induced aggregation was faster following 0,4 mg/kg than following 1 mg/kg MA-16N7C2 administration, despite parallel recovery of remaining binding sites.

Based on estimations of 65 ml blood volume/kg, 15% of the platelets being sequestered in the spleen (29) and the determinations of platelet count and number of remaining binding sites in pre- and 1 h post-injection samples, one can calculate that 77, 75, 63, 66, 69, 40 and 47% of injected antibody became bound to the platelets when 0.05, 0.1, 0.2 0.3, 0.4, 0.5 and 1.0 mg/kg MA-16N7C2 was administered respectively. This apparent saturation of platelet receptors was reflected in the peak plasma levels of murine antibodies measured 1 h after injection: low to undetectable plasma levels of murine antibody persisted until platelet binding sites were nearly saturated, at which point the plasma levels increased significantly.

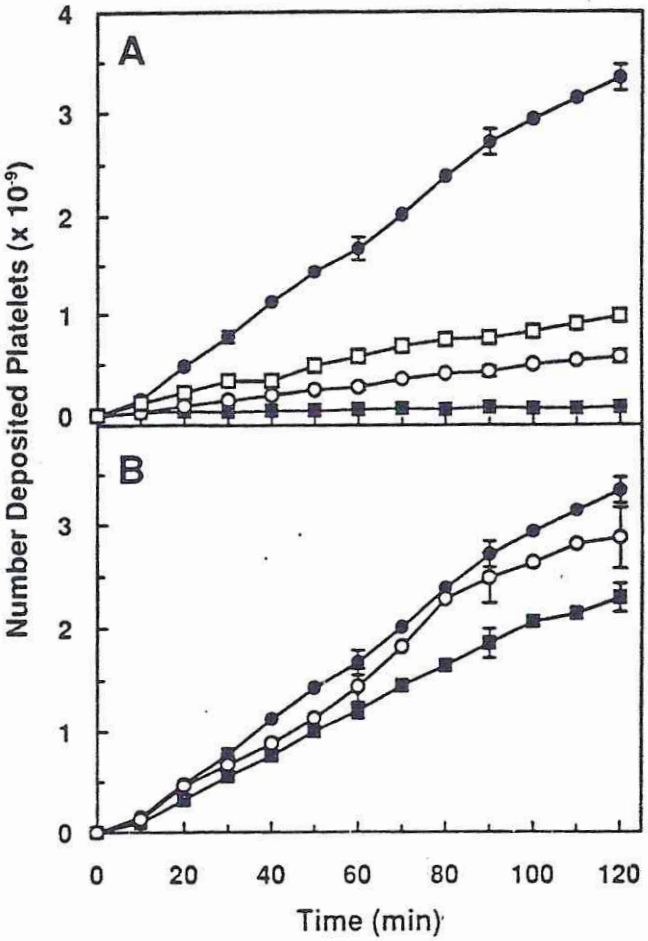

Fig. 2 Platelet depositions over a 2 h period onto Dacron vascular grafts incorporated into a permanent AV-shunt in baboons before (●), 15 min (□), 24 h (○) and 48 h (■) after IV-injection of 0.3 mg/kg (A) or 0.1 mg/kg (B) MA-16N7C2

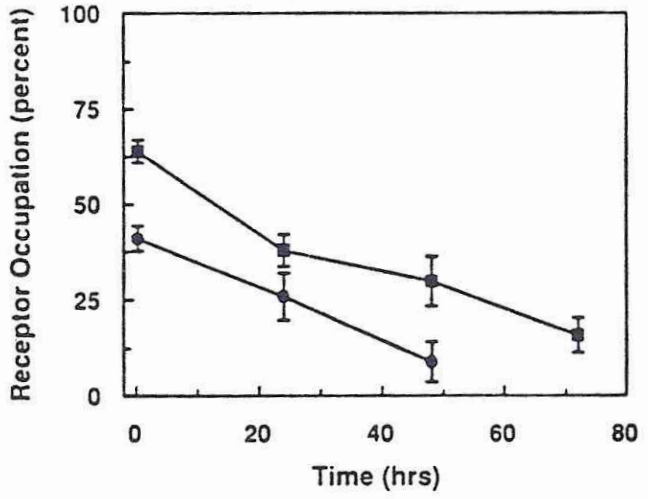

Fig. 3 Percentage occupied binding sites for ^{125}I-MA-16N7C2 on baboon platelets as a function of time following IV-administration of 0.3 mg/kg (■) or 0.1 mg/kg (●) of MA-16N7C2

2. *Thrombosis Studies*

Platelet deposition. Platelet deposition onto the thrombogenic device is shown in Fig. 2. In the control studies, platelets were rapidly deposited. After two hours of exposure of the flowing blood to the thrombogenic surface, 3.34 ± 0.34. 10^9 platelets were deposited. Treatment with 0.3 mg/kg MA-16N7C2 virtually abolished deposition on the day of treatment and the antibody still resulted in marked inhibition after 24 and 48 h (Fig 2A). After 24 h, deposition was still inhibited by approximately 83% vs control after two hours of placement of the thrombogenic device. The corresponding inhibition after 48 h was 71%. Treatment with 0.1 mg/kg antibody was not as effective as treatment with 0.3 mg/kg (Fig. 2B). On the day of treatment, deposition was inhibited by approximately 31% 2 h following placement of the device. The corresponding inhibition after 24 h was approximately 14%.

Platelet receptor occupation. Ten minutes following a bolus infusion of 0.3 mg/kg of MA-16N7C2, approximately 64% of the GPIIb/IIIa receptors were occupied by the antibody (Fig. 3). Occupation decreased almost linearly, reaching a value of approximately 16% 72 h after treatment. Extrapolation of the decrease of receptor occupation suggested that the antibody remained platelet bound for up to 61 h after injection. Treatment with 0.1 mg/kg MA-16N7C2 blocked approximately 41% of the GPIIb/IIIa receptors 10 min after injection. The decrease that followed also fitted a linear function best, and the estimate of antibody bound to the platelet was approximately 41 h.

Bleeding time and ex vivo platelet aggregation. The bleeding time was lengthened ten minutes following injection of 0.3 mg/kg MA-16N7C2, but returned to normal values 24 h later (Table 1).

MA-16N7C2 inhibited the ex vivo aggregation of platelets in response to ADP and collagen and these effects were dose-dependent (Table 1). The inhibitory effect also lasted for some time following injection, i.e. in the case of the high dose, normal aggregation was obtained approximately 72 h following treatment. In the case of the low dose, the aggregation response returned to normal 24 h following treat-

Table 1 Bleeding time and the ex vivo platelet aggregation response before and following treatment with 16N7C2 (ND = not done)

Measurement	Dose	Time				
		Pre	15 min	24 hours	48 hours	72 hours
Bleeding time (min)	0.3 mg/kg	2 ± 0.2	20 ± 7	5 ± 1	4 ± 1	3 ± 0.5
Ex vivo platelet aggregation response						
ADP	0.3 mg/kg	100	0	52 ± 10	83 ± 17	100
(20 μM)	0.1 mg/kg	100	75 ± 16	100	ND	ND
Collagen	0.3 mg/kg	100	21 ± 0.2	52 ± 11	74 ± 17	100
(0.05 g/l)	0.1 mg/kg	100	68 ± 19	100	ND	ND

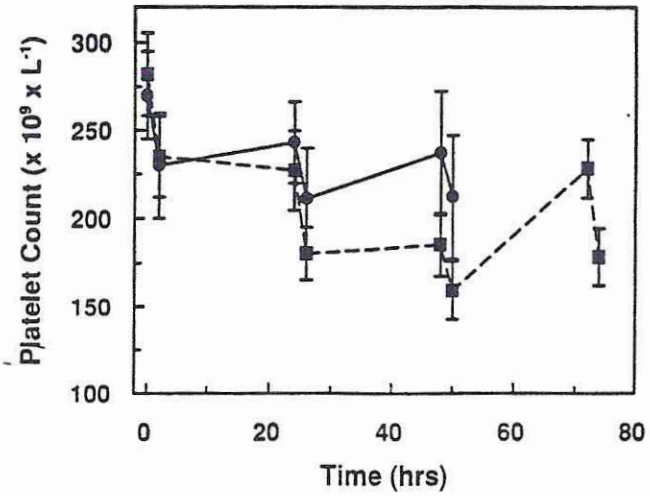

Fig. 4 Change in peripheral platelet counts in baboons treated with 0.3 mg/kg (■) or 0.1 mg/kg (●) of MA-16N7C2 as a function of time before and after placement of the thrombogenic device at 0, 24, 48 and 72 h

ment. The estimated half-life of inhibition of ADP-induced aggregation by 0.3 mg/kg antibody was approximately 12 h. The corresponding half-life of inhibition of collagen-induced aggregation was approximately 10 h.

Platelet count. The changes in platelet count as a result of the placement of thrombogenic devices and between placements are given in Fig. 4. In all studies, the platelet count decreased as a result of placement of devices irrespective of the presence of the antibody. The decrease ranged from 11 to 22%. The platelet count between placements of devices dropped reaching a minimum 48 h after antibody administration.

Coagulation measurements. Neither treatment with MA-16N7C2 nor placement of the thrombogenic devices affected the APTT or plasma concentration of fibrinogen markedly.

Discussion

In this study, we assessed the in vivo activity of the first described anti-GPIIb/IIIa monoclonal antibody with an echistatin-like RGD-peptide containing sequence (22) in baboons. The initial part of the study was done to determine the effects of different intravenous doses of MA-16N7C2 on GPIIb/IIIa-receptor occupation, bleeding time and the ex vivo aggregation of platelets in response to ADP and collagen. In the second part, the antithrombotic potential of MA-16N7C2 was assessed in a baboon model of platelet-dependent arterial-type thrombus formation.

Intravenous infusion of MA-16N7C2 caused a dose-dependent lengthening of the bleeding time and inhibition of ex vivo platelet aggregation, which took several days to recover. Plasma levels of murine monoclonal antibodies only started to increase when 60% to 70% of available GPIIb/IIIa-receptors were occupied, confirming a high affinity of the antibody for its target (22). In addition, the disappearance of MA-16N7C2 from the plasma preceded normalisation of the bleeding time, return of platelet aggregability and recovery of the number of binding sites on the platelet membrane.

The relationship between the number of GPIIb/IIIa receptors available after treatment with MA-16N7C2 and the inhibition of ex vivo platelet aggregation is in agreement with results obtained upon administration of 7E3, another anti-GPIIb/IIIa antibody, to humans (16): when approximately 70% of receptors are occupied, ex vivo platelet aggregation in response to ADP is abolished. 30-40% receptor occupancy results in an attenuated aggregation, whereas blocking 15-25% of the receptors did not affect aggregation.

As was observed with other anti-GPIIb/IIIa antibodies, such as 7E3, LJ-CP-8 and AP-2 when infused in humans or baboons (12, 14, 31), we also here could observe that bleeding times normalised before ADP-induced platelet aggregation. This implies that detection of an abnormal ex vivo aggregation response not necessarily predicts abnormal platelet haemostatic function in vivo (12).

A bolus infusion of MA-16N7C2 inhibited platelet-dependent thrombus formation in a dose- and time-dependent manner. Maximum inhibition was observed on the day of injection of 0.3 mg/kg MA-16N7C2. Following this dose, platelet deposition was still markedly inhibited 48 h later. Similarly, when LJ-CP-8 and AP-2 were studied in a comparable model, platelet deposition was markedly inhibited shortly after treatment and returned progressively to control values over the next 48 h (12, 27, 31). However, in the case of these antibodies, much larger doses were required to obtain equivalent effects. The effect of MA-16N7C2 therefore seems to be more powerful and longer lasting than that of the above mentioned antibodies.

In the dose-finding studies, administration of MA-16N7C2 caused a decrease of about 60% in the blood platelet count, which reached a nadir after approximately 48 h. The decrease in platelet number due to the placement of the thrombogenic device in untreated animals, was also found in the animals that received MA-16N7C2. This is in contrast with results obtained when baboons were treated with a thromboxane receptor blocker (BAY U3405, 27), a thrombin inhibitor (D-Phe-Pro-Arg chloromethylketone, 32, 33), a factor Xa inhibitor (rTAP, 34) and more particularly with other monoclonal anti-GPIIb/IIIa antibodies (12). The reason for this discrepancy is at present unclear. The decrease in the platelet number strongly suggests that platelets were consumed by the thrombogenic surface. However, in view of the relatively few platelets that were deposited, especially on the day of infusion of 0.3 mg/kg, one must conclude that the turnover rate of deposited platelets was high.

In contrast to other studies using anti-GPIIb/IIIa antibodies, we here could relate differences in platelet function to receptor occupation. The decrease in receptor occupation following the bolus injection fitted a linear function best, and extrapolation of these curves suggest that MA-16N7C2 remained platelet bound for between 41 and 61 h. Furthermore, the differences in inhibition of platelet deposition following 0.3 and 0.1 mg/kg administration in relation to GPIIb/IIIa receptor occupation are noteworthy. For example, on the day of injection of 0.1 mg/kg approximately 41% of receptors were occupied with MA-16N7C2, which resulted in about 31% inhibition of platelet deposition. In contrast, 48 h following injection of 0.3 mg/kg, receptor occupation was approximately 31%, while platelet deposition was inhibited by about 71%.

The reason for this difference in inhibition at similar levels of receptor occupation is not clear. A possible explanation may well be the interchange between extra- (50-80%) and intraplatelet (40-20%) pools of GPIIb/IIIa (18,19) and uptake of anti-GPIIb/IIIa antibodies and desintegrins into the intraplatelet pool (20, 21). With the high dose of MA-16N7C2, initially up to 65% of the external receptors were occupied. Over an extended time period, e.g. 48 h, these receptors can interchange with unoccupied internal receptors. Since receptor occupation was measured on unstimulated platelets, only the surface exposed GPIIb/IIIa are detected. However, when platelets become activated during thrombus formation, the intracellular pool of GPIIb/IIIa is

expresssed on the surface (35, 36). In the case of a high dose of antibody and after an extended period, the GPIIb/IIIa receptors are to some extent blocked and thus unable to contribute to platelet deposition.

When on the other hand only 0.1 mg/kg MA-16N7C2 was given and thrombus formation is measured shortly thereafter, i.e. before significant GPIIb/IIIa cycling can occur, an unaffected, functionally active pool of internal receptors became exposed. As a result, a larger thrombus formed. This assumption is further strengthened by the difference in recovery patterns obtained following infusion of 1 and 0.4 mg/kg MA-16N7C2: the number of free available GPIIb/IIIa-receptors recovered in parallel, whereas normalisation of the bleeding time, collagen-induced aggregation, and to a lesser extent ADP-induced aggregation was faster following a dose of 0.4 mg/kg than after a dose of 1 mg/kg.

An alternative hypothesis is that platelet α-granules may become loaded with antibody, which then would be secreted upon platelet activation, in a manner comparable to the normal mechanism of fibrinogen uptake and secretion. This hypothesis is strengthened by observation that transfusion of platelet concentrates from dogs 48 h after treatment with an i.v. bolus injection of 0.8 mg/kg 7E3-Fab, to untreated dogs, still prevented occlusion of an everted (inside out) carotid arterial segment that had been inserted in a transsected femoral artery (37).

In conclusion, our results confirmed the antithrombotic action of an anti-GPIIb/IIIa monoclonal antibody. We could also show that a putative "loading" of internal GPIIb/IIIa receptors can result in a higher than expected antithrombotic action. Whether the same holds true for smaller GPIIb/IIIa antagonists awaits further study.

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Antithrombotic Effect of Platelet Glycoprotein Ib–Blocking Monoclonal Antibody Fab Fragments in Nonhuman Primates

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Abstract—Platelet adhesion in arterial blood flow is mainly supported by the platelet receptor glycoprotein (GP) Ib, which interacts with von Willebrand factor (vWF) that is bound to collagen at the site of vessel wall injury. Antibody 6B4 is a monoclonal antibody (MoAb) raised against purified human GPIb. MoAb 6B4 inhibits both ristocetin- and botrocetin-induced, vWF-dependent human platelet agglutination. MoAb 6B4 furthermore blocks shear-induced adhesion of human platelets to collagen I. We studied the antithrombotic effect of this inhibitory murine MoAb 6B4 in a baboon model of arterial thrombosis. When injected into baboons, intact IgG and its $F(ab')_2$ fragments caused almost immediate thrombocytopenia, whereas injection of the Fab fragments alone did not. Fab fragments were subsequently used to investigate their in vivo effect on platelet deposition onto a thrombogenic device, consisting of collagen-rich, glutaraldehyde-fixed bovine pericardium (0.6 cm^2), at a wall shear rate ranging from 700 to 1000 s^{-1}. Baboons were either pretreated with Fabs to study the effect of inhibition on platelet adhesion or treated 6 minutes after placement of the thrombogenic device to investigate the effect on interplatelet cohesion. Pretreatment of the animals with bolus doses ranging from 80 to 640 μg/kg Fab fragments significantly reduced ^{111}In-labeled platelet deposition onto the collagen surface by ≈43% to 65%. Only the highest dose caused a significant prolongation (doubling) of the bleeding time. Ex vivo ristocetin-induced platelet agglutination was equally reduced. Treatment with a bolus of 110 μg/kg Fab fragments after a thrombus was allowed to form for 6 minutes had no effect on further platelet deposition. We therefore conclude that Fab fragments or derivatives of inhibitory anti-GPIb antibodies may be useful compounds to prevent thrombosis. **(*Arterioscler Thromb Vasc Biol*. 2000;20:1347-1353.)**

Key Words: platelet adhesion ■ platelet aggregation ■ thrombosis ■ glycoprotein Ib ■ monoclonal antibodies

Blood platelets, through the processes of adhesion, activation, shape change, the release reaction, and aggregation, form the first line of defense when blood vessels are damaged. Platelets form a hemostatic plug at the site of injury to prevent excessive blood loss. Extensive platelet activation, however, may overcome the normal thromboregulatory mechanisms that limit the size of the hemostatic plug. The platelets then become major prothrombotic offenders predisposing to vaso-occlusive disease.[1,2]

Platelet adhesion is regarded as the trigger for hemostasis and thrombosis. When subendothelial collagen is exposed at the site of vessel injury, circulating von Willebrand factor (vWF) binds to it and, under the influence of arterial blood flow, undergoes a conformational change enabling it to bind to its receptor, glycoprotein (GP) Ib, on the platelet membrane.[3] As a result, motion of the platelets on the collagen surface is decreased, which may then be followed by firm adhesion mediated by other collagen receptors.[4] After the initial adhesion, platelets will aggregate, an interaction that is assured through the binding of GPIIb-IIIa complexes with multivalent ligands, in particular, vWF and fibrinogen.[5] The platelet GPIb receptor is thus important for initiating a thrombus at the site of the exposed lesion. Abnormalities in GPIb, as seen in Bernard-Soulier syndrome, result in deficient platelet attachment to a site of vascular injury and a predisposition to clinical bleeding.[6]

Lately, much effort has been directed to develop antibodies and peptides that can block the binding of the adhesive proteins to GPIIb-IIIa, and many of these are being tested in clinical trials.[7–9] On the other hand, the development of compounds that interfere with the vWF-GPIb axis has lagged behind. Only a few in vivo studies that investigated the effects of inhibition of platelet adhesion on thrombogenesis are described. They include the use of anti-vWF monoclonal antibodies (MoAbs),[10–12] GPIb-binding snake venom proteins like echicetin[13] and crotalin,[14] aurintricarboxylic acid

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that binds to vWF,[15] and recombinant vWF fragments like VCL,[16–18] all of which inhibit vWF-GPIb interaction. All of these studies showed the potential feasibility of antithrombotic therapy based on inhibition of the GPIb-vWF axis.

A number of potent, inhibitory anti-GPIb antibodies have been produced and were extensively tested with respect to their in vitro effect under both static (platelet agglutination, vWF binding) and flow conditions.[19] We are aware of only 2 successful in vivo studies on guinea pigs, in which $F(ab')_2$ fragments of PG-1, a monoclonal anti–guinea pig GPIb antibody, were used.[20,21] These fragments were shown to significantly prolong the time to arterial microvascular graft thrombosis without prolonging the bleeding time.[21] In another guinea pig model, the fragments could effectively reduce thrombus formation on a laser-induced injury.[20] However this PG-1 antibody is specific for guinea pig platelets and does not cross-react with human platelets. Part of this rather surprising lack of in vivo studies is due to the low cross-reactivity of the anti-human GPIb MoAbs with platelets from commonly used laboratory animals. This situation necessitates the use of nonhuman primates as experimental animals. However, even then, attempts to perform in vivo studies are hampered because injection of the anti-GPIb MoAbs, as well as the snake venom protein echicetin that reacts with GPIb, invariably causes severe thrombocytopenia.[10,13,22,23]

We have studied the antithrombotic efficacy of a novel murine MoAb, 6B4 (IgG1), raised against human platelet GPIb, in in vitro and in vivo studies. In vitro, 6B4 and its $F(ab')_2$ and Fab fragments all potently inhibited the binding of vWF to human GPIb under both static and flow conditions, and they also bound to baboon platelets. In the in vivo studies in which 6B4 was injected into baboons, the intact MoAb caused immediate and severe thrombocytopenia; injection of its $F(ab')_2$ fragments resulted in a moderate decrease in platelet count, whereas the Fab fragments did not have a major effect on platelet count. Furthermore, the Fab fragments were studied in a baboon model of platelet-dependent arterial thrombosis. Platelet deposition onto collagen-rich bovine pericardium was inhibited when the fragments were injected into the baboons before a thrombus was generated. On the other hand, when the Fab fragments were injected after a thrombus was allowed to form, no inhibition of further thrombosis was observed.

Methods

Preparation and Purification of Intact MoAb 6B4, $F(ab')_2$, and Fab Fragments

The antibody used in these studies, 6B4 (subtype IgG1), is a murine MoAb raised against purified human GPIb. GPIb was purified from outdated platelet concentrates collected by the Belgian Red Cross in Leuven, Belgium, according to the method described by Wicki and Clemetson.[24] BALB/c mice were immunized by intraperitoneal injection with the purified GPIb. Murine antibodies were prepared by conventional hybridoma technology as previously described,[25] and generated hybridoma cells were screened for antibody production in an ELISA by using purified GPIb. Hybridoma cells producing anti-GPIb MoAbs were grown and subsequently injected into pristane-primed BALB/c mice. After 10 days ascites fluid was collected. The IgG was extracted from the ascites by using protein A–Sepharose CL-4B (Pharmacia). These antibodies were screened for inhibition of ristocetin-induced human platelet aggregation as described below. MoAb 6B4 IgG totally abolishes both the ristocetin- and the botrocetin-induced aggregation of human platelet-rich plasma (PRP).

To prepare $F(ab')_2$ fragments, MoAb 6B4 was dialyzed overnight against a 0.1 mol/L citrate buffer (pH 3.5). The antibody was digested by incubation with pepsin (Sigma Chemical Co; 1 part pepsin to 200 parts MoAb) for 1 hour at 37°C. Digestion was stopped by adding 1 volume of a 1 mol/L Tris-HCl buffer (pH 9) to 10 volumes of antibody solution.

Monovalent Fab fragments were prepared by papain digestion. A 1:10 (vol/vol) solution of 1 mol/L phosphate buffer (pH 7.3) was added to the antibody. Papain (Sigma) was added at a ratio of 1 volume papain to 25 volumes of the phosphate buffer containing the MoAb, 10 mmol/L L-cysteine-HCl (Sigma), and 15 mmol/L EDTA. After incubation for 3 hours at 37°C, digestion was stopped by adding freshly prepared iodoacetamide solution (Sigma) to a final concentration of 30 mmol/L, which was then kept in the dark at room temperature for 30 minutes.

Both $F(ab')_2$ and Fab fragments were further purified from contaminating intact IgG and Fc fragments by using protein A–Sepharose. The purified fragments were finally dialyzed against PBS. The purity of the fragments was determined by SDS–polyacrylamide gel electrophoresis, and the protein concentration was measured by using bicinchoninic acid protein assay reagent A (Pierce Chemical Co).

Further Antibody Characterization

MoAb 6B4 binds to a (His1–Val289) recombinant GPIbα (rGPIbα) fragment expressed by Chinese hamster ovary cells,[26] indicating that its epitope is localized within the amino-terminal region of GPIbα. MoAb 6B4 Fabs were further tested for inhibition of ristocetin- and botrocetin-induced binding of vWF to the rGPIbα fragment by ELISA.[27] Microtiter plates were coated with 5 μg/mL MoAb 2D4 for 48 hours at 4°C. MoAb 2D4, an anti-GPIb MoAb that we produced, binds to the rGPIbα fragment but does not block vWF binding. Nonabsorbed sites were blocked with 3% skimmed milk, whereafter the plates were washed with Tris-buffered saline containing 0.1% Tween 20. Purified rGPIbα fragments were immobilized on MoAb 2D4 by incubating 2 μg/mL rGPIbα for 2 hours at 37°C. After being washed with Tris-buffered saline–Tween 20, increasing concentrations of 6B4 Fab fragments diluted in Tris-buffered saline–Tween 20 were added, followed by 1.25 or 0.6 μg/mL purified human vWF, respectively, when ristocetin (300 μg/mL) or botrocetin (0.5 μg/mL) was used as a modulator. Binding of vWF was determined by incubating the resulting mixture for 1 hour with horseradish peroxidase–conjugated polyclonal anti-vWF antibody (Dako), diluted 1/3000 in Tris-buffered saline–Tween 20. The color reaction, stopped by addition of 4 mol/L H_2SO_4, was generated with *o*-phenylenediamine (Sigma). Ristocetin was supplied by abp, and purified vWF was purchased from the Red Cross, Belgium. The purification of botrocetin from crude *Bothrops jararaca* venom (Sigma) was performed as previously described.[28]

The effect of 6B4 Fab on shear-induced platelet adhesion to collagen was tested in a Sakariassen-type parallel-plate flow chamber at shear rates of 650, 1300, and 2600 s^{-1} as previously described.[29] Human collagen type I (Sigma) was dissolved in 50 mmol/L acetic acid (1 mg/mL), dialyzed for 48 hours against PBS, subsequently sprayed onto plastic Thermanox coverslips, and stored at room temperature overnight before use. Twelve milliliters of blood anticoagulated with low-molecular-weight heparin (25 U/mL; Clexane, Rhône-Poulenc Rorer) was preincubated with 6B4 Fab fragments at 37°C for 5 minutes. This was then used to perfuse the collagen-coated coverslips. After 5 minutes of perfusion, the platelets were fixed with methanol and the coverslips stained with May-Grünwald/Giemsa. Platelet adhesion (percent of total surface covered with platelets) was evaluated with a light microscope connected to an image analyzer. An average of 30 fields per coverslip were analyzed. Platelet adhesion was expressed as percent maximal platelet adhesion obtained in the absence of inhibitor.

Animal Studies

Normal male baboons (*Papio ursinus*) were used. The animals weighed between 10 and 15 kg and were disease-free for at least 6 weeks before the experiments. All procedures were approved by the Ethics Committee for Animal Experimentation of the University of the Free State in accordance with the National Code for Animal Use

in Research, Education, Diagnosis and Testing of Drugs and Related Substances in South Africa.

The baboons had permanent polytetrafluoroethylene (Teflon)–silicone rubber (Silastic) arteriovenous shunts implanted in the femoral vessels.[30] Blood flow through the shunts varied between 100 and 120 mL/min, resulting in wall shear rates between 700 and 1000 s^{-1}, which compares well with the shear rates found in medium-size arteries.[30] Handling of the baboons was achieved through anesthesia with ketamine hydrochloride (≈10 mg/kg IM; Anaket-V, Centaur Laboratory).

Study Protocol

To test the effect of the MoAb on platelet count, 6B4, its $F(ab')_2$ fragments, or its Fab fragments were administered to 3 different baboons. The plasma volume was calculated by assuming a blood volume of 65 mL/kg body mass and correcting for the hematocrit.[31] Platelet-dependent arterial thrombus formation was induced by using bovine pericardium (0.6 cm^2 fixed in buffered glutaraldehyde).[32] The pericardium was built into the wall of Silastic tubing (3-mm inside diameter). The method of preparation of the thrombogenic device has been described in detail,[31] except that fixed bovine pericardium was used instead of Dacron vascular graft material. In each experiment, a thrombogenic device prefilled with saline to avoid a blood-air interface was incorporated as an extension segment into the permanent arteriovenous shunt by means of Teflon connectors.[30] Two approaches were followed to determine the effect of 6B4 Fab fragments on platelet adhesion and interplatelet cohesion onto the collagen of the bovine pericardium.

Dose-Response Effect of 6B4 Fab Fragments on Platelet Adhesion and Deposition

Seven baboons were used in the first studies; in total 13 perfusion experiments were performed. In the first 5 experiments (3 baboons), a thrombogenic device was placed to determine the deposition of platelets (see the section below on graft imaging and quantification of platelet deposition). After 30 minutes, the device was removed and blood flow through the permanent arteriovenous shunt reestablished. Fifteen minutes after removal of the device, each baboon was treated with a bolus of 80 μg/kg 6B4 Fab fragments (in 2 mL saline), and 15 minutes after treatment, a second thrombogenic device was placed for 30 minutes to determine the effect of the Fab fragments on thrombogenesis. The device was again removed and blood flow through the permanent shunt established. This step was followed by a second bolus injection of Fab fragments (80 μg/kg) to attain a cumulative dose of 160 μg/kg. After 15 minutes, a third thrombogenic device was placed for 30 minutes and platelet deposition was measured. In 4 other experiments (2 baboons), the same study protocol was used, except 2 doses of 320 μg/kg were administered. Sham studies were done in 4 other experiments (2 baboons). In these studies, the same protocol of placement of thrombogenic devices was followed, but the baboons were not treated with Fab fragments.

Effect of Anti-GPIb 6B4 Fragments on Interplatelet Cohesion

In a second series of 12 experiments, 6 baboons were used. In all baboons, a thrombogenic device was placed for 24 minutes. In 6 experiments (3 baboons), the baboons received a bolus injection of Fab fragments of 110 μg/kg. The fragments were injected 6 minutes after placement of the thrombogenic device to allow enough platelets to be deposited to cover the collagen surface. In the other 6 experiments (3 baboons), the baboons did not receive any Fab fragments.

In both approaches, blood was collected at different time points to determine platelet count and hematocrit (EDTA), circulating and platelet-associated radioactivity, the ex vivo aggregation of platelets in response to ristocetin, and the plasma concentrations of Fab fragments (see the section on laboratory measurements). The time points at which the blood was collected are given in the figures.

Graft Imaging and Quantification of Platelet Deposition

Autologous blood platelets were labeled with ^{111}In-tropolone,[33] and imaging and quantification of the deposition of the ^{111}In-labeled platelets were done as described in detail.[30,31] In brief, image acquisition of the grafts, including proximal and distal Silastic segments, was done with a large-field-of-view scintillation camera fitted with a high-resolution collimator. The images were stored on and analyzed with a Medical Data Systems A^3 computer (Medtronic) interfaced with the scintillation camera. Dynamic 2-minute image acquisition was started simultaneously with the start of blood flow through the devices. A 2-minute image (128×128-byte mode) of a 3-mL autologous blood sample collected in EDTA was also acquired each time that the grafts were imaged to determine circulating blood radioactivity (blood standard). A region of interest of the graft segment was selected to determine the deposited and circulating radioactivity in each of the dynamic images. Radioactivity in a region of similar size in the proximal segment of the extension tubing was determined and subtracted from the radioactivity in the graft region to calculate deposited radioactivity. Platelet deposition was expressed as the total number of platelets deposited. The method to calculate this has been described in detail.[30]

Laboratory Measurements

Receptor Binding Studies

6B4, its $F(ab')_2$ fragments, or Fab fragments were labeled with $Na^{125}I$ (Amersham) by using the Iodo-Gen method.[34] Iodo-Gen was purchased from Pierce. Baboon PRP, adjusted with autologous plasma to a count of 100 000 platelets/μL, was incubated with different concentrations of iodinated 6B4, $F(ab')_2$ fragments, or Fab fragments for 15 minutes at room temperature. The mixture was layered onto 20% sucrose buffer (wt/vol) containing 0.1% (wt/vol) BSA and centrifuged for 4 minutes at 10 000*g* in Eppendorf tubes. The top fluid, including the plasma, was removed and the pellets were counted in a gamma counter. Binding studies were performed in duplicate on the PRP of 2 baboons.

In Vitro and Ex Vivo Platelet Aggregation

The aggregation of platelets in response to ristocetin (ABP) was done on 10 mL of blood collected in 1 mL of 3.2% trisodium citrate. PRP was prepared by differential centrifugation[35] and the platelet count adjusted to 200 000 platelets/μL with autologous plasma. The aggregation response was measured in a Monitor IV Plus aggregometer (Helena Laboratories) and recorded for 5 minutes. The percent aggregation at 5 minutes was calculated as the difference in light transmission between PRP and platelet-poor plasma.

In in vitro studies, the PRP was preincubated for 5 minutes with serial dilutions of intact IgG 6B4, $F(ab')_2$ fragments, or Fab fragments before aggregation was initiated. Inhibition of aggregation was calculated from the difference in the aggregation response of platelets without and with antibody or fragments. In the ex vivo determinations, inhibition was calculated from the difference in the aggregation response of platelets before and after treatment of the baboons.

Plasma levels of 6B4 Fab fragments were measured with a sandwich ELISA. In brief, microtiter plates were coated overnight at 4°C with 5 μg/mL polyclonal goat anti-mouse IgG (Sigma). After unoccupied binding sites were blocked with BSA, serial dilutions of baboon plasma were added to the wells and incubated for 2 hours. Bound 6B4 Fab fragments were detected by using goat anti-mouse IgG (Fab-specific) conjugated to peroxidase (Sigma) and developed by using *o*-phenylenediamine as described above. Standard curves were constructed by adding known amounts of 6B4 Fab fragments to baboon plasma.

Bleeding Time

The bleeding time was determined by using the Simplate II device (Organon Teknika) according to the instructions of the manufacturer. The volar surface of the forearm of the baboons was shaved, and a pressure cuff was applied and inflated to 40 mm Hg.

Statistical Analysis

Student's *t* test for paired data was used to test for statistically significant differences. Data given in the text are mean±SE. Probability values <0.05 were considered statistically significant.

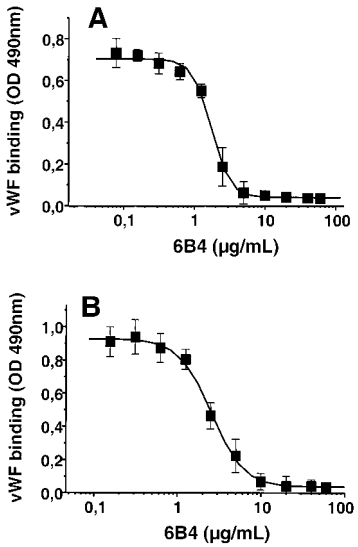

Figure 1. Semilog plots showing inhibitory effect of anti-GPIb 6B4 Fab fragments on ristocetin- (0.3 mg/mL, A) or botrocetin- (0.5 μg/mL, B) induced vWF binding to an rGPIbα (His1–Val289)-fragment. The rGPIbα fragment (2 μg/mL) was immobilized on MoAb 2D4 (5 μg/mL) coated onto microtiter plates. vWF (1.25 or 0.6 μg/mL, respectively, when binding was induced by ristocetin or botrocetin) was added together with increasing concentrations of 6B4 Fab fragments. vWF binding was detected. Data are mean±SD (n=3). OD indicates optical density.

Results

In Vitro Effect of 6B4 Fab Fragments on Human Platelets

MoAb 6B4 Fab fragments blocked the ristocetin- (1 mg/mL) and botrocetin- (0.5 μg/mL) induced human platelet agglutination, with an IC_{50} of 1.2±0.3 μg/mL (24±6 nmol/L) and 2.0±0.5 μg/mL (40±10 nmol/L), respectively. For the intact 6B4 IgG, these values were 0.3 μg/mL (2.0 nmol/L) and 0.8 μg/mL (5.3 nmol/L), respectively. 6B4 binds to an epitope localized on the amino-terminal part (His1–Val289) of GPIbα. The inability of 6B4 to bind to denatured GPIb during Western blotting (not shown) strongly suggests that this antibody binds to a conformation-sensitive epitope on GPIbα. The 6B4 Fab fragments dose-dependently inhibited both the ristocetin- (300 μg/mL) and botrocetin- (0.5 μg/mL) induced binding of vWF to the (1–289) rGPIbα fragment (Figure 1), with an IC_{50} of 1.8 μg/mL (36 nmol/L) and 2.5 μg/mL (50 nmol/L), respectively.

The 6B4 Fab fragments inhibited platelet adhesion to human collagen type I in a concentration-dependent manner at shear rates of 650, 1300, and 2600 s^{-1} (Figure 2), with a 50% reduction of surface coverage obtained at a concentration of 3.5 μg/mL (70 nmol/L), 1.1 μg/mL (22 nmol/L), and 0.5 μg/mL (10 nmol/L), respectively.

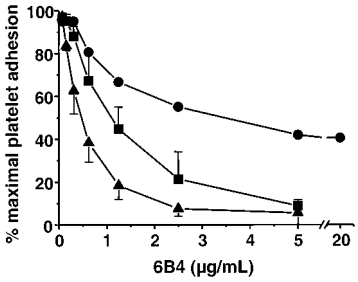

Figure 2. Effect of anti-GPIb 6B4 Fab fragments on shear-induced adhesion of human platelets to human collagen I–coated coverslips. Blood was perfused at a constant shear rate of 650 s^{-1} (●, n=2), 1300 s^{-1} (■, n=3), and 2600 s^{-1} (▲, n=3) for 5 minutes. Data are mean±SD and are expressed as percent of maximal platelet adhesion relative to control value in the absence of 6B4 Fab (35%, 33%, and 29%, respectively, at 650, 1300 and 2600 s^{-1}).

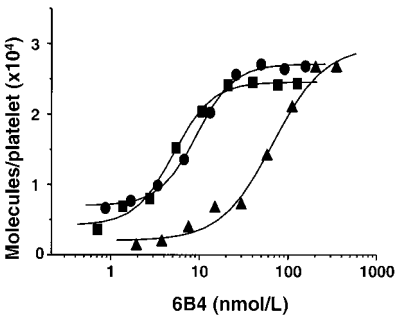

Figure 3. Semilog binding curves of anti-GPIb ^{125}I-6B4 IgG (■), ^{125}I-F(ab′)$_2$ (●), and ^{125}I-Fab fragments (▲) to baboon platelets in plasma. Data are mean of duplicate measurements in PRP from 2 baboons.

In Vitro Effect of MoAb 6B4 and Its F(ab′)$_2$ and Fab Fragments on Baboon Platelets

Binding of the antibody and its fragments to baboon platelets was dose dependent and saturable. Half saturation (K_{D50}) was obtained with 4.7 nmol/L for 6B4 IgG, 6.4 nmol/L for F(ab′)$_2$, and 49.2 nmol/L for Fab (Figure 3). At saturating concentrations, ristocetin-induced aggregation was completely abolished (Figure 4). The IC_{50} of platelet aggregation was obtained at 4.5 nmol/L, 7.7 nmol/L, and 40 nmol/L for 6B4 IgG, F(ab′)$_2$, and Fab fragments, respectively.

Effect of Injection of MoAb 6B4, F(ab′)$_2$, and Fab Fragments on Peripheral Platelet Count in Baboons

In 1 baboon, 100 μg/kg intact antibody caused a profound decrease in the blood platelet count ($<30\times10^9$/L) within 10 minutes after injection. After 48 hours, the platelet count was still $<100\times10^9$/L. When 100 μg/kg 6B4 F(ab′)$_2$ fragments was injected into 2 baboons, the platelet count decreased rapidly to between 120 and 150×10^9/L, ie, by ≈60%, but then reached preinfusion values within 24 hours. Finally, when 80 to 320 μg/kg of the monovalent 6B4 Fab fragments was injected, the platelet count (45 minutes after injection) decreased by only ≈10% to 20% and by only 26% when 640

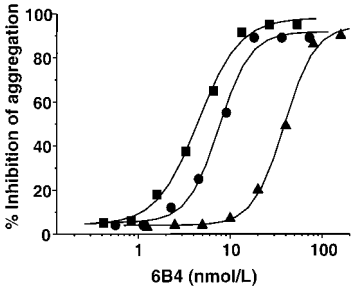

Figure 4. Semilog plot showing effect of anti-GPIb 6B4 IgG (■), F(ab′)$_2$ (●), and Fab fragments (▲) on ristocetin-induced baboon platelet aggregation. Data, expressed as percent inhibition, are mean of duplicate measurements in PRP from 2 baboons.

Platelet Counts, Plasma Levels of 6B4 Fab Fragments, Ex Vivo Ristocetin-Induced Platelet Agglutination, and Bleeding Times After 6B4 Fab Administration

Dose, μg/kg	Time, min	Platelet Counts ×10³/μL	Platelet Counts % Decrease	Plasma Levels, μg/mL	% Inhibition of Ex Vivo Ristocetin-Induced (1.5 mg/mL) Platelet Agglutination	Bleeding Times, s
n=5						
0	Pre	307±32	0	0.07±0.03	0	190±20
80	90	272±22	11	1.72±0.14	26±9	160±33
160	150	248±19	19	4.84±0.56	47±12*	250±45
	270	315±31		0.45±0.09	8±3	ND
n=4						
0	Pre	283±23	0	0.02±0.01	0	232±42
320	90	219±10	23	9.13±0.48	25±21	340±63
640	150	210±13	26	15.35±1.38	80±9*	405±45*
	270	238±20	16	1.19±0.09	15±9	ND
	24 h	236±13	17	0.04±0.01	7±3	ND

ND indicates not determined. Administration of 80–640 μg/kg 6B4 Fab fragments to baboons was performed. Values are given as mean±SE.

Statistical comparisons were made by Student's *t* test for paired sample groups: *$P<0.05$.

μg/kg was injected (the Table). On the basis of this result, the 6B4 Fab fragments were used for further studies.

Effect of Different Doses of MoAb 6B4 Fab Fragments on Platelet Deposition

Platelet adhesion and deposition onto thrombogenic devices sequentially placed 30 minutes apart are summarized in Figure 5. In the sham studies (Figure 5A), placement of the previous graft had no significant effect on platelet deposition that formed on subsequent grafts.

In the treatment studies (Figure 5B), dosages of 80 and 160 μg/kg (2×80 μg/kg 6B4 Fab) significantly inhibited platelet deposition in comparison with control by ≈43% and 53%, respectively. Doses of 320 and 640 μg/kg (2×320 μg/kg 6B4 Fab) significantly reduced platelet deposition by 56% and 65%, respectively.

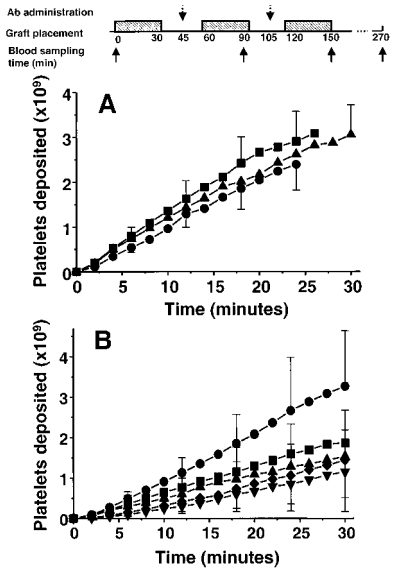

Figure 5. Platelet deposition onto 3 thrombogenic devices containing bovine pericardium placed consecutively at times 0 (●), 60 (■,♦), and 120 (▲,▼) minutes for 30 minutes (top shaded bars). A, Sham experiments (n=4). B, After injection of 0 (●) (n=9), 80 (■), and 160 (▲) μg/kg 6B4 Fab fragments (n=5) or 320 (♦) and 640 (▼) μg/kg (n=4). Values are given as mean±SE.

Plasma levels of 6B4 Fab fragments and inhibition of ex vivo agglutination determined on samples obtained 45 minutes or 2 hours after administration changed in both a dose- and time-dependent manner (the Table). Ex vivo ristocetin-induced platelet aggregation was significantly inhibited at doses of 160 and 640 μg/kg. Both ristocetin-induced platelet aggregation and plasma values returned to baseline within 3 hours after antibody injection.

Bleeding times determined in the treatment studies before and 45 minutes after injection of 80 to 320 μg/kg 6B4 Fab fragments were not significantly prolonged. Only a dose of 640 μg/kg significantly prolonged the bleeding time.

Effect of 6B4 Fab Fragments on Interplatelet Cohesion

Treatment of the baboons with 110 μg/kg 6B4 Fab did not affect platelet deposition when the animals were injected after a thrombus was allowed to form for an initial 6 minutes (Figure 6).

Discussion

The initial step in platelet adhesion consists of vWF binding to GPIb. We investigated the effect of inhibiting this interaction on platelet function both in vitro and in vivo. A murine MoAb, 6B4, that binds to a conformational epitope in the amino-terminal part of GPIbα was used. The antibody and its fragments potently inhibited the binding of vWF to an rGPIbα fragment (His1–Val289) and dose-dependently inhibited vWF-dependent human platelet agglutination. The intact antibody and its fragments also dose-dependently inhibited human platelet adhesion to type I collagen in a flow chamber at wall shear rates of 650, 1300, and 2600 s^{-1}. This inhibition was shear dependent, ie, more pronounced at higher shear.

Of interest was the finding that 6B4 did not react with platelets from dogs, hamsters, pigs, or guinea pigs but did

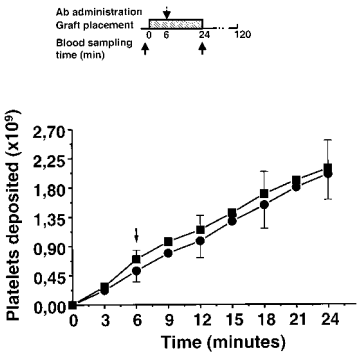

Figure 6. Influence of late treatment of baboons with 6B4 Fab fragments on platelet deposition. The thrombogenic device was placed at time 0 and platelet deposition determined for 24 minutes (top shaded bar). After 6 minutes (arrow), animals were either left untreated (■) or treated with a bolus of 110 (●) μg/kg 6B4 Fab fragments (n=6). Values are given as mean±SE.

bind to baboon platelets with much the same characteristics as to human platelets. As a result, baboons were used for in vivo and ex vivo studies. Both the intact antibody as well as its $F(ab')_2$ fragments caused immediate thrombocytopenia, similar to what was seen when other anti-GPIb MoAbs were injected into different experimental animals.[10,22,23] On the other hand, the Fab fractions had only a moderate effect on the blood platelet count, and we therefore decided to use the Fab fractions to assess the antithrombotic effect of 6B4 in a baboon model of arterial thrombosis.[30] The glutaraldehyde-fixed bovine pericardium was highly thrombogenic: after 30 minutes of exposure to native flowing blood, $\approx 3\times10^9$ platelets deposited on the area of 0.6 cm^2. In similar studies, only $\approx 0.7\times10^9$ platelets accumulated in 30 minutes on Dacron vascular graft material (0.9 cm^2).[31] It is therefore also not surprising that a number of control thrombogenic devices became occluded before 30 minutes of exposure to flowing blood.

Treatment of baboons with 6B4 Fab fragments inhibited platelet deposition on the thrombogenic devices by 43% to 65%. The observed effect must be ascribed to the effect of the antibody, because sequential placement of thrombogenic devices in untreated baboons caused no decreased deposition. No complete inhibition of platelet deposition was observed, even at high doses.

It is possible that this incomplete inhibition was due to the medium shear rates (700 to 1000 s^{-1}) used in this study. In general, in flow chambers a more effective inhibition with vWF-GPIb inhibitors is obtained at higher shear rates,[11,12,36] which we also demonstrated here by finding a more pronounced effect of 6B4 on platelet adhesion to collagen when a higher shear was applied in a flow chamber; also, in vivo an arterial thrombus was more readily prevented than a venous one.[11] This implies that inhibition of GPIb would result especially in arterial effects, which in addition could result in less bleeding risk.

The doses used indeed caused a significant reduction in thrombus size without apparently increasing the risk of bleeding since bleeding time was only mildly prolonged at the highest dose. This finding supports data obtained with other GPIb-vWF–blocking agents (VCL, anti-vWF MoAb AJvW-2) that lengthened the bleeding time only moderately.[11,12,17,18] This finding is important and might provide a major advantage in the development of antithrombotic agents compared with GPIIb-IIIa antagonists, like ReoPro.[37] On the other hand, the GPIb-vWF interaction in contrast to the GPIIb-IIIa–fibrinogen interaction is the ultimate first step in platelet adhesion under fast blood flow. Because binding of vWF to GPIb also activates platelets,[38,39] it is reasonable to assume that by inhibiting vWF-GPIb binding, fewer platelets will be activated. The smaller thrombus that finally forms may therefore be a consequence of both fewer platelets that adhere to collagen and less platelet aggregation. Thus, where GPIIb-IIIa blockers mainly prevent platelet aggregation, interruption at an earlier stage by a GPIb blocker is expected not only to limit the platelet plug that is formed but also to reduce additional platelet-dependent effects, such as granule release, thought to play a role in the development of arteriosclerosis and restenosis.[40,41]

There are also indications that the GPIb-IX-V complex is involved in platelet-platelet interactions. Ruggeri et al[42] recently reported that blocking the GPIb-vWF interaction, after platelets from PPACK-anticoagulated blood had adhered to bovine collagen in vitro for 100 seconds at 1500 s^{-1}, prevented further thrombus growth measured after another 740 seconds, even at low shear rates that do not normally initiate vWF-dependent platelet adhesion. To test this concept in vivo, we performed a second series of studies to investigate the role of GPIb in platelet-platelet interactions at intermediate shear rates. A thrombogenic device was placed as an extension segment in the permanent arteriovenous shunt and exposed to native flowing blood. After 6 minutes the baboons were treated with the Fab fractions of 6B4. We postulated that a 6-minute exposure (number of platelets deposited was $\approx 0.6\times10^9$) was sufficient to allow ample coverage of the pericardium with adhering platelets. Inhibition of platelet deposition due to treatment, when compared with sham studies, would therefore reflect inhibition of platelet-platelet interactions. Because no such effect was seen, it strongly suggested that GPIb does not play a major role in in vivo platelet-platelet interactions under the conditions used in this study.

In conclusion, we have reported on the first anti-human GPIb antibody that can be used successfully to prevent platelet adhesion and thrombus formation in vivo, thereby confirming the predominant role of GPIb in platelet adhesion in vivo. Our studies, however, do not support the hypothesis that GPIb also plays a part in platelet-platelet interactions in vivo. On the basis of our results on bleeding times and the inhibition of thrombogenesis, we propose that the Fab fragments or derivatives of the anti-GPIb MoAb 6B4 may be useful compounds in preventing arterial thrombosis in those patients in whom thrombosis is expected, ie, after vascular engraftment, endarterectomy, or balloon catheterization.

Acknowledgments

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Animal Models

The humanized anti-glycoprotein Ib monoclonal antibody h6B4-Fab is a potent and safe antithrombotic in a high shear arterial thrombosis model in baboons

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Summary

The Fab-fragment of 6B4, a murine monoclonal antibody targeting the human platelet glycoprotein (GP) Ibα and blocking the binding of von Willebrand factor (VWF), is a powerful antithrombotic. In baboons, this was without side effects such as bleeding or thrombocytopenia. Recently, we developed a fully recombinant and humanized version of 6B4-Fab-fragment, h6B4-Fab, which maintains its inhibitory capacities *in vitro* and *ex vivo* after injection in baboons. We here investigated the antithrombotic properties, the effect on bleeding time and blood loss and initial pharmacokinetics of h6B4-Fab in baboons. The antithrombotic effect of h6B4-Fab on acute platelet-mediated thrombosis was studied in baboons where thrombus formation is induced at an injured and stenosed site of the femoral artery, allowing for cyclic flow reductions (CFRs) which are measured on an extracorporeal femoral arteriovenous shunt. Injection of 0.5 mg/kg h6B4-Fab significantly reduced the CFRs by 80%, whereas two extra injections, resulting in cumulative doses of 1.5 and 2.5 mg/kg, completely inhibited the CFRs. Platelet receptor occupancy, plasma concentrations and effects *ex vivo* were consistent with what was previously observed. Finally, minimal effects on bleeding time and blood loss, no spontaneous bleeding and no thrombocytopenia were observed. We therefore conclude that h6B4-Fab maintains the antithrombotic capacities of the murine 6B4-Fab, without causing side effects and therefore can be used for further development.

Keywords

Antithrombotic, glycoprotein Ib, humanized antibody, platelet adhesion, thrombosis

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Introduction

Platelets are a key factor in the maintenance of normal haemostasis (1). However, in some pathological situations, such as stroke or myocardial infarction, vessel damage and enhanced shear rates cause platelet activation and thrombus formation leading to vessel occlusion. The initial stage of this process depends on the binding of platelet glycoprotein Ibα (GPIbα) to von Willebrand factor (VWF), bound to the collagen matrix exposed to the blood flow as a consequence of the vessel damage.

Previously, we prepared and characterized a murine monoclonal antibody (mAb) against human GPIbα, designated as 6B4. This mAb inhibits VWF-dependent platelet adhesion to collagen under high shear-stress conditions, as was shown in flow chambers (2). Injection of 6B4-Fab-fragments has a potent antithrombotic effect *in vivo* in baboons (3, 4) but also strongly inhibits ristocetin-induced platelet aggregation *ex vivo* (3). In contrast to most antithrombotic drugs, but in line with observations with other experimental compounds that interfere with the collagen-VWF-GPIb axis (5), 6B4-Fab administration does not induce a significant prolongation of the bleeding time. The epitope recognized by 6B4 was first mapped using chimera human/dog rGPIbα to reside within the C-terminal flanking region, between residues 201 and 268 of GPIbα (6). More recently, by using computer docking and mutagenesis we further could identify Asp235 and Lys237 as dominant epitope residues, whereas the paratope of 6B4 is mainly formed by residues Tyr31, Lys32, Asp33 and Glu98 of the light chain CDR1 and 3 respectively and Tyr106 of the heavy chain CDR3 (7). From these data, it is clear that 6B4 directly competes with the VWF A1-domain binding

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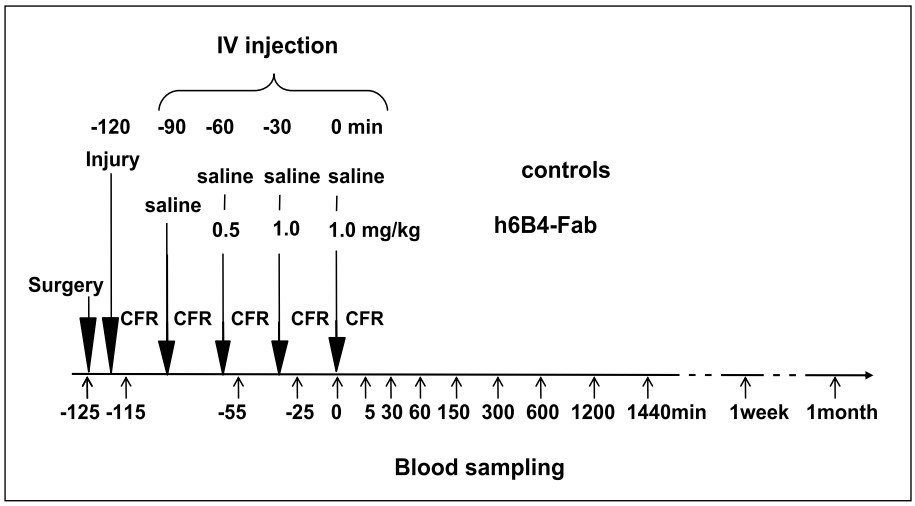

Figure 1: Time schedule for the study. The first blood sample is drawn before the start of surgery. Blood flow is recorded 15 min before damaging the artery (injury). The first series of CFRs is recorded for 30 min (–120 to –90). During this period the second blood sample is drawn (-115 min). Saline is next injected and CFRs are recorded (-90 to –60). After the saline control, consecutive doses of h6B4 Fab or saline are administered to treated or control animals respectively and CFRs are recorded for 30min, (-60 to -30, -30 to 0 and 0 to 30 min). Each time a blood sample is drawn 5 min later, whereas consecutive samples are obtained at 30, 60, 150, 300, 600, 1200, 1440 min, 1 week and 1 month to determine full blood count, ristocetin-induced platelet aggregation, receptor occupancy and plasma concentration.

site within GPIbα (8, 9). As a further step in the development of 6B4, we prepared a fully recombinant and humanized version of the 6B4-Fab-fragment, named h6B4-Fab (10). This molecule has inhibitory capacities *in vitro* and *ex vivo* equivalent to the parental murine 6B4-Fab-fragment, and with a comparable Kd of 0.8 ± 0.1 x 10^{-8} (10).

In the present study, we validated the antithrombotic properties of h6B4-Fab in a baboon thrombosis model by studying the antiplatelet effects in the injured and stenosed femoral artery under high-shear stress conditions (Roodt et al., submitted) and monitored the bleeding tendency and platelet counts.

Materials and methods

Production and purification of murine and humanized recombinant 6B4-Fab fragments

The murine and humanized 6B4-Fab-fragments were produced by transient expression with the FreeStyle™ 293 Expression System (Invitrogen, Carlsbad, CA, USA) with the 293 FreeStyle™ cells as previously described. Briefly, cells were cultured to obtain 1.1x10^6 cells per ml on the day of the transfection. The cells were pelleted before resuspension in fresh, pre-warmed FreeStyle™ 293 Expression medium. Plasmid DNA coding for the recombinant 6B4-Fab fragment was diluted into OptiMEM® in the presence of 293fectin™. After incubation for 30 minutes (min) at room temperature to allow the DNA-293fectin™ complexes to form, the mixture was added to the cell suspension. The transfected suspension was further incubated for 120 hours (h) at 37°C. The cells were discarded and the secreted 6B4-Fab-fragments were dialyzed, concentrated and purified by ion-exchange chromatography, first on a Phenyl Sepharose fast flow column (Pharmacia Biotech, Uppsala, Sweden) followed by a second purification on a Q-Sepharose Fast Flow column, (Pharmacia Biotech) to remove contaminants. 6B4-Fab-fragments present in the flowthrough were next dialyzed against PBS and their concentration determined. The molecules were kept at –20°C before use.

Animal study

Housing, treatment, surgery and care for the animals used in the study was approved by the Control Committee on Animal Experimentation of the University of the Free State, South Africa. Seven healthy baboons (*Papio hamadryas*) of either sex, weighing between 8 to 13 kg, were used in this study. Animals were anaesthetized with ketamine (0.1 ml/kg IM/30 min) prior to surgery. Dissection was made on the leg to expose 4–5 cm of the femoral vessels. All nearby branches in the femoral artery and vein were ligated. Next a small incision in both artery and vein was made in which a catheter was inserted. To the catheter tips, silicone tubing was connected, thus shunting the femoral artery to the femoral vein. This setup increases blood flow 3– to 5-fold, yielding flow rates of between 150 and 300 ml/minute. Blood flow was measured by a probe (Transonic systems TS410, probe: ME3PXL1OO8) attached on the silicone tubing. After a latency phase of 15 min to stabilize the flow, the endothelium of the femoral artery was injured proximally to the vessel tip with a Martin needle holder (Hegar-Baumgartner TC Gold 14cm Product code 20.634.14) by pressing on the vessel for 10 seconds at maximum depression. Two overlapping injuries are made.

Arterial stenosis was next applied by adjusting a plastic constrictor device over the injury site to reduce the flow to 30 ml/min (80 to 90% stenosis). Injury and stenosis resulted in thrombus formation in the femoral artery, detected as a reduction in the flow. When blood flow reached almost baseline, the thrombus was dislodged by opening the constrictor. After this, the external stenosis was restored inducing a new thrombus formation. This repeated process results in cyclic flow reductions (CFR's). Baseline CFR's were recorded for 30 min. Saline was injected and CFR's were monitored for a further 30 min which served as an internal control. In five baboons, the effect of a first injection of 0.5 mg/kg of h6B4-Fab, followed by two injections of 1 mg/kg of h6B4-Fab separated by a 30-min interval, on the CFR's was monitored each time for 30 min. Control baboons (n=2) were injected with saline instead. Blood samples were drawn at set times (Fig. 1) to determine full blood count, GPIbα receptor occupancy, bleeding time, blood loss, platelet aggregation *ex vivo* induced by ristocetin and h6B4-Fab plasma levels. As a control

compound, Clopidogrel (Plavix™), purchased from Bristol-Myers Squibb/Sanofi Pharmaceuticals (New York, NY, USA), was pulverised and suspended in methanol (150 mg/ml). This solution was further diluted in saline to a concentration of 10 mg/ml and passed through a 0.22 μm filter. Oral administration does not allow the performance of baseline measurements or the use of stepping doses and as the active metabolite was not available, we decided to follow the procedure described by Yao et al . (11), which was done with the co-operation of the Sanofi Research department.

Platelet count and blood loss

The full blood count including platelet numbers was evaluated before the surgery, 5 min after each bolus injection of h6B4-Fab, and 30 min, 1, 2.5, 5, 10, 20, 24 h and one week after the last injection. As described elsewhere (10), blood samples were collected in 3.13% trisodium citrate and measured on a Technicon H3 blood cell analyzer (Bayer AG, Leverkusen, Germany). Parameters related to bleeding tendency were monitored in two ways: the skin template bleeding time was determined at the surface of the shaved forearm as the time needed from the skin incision to cessation of bleeding, as previously described (10). In addition, we evaluated the blood loss during 30 min from a standardized incision of 2 cm long and 0.8 cm deep, made by using a Swann-Morton #15 (Roodt et al, submitted). Pre-weighed and standardized gauzes were inserted in the wound and replaced after 30-min periods. Blood loss was calculated by subtracting gauze weight before and after use, which was more straightforward and more reproducible than measuring haemoglobin. Results were expressed as ratio of the saline control to account for variation among individuals.

Ristocetin-induced platelet aggregation

Platelet aggregation *ex vivo* was investigated as previously described (10). Briefly, a baboon platelet suspension was prepared at $300x10^3$ platelets/μl by mixing platelet-rich plasma (PRP) and autologous platelet-poor plasma (PPP) prepared by differential centrifugation from citrated blood. Platelet aggregation was induced by ristocetin A (ABP corp., Marlton, NJ, USA) and was performed at 37°C, 1,000 rpm on a C560CA aggregometer (Chrono-log corp, Halfway House, South Africa). A higher concentration of ristocetin (3 mg/ml) than used with human platelets (1 mg/ml) was needed to make sure that platelets of all baboons would agglutinate. The extent of aggregation was estimated quantitatively by measuring the curve amplitude, and expressed in percentage of the value from the pretreatment sample.

Receptor occupancy

GPIbα receptor occupancy by h6B4-Fab-fragment was estimated using flow cytometry by competition with a fixed concentration of biotinylated 6B4 (6B4-b) (home made, [10]) in combination with streptavidin/phycoerythrin (PE) (BD Biosciences, Erembodegem, Belgium) as described previously. Baboon platelets ($7x10^6$), prepared from citrated blood, were first incubated with 10 ng of 6B4-b followed by 5 μl of streptavidin/PE. Both incubations were done for 15 min at room temperature. Samples were finally fixed with 0.2% formaldehyde, 0.9% NaCl before determining platelet-associated fluorescence by flow cytometry (FACSCalibur™, BD Biosciences). The percentage of 6B4-b bound to baboon platelets was calculated, with 100% set in the absence of h6B4-Fab. Receptor occupancy was determined by subtracting the percentage of labeled molecules bound to the platelets after treatment with h6B4-Fab from that obtained before treatment.

Plasma concentration

h6B4-Fab plasma concentrations were determined in an ELISA assay as described previously (10). Briefly, rGPIbα was captured by mAb 2D4 precoated on an ELISA plate. Baboon plasma was then added in a dilution series to the wells. After 1.5 h incubation at RT, unbound proteins were removed and bound h6B4-Fab, was detected with Fab-specific goat anti-human IgG HRP-labeled (Sigma, St.Louis, MO, USA) and color development by addition of H_2O_2 and orthophenylenediamine (OPD, Sigma). The reaction was stopped with H_2SO_4 before optical density determination (490–630 nm) on a microplate reader EL340 (Biotek instrument, Winooski, VT, USA). The plasma concentration of h6B4-Fab in each sample was calculated using exponential increase first order equations from standard curves. These standard curves were constructed by in-vitro addition of known concentrations of h6B4-Fab to baboon plasma.

Statistical analysis

The different parameters tested were compared by the Student t-test and the differences were considered to be statistically significant when $p < 0.05$.

Results

Haematological parameters and thrombocytopenia

The full blood count and platelet number were evaluated before the surgery, 5 min after each bolus injection of h6B4-Fab, but also 30 min, 1, 2.5, 5, 10, 20, 24 h, one week and one month after the last injection. The white blood cells (WBC) increased from 6.2 ± 0.3 10^3/μl up to 14.7 ± 0.6 10^3/μl with a predominance of neutrophils (Table 1); however, they were almost back to normal after one day. This increase was also observed in the controls (and in baboons treated with other drugs, J Roodt unpublished data) albeit to a somewhat lesser extent, implying that the increase is likely due to the surgical procedure.

No marked thrombocytopenia was observed throughout the experiment (Table 1). A small decrease (max. 30%) in the platelet count was observed in both treated and control animals near the end of the thrombosis study which would indicate that a non-negligible platelet consumption occurs in the model. Later on, platelet counts increased and normalized within a day.

Antithrombotic effect: CFR inhibition

After the surgical procedure the CFRs were recorded for 30 min to ascertain stable periods. Injection of saline had no effect on the CFRs compared to the control before injection. On average 10,0 CFRs were recorded during the 30-min period with a standard deviation of 2.9 (n= 8), indicating that both the damage induced to the vessel and the artificial stenosis applied, resulted in a reproducible model. In contrast, administration of 0.5 mg/kg

Table 1: White blood cell and platelet counts in baboons treated with saline or h6B4-Fab in function of time. Values are range (saline n=2) and mean ± SEM (h6B4-Fab n= 5). Percentage of platelet count is between brackets. * statistically different p<0.05 t-test for paired sample groups.

Time	–125 min	–115 min	–55 min	–25 min	5 min	30 min	60 min	150 min	300 min	600 min	1200 min	1440 min	1 week
	control	baseline	0.5mg/kg	1.5mg/kg	2.5mg/kg	-	-	-	-	-	-	-	-
White Blood Cells (10^3/µl)													
Saline	5.8–7.0	5.0–7.8	3.7–10.4	4.3–10.6	5.8–8.5	6.8–8.1	nd	nd	nd	nd	nd	nd	nd
h6B4-Fab	6.2 ± 0. 3	5.7 ± 0.4	7.8 ± 0.4	9.3 ± 0.6	11.5 ± 0.5*	13.4 ± 0.8*	13.9 ± 0.7*	14.0 ± 0.6*	14.0 ± 0.8*	14.0 ± 0.8*	14.7 ± 0.6*	12 ± 0.4*	8.9 ± 0. 4
Platelets (10^3/µl)													
Saline	280–311 -	281–361 (100 ± 0)	265–276 (85.4 ± 6.3)	221–249 (73.8 ± 3.4)	231–239 (74.2 ± 5.6)	231–250 (76.5 ± 8.8)	nd	nd	nd	nd	nd	nd	nd
h6B4-Fab	403.8 ± 21.2 -	363.2 ± 3. 2 (100 ± 0)	328.8 ± 14. 5 (91.3 ± 4.9)	309.8 ± 11. 9 (85.8 ± 4)	292.0 ± 10.3 (80.9 ± 3.5)	267.2 ± 13.4* (74.2 ± 4.3)	298.6 ± 9.9 (82.7 ± 3.5)	312.4 ± 11.2 (86.6 ± 3.9)	327.0 ± 13.7 (90.9 ± 4.8)	330.4 ± 7.8 (91.5 ± 3.1)	353.4 ± 11.3 (98 ± 4.1)	336.2 ± 9.1 (93.2 ± 3.5)	537.2 ± 18.9 (148.6 ± 5.6)

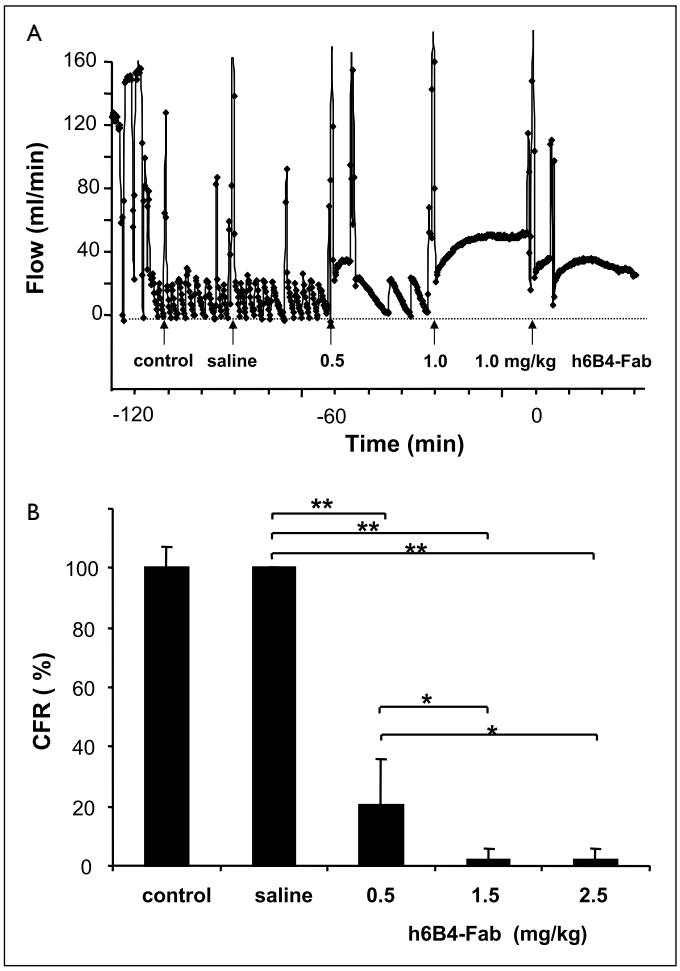

Figure 2: Cyclic flow reductions. Representative recording of the blood flow in the arteriovenous shunt (A). Every injection is marked by an arrow. Histogram of the CFRs (B). Values of the CFRs during 30 min recording are expressed as percentage of the number of CFRs following saline injection. Mean values ± SD (n=5). * statistically different p<0.05, ** p<0.005 t-test for paired sample groups.

h6B4-Fab significantly reduced the number of CFRs to only 20% of the control (Fig. 2A, B). Two extra injections of 1 mg/kg h6B4-Fab completely inhibited the CFRs, resulting in a continuous patent artery.

Bleeding time and blood loss

The bleeding time was not significantly affected by injections of h6B4-Fab (Table 2), except for a mere two-fold, but statistically significant, increase 60 min after the highest dose, which returned to baseline within 24 h.

Blood loss was determined by measuring the blood adsorbed by a standardized gauze in a standardized incision, over 30 min. As this parameter was relatively variable between but much less within baboons, the blood loss was expressed as the fold increase over the value seen at baseline in the same animal (Table 2). The maximum increase was about 2.6 at the cumulative dose of 1.5 mg/kg h6B4-Fab, which was statistically significantly different compared to saline at the same time point, and did not further increase when a full antithrombotic effect was obtained with

Table 2: Bleeding parameters in baboons treated with saline or h6B4-Fab in function of time. Template bleeding time is expressed in seconds. nd indicates not determined. Blood loss is expressed as the ratio of the volume of blood that was adsorbed during 30 min by a gauze from a standardized incision upon saline administration. Values are range (saline n=2) and mean ± SEM (h6B4-Fab n= 5). * statistically different p<0.05 t-test for paired sample groups.

Time	–125 min	–115 min	–55 min	–25 min	5 min	30 min	60 min	600 min	1200 min	1440 min
	control	baseline	0.5mg/kg	1.5mg/kg	2.5mg/kg	-	-	-	-	-
Bleeding time (s)										
Saline	225–345	nd	150–240	135–165	180–210	105–270	nd	nd	nd	nd
h6B4-Fab	84 ± 2.7	nd	156 ± 14.5	165 ± 21	177 ± 13.8	126 ± 7.5	165 ± 25.7*	132 ± 7.5	120 ± 12.7	99 ± 8.1
Blood loss (ratio over baseline)										
Saline	0.5–2.6	1.0–1.0	0.5–1.0	0.6–0.4	0.3–0.4	nd	nd	nd	nd	nd
h6B4-Fab	3.5 ± 0.7	1.0 ± 0.0	2.1 ± 0.4	2.6 ± 0.3*	2.2 ± 0.4	nd	nd	nd	nd	nd

2.5 mg/kg (Fig. 3A). A similar full antithrombotic effect was seen after administration of 2.5 mg/kg clopidogrel where; however, a 4.3 ± 2.6 fold increase in blood loss was observed, that even further increased to 8.0 ± 5.0 -fold at 5 mg/kg (Fig. 3B). Also 1.6 mg/kg abciximab gave a full inhibition of the CFRs; however, it increased the blood loss by some 20-fold (Roodt et al., unpublished data).

6B4-Fab plasma concentration

Intravenous administration of 0.5 mg/kg h6B4-Fab resulted in a plasma concentration of 6.3 ± 1.1 µg/ml 5 min after its injection (Fig. 4A). An additive dose of 1 mg/kg, 30 min after the first one, increased the plasma level of the drug to 25.9 ± 3.5 µg/ml. Finally, the last injection of h6B4-Fab further increased the plasma level to 28.7 ± 3.2 µg/ml. The presence of the antibody in the blood stream then decreased rapidly and reached baseline in 24 h. Based on these measurements in function of time after the last dose, an initial pharmacokinetic analysis was performed using WinNonlin® Compartmental Modeling Analysis (Pharsight, Mountain View, CA, USA): h6B4-Fab is cleared from circulation with an initial half-life of approximately 15.5 min, with the predominant clearance mechanism occurring for 96% via a single compartment, indicating that the secondary half-life is not relevant for 6B4 pharmacokinetics.

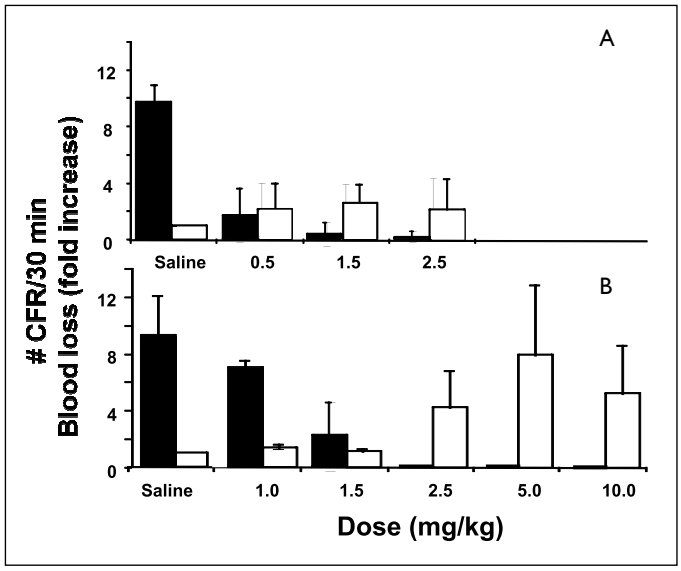

Figure 3: Relationship between antithrombotic effect and blood loss. The influence of increasing doses of h6B4 (a, n= 5) and of clopidogrel (b, n= 3) on the number of CFRs per 30 min (black bars) and the relative increase of blood loss over saline (white bars) (mean ± SD).

Receptor occupancy

The first dose of h6B4-Fab only resulted in a partial GPIbα occupancy with a maximum of approximately 52% after 5 min (Fig. 4B). Injection of the two doses of 1 mg/kg resulted in 66% and 64% (Fig. 3B) of the GPIbα receptors occupied by h6B4-Fab. After 1 h, the remaining receptor occupancy was 27.8 ± 4.9% and continued to decrease to 19.2 ± 3.4% 300 min post dose. Interestingly, the receptor occupancy increased again 10 h (600 min) after the last injection and to a lower extent than for the main peak. At this stage the receptor occupancy could be estimated to be approximately two times less important than for the initial peak with 35% and 65%, respectively. Finally, after 24 h, the GPIbα occupancy was still 16.5 ± 4%.

Platelet activity: Ristocetin-induced platelet aggregation

Already the injection of a cumulative dose of 1.5 mg/kg h6B4-Fab completely inhibited ristocetin-induced platelet aggregation (RIPA) after 5 min (Fig. 4C). After the cumulative dose of 2.5 mg/kg, the RIPA was fully inhibited for 30 min more and then recovered slowly within 24 h.

Expressing h6B4-Fab plasma concentration versus percentage of ristocetin-induced platelet aggregation, shows that 10 µg/ml is enough to fully inhibit ristocetin-induced platelet aggregation (Fig. 5A). At this same concentration also the GPIbα occupancy was maximal. The relationship between the receptor occupancy and ristocetin-induced platelet aggregation revealed that an estimated receptor occupancy of ~ 50% is enough to fully inhibit the ristocetin-induced platelet aggregation (Fig. 5B).

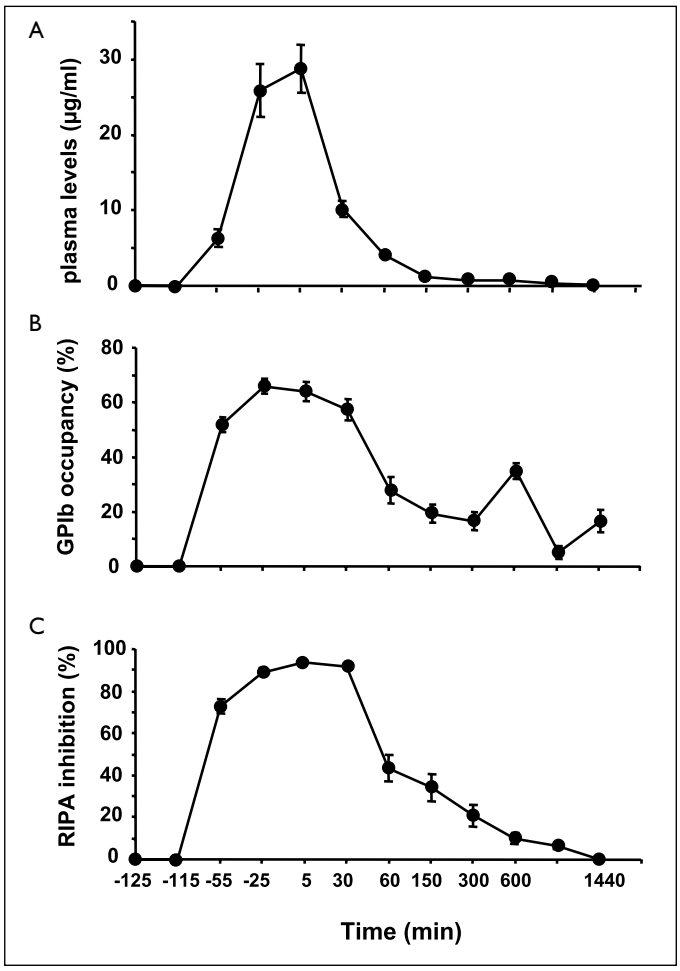

Figure 4: Ex-vivo effects of h6B4-Fab administration to baboons. Successive doses of h6B4-Fab were administered to baboons and antibody plasma levels (A), GPIbα occupancy (B) and ex-vivo ristocetin-induced platelet aggregation (C) were determined as described in materials and methods. Data represent the mean ± SEM calculated from five independent experiments.

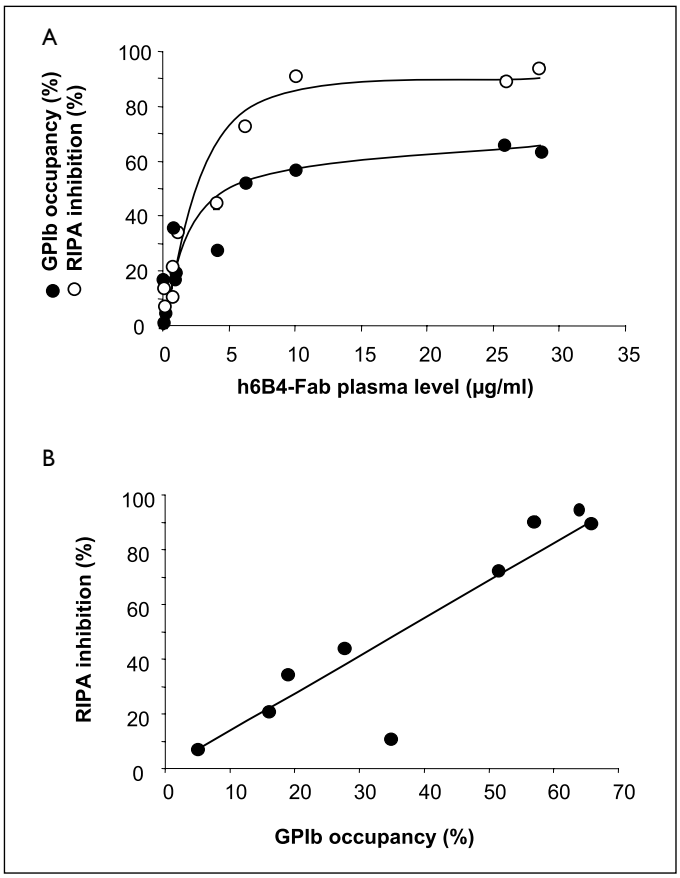

Figure 5: Relationship between h6B4-Fab plasma level, the percentage of GPIbα occupancy (●) and ristocetin induced platelet aggregation (RIPA) (○) (A) and between percentage of GPIbα occupancy and RIPA (B). To construct the curves, mean values for each parameter were used.

Discussion

Cardiovascular diseases remain the main cause of death in Western countries (12). Treatment normally consists of anticoagulants and/or antiplatelet drugs, often in conjunction with percutaneous interventions. The antiplatelet agents commonly used are aspirin and clopidogrel. Clinical trials have demonstrated significant efficacy of these drugs and their combination results in improved outcomes, albeit with a small but significant inherent risk of increased bleeding (13). The development of GPIIb/IIIa blockers has been a most remarkable improvement to antiplatelet approaches; however, after many clinical trials, it seems that this class of blockers only can be used effectively in the prevention of ischemic complications and mortality after percutaneous coronary intervention (14). These studies also demonstrated that e.g. abciximab injection can induce thrombocytopenia and bleeding. Limited efficacy is likely due in part to the inability to reach maximal therapeutic efficacy limited by the increasing risk of bleeding (13).

We have tackled this problem via an alternative new approach. Aspirin, clopidogrel or GPIIb/IIIa blockers prevent the development of thrombi, however, not exclusively under high shear stress conditions as seen in an atherosclerotic, stenotic environment. On the other hand, it is known that bridge formation between platelets and collagen by VWF is a prerequisite to allow platelets to withstand the elevated shear forces, whereas the role of VWF at lower shear is redundant at best. So interfering with this adhesion pathway might result in a specific targeting of the antithrombotic action to stenotic arteries, leaving the rest of the circulation intact, with presumably less risk for spontaneous bleeding.

We have shown that the monoclonal anti-human GPIbα antibody, 6B4, inhibits platelet adhesion to human collagen type I in a perfusion flow chamber and this more efficiently with increasing shear rate (15). The antithrombotic property of the mAb was demonstrated in baboon models of arterial thrombosis (3, 4) in which intravenous injection of 6B4-Fab-fragment at 0.6 mg/kg reduced significantly and at 2 mg/kg completely blocked platelet dependent thrombus formation. This antithrombotic effect was not accompanied by a prolongation of the bleeding time, in contrast to what is normally found with regular antiplatelet drugs (13). In view of these promising results, we recently prepared a

What is known about this topic?
– The murine anti-glycoprotein Ib antibody 6B4 is antithrombotic in baboons
– and does not prolong the template bleeding time.

What does this paper add?
– The humanised anti-glycoprotein Ib antibody 6B4 is antithrombotic in baboons
– and does neither prolong the template bleeding time nor increase the blood loss from a standardised incision.
– A new cyclic flow reduction model in the baboon is used, where the damage and stenosis is applied to the femoral artery, and the changes in blood flow are determined on an extracorporeal femoral arteriovenous shunt.

fully recombinant and humanized version of 6B4 called h6B4-Fab (10) with similar affinity and in-vitro and ex-vivo efficacy in different models as the murine Fab. In line with that study, we here demonstrated again that h6B4-Fab causes a dose- and time-dependent inhibition of ristocetin-induced platelet aggregation *ex vivo*. As we previously observed with the murine 6B4-Fab (4), 60% of GPIbα occupied by h6B4-Fab is enough to totally inhibit the thrombus formation but also to inhibit the ex-vivo platelet aggregation induced by 3 mg/ml ristocetin. This is in perfect agreement with the findings of van Zanten et al. (16), who reported that the minimal GPIbα number needed to affect platelet adhesion under flow conditions is 50%. The value of 50% seems to be really critical, since with a receptor occupancy of 40%, the inhibition of the RIPA is almost negligible. In the same way Kageyama et al. (17) showed that 50% VWF occupied by AJW200 was enough to totally inhibit the CFRs but also the botrocetin-induced platelet aggregation *ex vivo*. Finally, GPIbα remains partially occupied (16.5%) 24 h post injection, which again is in line with what we previously observed with h6B4-Fab and murine 6B4-Fab (4, 10). It nevertheless is clear that the actual receptor occupancy likely might be somewhat underestimated, since the biotinylated 6B4, used to determine the remaining free binding sites, will to some extent compete with the bound h6B4-Fab. Another explanation why even at the highest doses never an occupancy of 100% is reached could be that as a consequence of antibody binding GPIbα may become internalized or shed (18, 19), as is e.g. seen with antibodies against GPVI (20); this, however, awaits further verification.

In the present study, we now furthermore studied the antithrombotic effect of h6B4-Fab-fragment in injured and stenosed baboon femoral arteries, a further modification of the original canine model of Folts (21). Wu et al. (4) previously published that a full inhibition of the CFRs was obtained by injecting 2 mg/kg of murine 6B4-Fab in baboons. In our study, a cumulative dose of 1.5 mg/kg h6B4-Fab already resulted in a complete inhibition of the CFRs. Therefore, the humanized version of 6B4-Fab fragment retains the antithrombotic effect of the parental murine 6B4, and this without a marked prolongation of the bleeding time or increased blood loss, which may be indicators of enhanced bleeding tendency. The blood loss is increased over baseline; however, about 4 and 10 times less than observed with clopidogrel and abciximab, respectively (Roodt et al., submitted). The lack of effect on the bleeding time is in agreement with results obtained with different blockers of the GPIb/VWF axis such as VCL, a recombinant VWF-A1 domain (22, 23), AjvW-2-Fab, an inhibitory antibody against the VWF-A1 domain, or its humanized version, AJW200 (17, 24) or 82D6A3, an anti-VWF-A3 antibody that prevents VWF binding to collagen (25). Prolonged bleeding times, however, could be induced by using doses higher than needed for the antithrombotic effect (17, 26). Although it is indeed impossible to extrapolate bleeding risk from bleeding times, very encouraging results recently were obtained with an inhibitory Fab-fragment against GPIbα in a murine model of acute experimental stroke, where reduced brain infarct volumes and improved neurological status were not accompanied by an increase in bleeding complications (27).

In conclusion, the present study demonstrates that the humanised 6B4-Fab is a powerful inhibitor of the GPIbα/VWF axis and again proves that inhibiting platelet GPIbα is an efficient way to prevent thrombus formation in the injured and stenosed baboon femoral artery without causing prolongation of the bleeding time or increased blood loss. All together this results in a much larger therapeutic window than available with the current antithrombotics. Accordingly, inhibition of GPIbα by compounds such as h6B4-Fab, is a promising approach that needs to be validated in clinical trials aimed to prevent acute arterial thrombotic syndromes.

Acknowledgements

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Thrombosis

Inhibition of Platelet Glycoprotein Ib, Glycoprotein IIb/IIIa, or Both by Monoclonal Antibodies Prevents Arterial Thrombosis in Baboons

Dongmei Wu, Muriel Meiring, Harry F. Kotze, Hans Deckmyn, Nancy Cauwenberghs

Abstract—The antithrombotic efficacy of the monoclonal antibodies 6B4-Fab and MA-16N7C2 against platelet glycoprotein (GP) Ib and GP IIb/IIIa, respectively, on acute platelet-mediated thrombosis was evaluated in a baboon model of femoral artery stenosis, which is a modification of the original Folts model: platelet thrombi form on the injured stenosed artery, producing cyclic flow reductions (CFRs). A dose of 0.6 mg/kg 6B4-Fab significantly reduced the CFRs by 59±15%, whereas 2 mg/kg 6B4-Fab completely abolished the CFRs without prolongation of the bleeding time. MA-16N7C2 inhibited CFRs by 43±8% at a dose of 0.1 mg/kg and abolished the CFRs at a dose of 0.3 mg/kg but with a significant prolongation of the bleeding time. Finally, the combination of 0.6 mg/kg 6B4-Fab and 0.1 mg/kg MA-16N7C2 fully prevented the CFRs without prolongation of the bleeding time. The present study demonstrates that the inhibition of platelet GP Ib function by 6B4-Fab is a powerful intervention to prevent platelet thrombus formation in injured arteries without prolongation of the bleeding time; the latter is in contrast to the result after the inhibition of GP IIb/IIIa. Moreover, we demonstrate that combining a GP Ib blocker with a GP IIb/IIIa blocker can achieve a strong antithrombotic effect without increasing the bleeding time. This provides new information that will be beneficial in designing clinical therapeutic approaches. **(*Arterioscler Thromb Vasc Biol*. 2002;22:323-328.)**

Key Words: platelet glycoprotein Ib ■ platelet glycoprotein IIb/IIIa ■ cyclic flow reductions ■ antithrombotic agents ■ bleeding time

Platelets adhere to the subendothelium of damaged blood vessels through the collagen–von Willebrand factor (vWF)–platelet glycoprotein (GP) Ib axis. vWF forms the bridge between platelets and collagen in the vessel wall (especially under high-shear conditions) and in stenosed arteries and microvessels, where it initiates the formation of platelet aggregates. When vWF binds to GP Ib, platelets are slowed down, allowing direct platelet–collagen receptor interactions via, for example, integrin $\alpha_2\beta_1$ and glycoprotein VI, which activate platelets, finally resulting in a conformational change in the GP IIb/IIIa receptor to enable binding to fibrinogen and vWF, leading to the formation of platelet aggregates.[1,2] After extensive vessel injury or rupture of atherosclerotic plaques with subsequent exposure of thrombogenic surfaces, platelet aggregation can progress to result in complete thrombotic occlusion of the injured vessel.[3,4]

Various clinical studies and studies in experimental animals have clearly shown that inhibition of platelet aggregation, through prevention of the binding of the adhesive proteins to GP IIb/IIIa, is an effective approach to prevent thrombosis.[5–8] Unfortunately, this approach increases the risk of bleeding, especially at the doses that are effective in preventing thrombotic episodes.[8]

Blocking GP Ib[9–11] or vWF[12] results in an inability of the platelets to attach to the exposed subendothelium. Therefore, the GP Ib–vWF axis is an attractive target on which to focus for the prevention of thrombus formation in stenosed arteries.

In a recent study in baboons,[13] we showed that inhibition of platelet adhesion, through prevention of the binding of vWF to GP Ib by monoclonal antibody 6B4-Fab fragments, markedly inhibited thrombosis under relatively low shear conditions, without causing thrombocytopenia or major lengthening of the bleeding time. 6B4 is a potent GP Ib blocker, which, by interacting with GP Ibα (amino acids 201 to 268),[14] prevents vWF binding induced by either ristocetin, botrocetin, or shear. In the present study, we investigated the effect of inhibition of platelet adhesion in a modified Folts model,[15] in which the cyclic flow reductions are caused by platelet-dependent thrombi that form under high-shear conditions at injured stenosed sites of an artery. The model has been described to represent some of the events that occur in patients with unstable angina and is widely accepted to be effective and clinically relevant for testing potential antithrombotic agents.[16,17]

The aims of the study were 2-fold. First, we evaluated the antithrombotic efficacy of 6B4-Fab in high-shear conditions

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in baboons and compared it with the efficacy of the GP IIb/IIIa blocking monoclonal antibody MA-16N7C2[7,18] under the same conditions. The second aim was to determine whether the combination of an anti–GP Ib and anti–GP IIb/IIIa treatment could result in a synergistic antithrombotic effect and a lowering of the bleeding time prolongation associated with the anti–GP IIb/IIIa agent.

Methods

Preparation of Monoclonal Antibodies 6B4-Fab and MA-16N7C2

Antibodies 6B4, raised against human GP Ib,[13,19] and MA-16N7C2, directed against GP IIb/IIIa,[7,18] were purified from ascitic fluid from BALB/c mice on protein A Sepharose. 6B4-Fab was prepared from the IgG by papain digestion, as previously described.[13]

In Vitro Flow Studies

The effects of 6B4-Fab and MA-16N7C2 on platelet adhesion to collagen was studied in a Sakariassen-type parallel-plate flow chamber at a shear rate of 1500 s^{-1}, as previously described.[19] Blood was collected from healthy volunteers by using LMW-heparin (25 U/mL, Clexane) as an anticoagulant. Glass coverslips were coated with human collagen type I (Sigma Chemical Co), dissolved in 50 mmol/L acetic acid (1 mg/mL), and dialyzed against PBS for 48 hours. Fifteen milliliters of blood, preincubated with vehicle or compound at 37°C for 5 minutes, was perfused in the flow chamber for 5 minutes at 37°C. The platelets were fixed with methanol and stained with May-Grünwald–Giemsa. Platelet surface coverage was analyzed by using an image analyzer. An average of 10 fields per coverslip were analyzed. The results were expressed as percentage of total surface covered with platelets.

Folts Model in Baboons

Nineteen baboons (*Papio ursinus*) of either sex, weighing 12 to 18 kg, were used. All procedures were approved by the Ethics Committee for Animal Experimentation of the University of the Orange Free State in accordance with the National Code for Animal Use in Research, Education, Diagnosis and Testing of Drug and Related Substances in South Africa.

The experimental procedure was modified from the one described by Folts.[15] Baboons were anesthetized with ketamine hydrochloride (10 mg/kg IM), intubated with a cuffed endotracheal tube, and ventilated by a respirator with oxygen supplemented with 0.5% Fluothane to maintain anesthesia. Body temperature was maintained at 37°C with a heating table. A catheter was placed in a femoral vein for drug administration and blood sampling. A segment of another femoral artery was gently dissected free from surrounding tissue, and a perivascular ultrasonic flow probe (Transonic Systems Inc) was placed around the distal dissection site. The mean and phasic blood flow were recorded continuously throughout the experiment. Baboons were allowed to stabilize for 30 minutes. The proximal dissection site of the femoral artery was injured by applying 3 occlusions of the artery for 10 seconds at 2-mm intervals with the use of a spring-loaded forceps. A spring-loaded clamp was placed in the middle of the injured site to produce an external stenosis of 65% to 80%. A gradual decline in blood flow due to platelet adhesion and aggregation was observed. When flow reached zero, blood flow was restored by pushing the spring of the clamp to mechanically dislodge the platelet-rich thrombus. This repetitive pattern of decreasing blood flow after mechanical restoration is referred to as cyclic flow reductions (CFRs). Additional endothelial injury and appropriate external stenosis selections were repeated if needed. Finally, stable CFRs were obtained in 17 of 18 baboons.

After a 60-minute control period, test agents were given via an intravenous bolus injection, and monitoring was continued for up to 60 minutes after drug administration. The antithrombotic effect was quantified by comparing the number of CFRs per hour before and after drug administration. Blood samples for the different laboratory measurements (platelet count, coagulation, platelet aggregation, receptor occupation, and plasma levels) were drawn at −60, 0, 30, 60, 150, and 300 minutes and 24 hours after treatment.

Baboons were divided into 6 groups, and drug treatment was as follows: (1) vehicle (saline), n=2; (2) 6B4-Fab at 0.6 mg/kg, n=5; (3) 6B4-Fab at 2.0 mg/kg, n=3; (4) MA-16N7C2 at 0.1 mg/kg, n=3; (5) MA-16N7C2 at 0.3 mg/kg, n=2; and (6) 6B4 at 0.6 mg/kg plus MA-16N7C2 at 0.1 mg/kg, n=2. All agents were diluted in saline for injection. In addition, 4 mg/kg 6B4-Fab was injected in 1 baboon to determine the effect of such a high dose on platelet count, receptor occupation, bleeding time, and platelet aggregation. CFRs were not measured in this baboon.

Platelet Count, Coagulation, and Bleeding Time

All blood samples were collected in 0.32% (f.c.) trisodium citrate. The platelet count was determined by using a Technicon H_2 blood cell analyzer (Bayer Diagnostics). Prothrombin time and activated partial thromboplastin time were measured at 37°C by use of a coagulometer (Clotex II, Hyland).

The template bleeding time was measured at the surface of the forearm by using a Simplate II device (Organon Teknika). The volar surface of the forearm was shaved, and a pressure cuff was applied and inflated to 40 mm Hg. The time elapsed until the visual cessation of blood loss was recorded as the bleeding time. The wound was carefully dabbed every 15 seconds with filter paper.

Platelet Aggregation

Ex vivo platelet aggregation studies were performed on citrated blood with an aggregometer (Elvi 840, Pabisch). Platelet-rich plasma and platelet-poor plasma were prepared by differential centrifugation. The platelet count in platelet-rich plasma was adjusted to 200 000 platelets per microliter with autologous platelet-poor plasma. Platelet aggregation was induced by 1.5 mg/mL ristocetin (ABP), 20 μmol/L ADP (Sigma), or 50 μg/mL collagen (Nycomed Arzneimittel GmbH) and measured by the change in light transmission.

Plasma Concentration

Plasma levels of antibodies were measured by ELISA with the use of plates coated with 5 μg/mL goat anti-mouse IgG (Sigma) and developed with peroxidase-conjugated goat anti-mouse Fab-specific IgG (Sigma). The plasma concentration of the antibodies in each sample was calculated from standard curves constructed by adding known amounts of these antibodies to baboon plasma.

Receptor Occupancy

Ex vivo binding of ^{125}I-labeled 6B4-Fab or MA-16N7C2 IgG was used to determine the number of receptors occupied by 6B4-Fab and MA-16N7C2 IgG, respectively. Labeling of the antibodies with sodium ^{125}I (Amersham) was performed by using Iodogen (Pierce Chemical Co). Platelet-rich plasma (200 000 platelets per microliter) was incubated with a near saturating dose (2 μg/mL) of ^{125}I-MA-16N7C2 or ^{125}I-6B4-Fab for 15 minutes at room temperature. A fraction of this mixture was then layered onto 20% sucrose buffer (wt/vol) containing 0.1% BSA in Eppendorf tubes and centrifuged for 4 minutes at 10 000*g*. Platelet pellet–associated radioactivity was determined, and the number of radiolabeled molecules bound per platelet was calculated. The results were calculated by subtracting the number of labeled molecules bound to the platelets after treatment from that obtained before treatment and are expressed as the percentage of receptors occupied.

Statistical Analysis

Data are expressed as mean±SEM. The Student *t* test (2-tailed) or 1-factor ANOVA followed by the Fisher test was used for statistical evaluation. A value of $P<0.05$ was considered to be statistically significant.

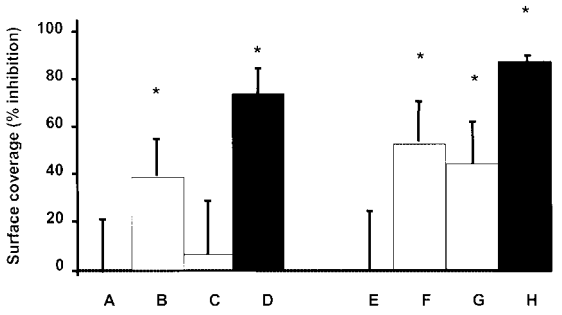

Figure 1. Inhibition by 6B4-Fab and MA-16N7C2 of platelet-dependent surface coverage of collagen-coated coverslips as determined after 5-minute perfusion of whole blood at 1500 s^{-1}. A and E, PBS control. B, 6B4-Fab (1.5 μg/mL). C, MA-16N7C2 (0.5 μg/mL). D, 6B4-Fab (1.5 μg/mL) plus MA-16N7C2 (0.5 μg/mL). F, 6B4-Fab (2.25 μg/mL). G, MA-16N7C2 (0.75 μg/mL). H, 6B4 Fab (2.25 μg/mL) plus MA-16N7C2 (0.75 μg/mL). Data are given as mean±SEM (n=4). *$P<0.05$ vs PBS control.

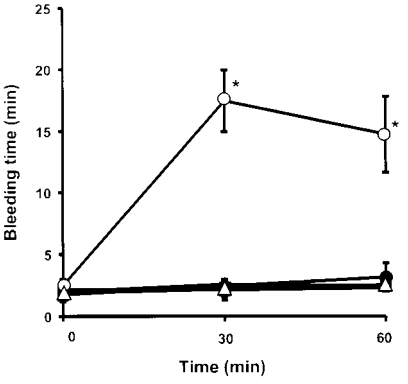

Fig 2. Effect of 6B4-Fab and MA-16N7C2 administration on template bleeding time in baboons. Open square indicates 0.6 mg/kg 6B4; solid square, 2.0 mg/kg 6B4; solid circle, 0.1 mg/kg MA-16N7C2; open circle, 0.3 mg/kg MA-16N7C2; and open triangle, 0.6 mg/kg 6B4+0.1 mg/kg MA-16N7C2. Data represent mean±SEM. *$P<0.05$ vs pretreatment value.

Results

In Vitro Flow Studies in the Flow Chamber

6B4-Fab completely inhibits platelet adhesion to collagen in flow at 5 μg/mL at 1300 s^{-1} and 2.5 μg/mL at 2600 s^{-1}.[13] In the present study, we analyzed the combined effects of partially inhibitory concentrations of 6B4-Fab and MA-16N7C2 on human platelet adhesion to human collagen type I in vitro after 5 minutes of perfusion at 1500 s^{-1} in a flow chamber. Concentrations of 1.5 and 2.25 μg/mL 6B4-Fab inhibited platelet adhesion by 38% and 53%, respectively, whereas 0.5 and 0.75 μg/mL MA-16N7C2 inhibited the surface coverage by platelets by 7% and 44%, respectively, mainly by reducing platelet aggregate formation. A combination of 1.5 μg/mL 6B4-Fab and 0.5 μg/mL MA-16N7C2 inhibited surface coverage by 76%, whereas 88% inhibition was achieved when 2.25 μg/mL 6B4-Fab and 0.75 μg/mL MA-16N7C2 were given in combination (Figure 1).

Platelet Count, Coagulation, and Bleeding Time

The platelet count was not significantly affected by injection of up to 4 mg/kg 6B4-Fab or MA-16N7C2 (in agreement with previous observations)[7,13] or the combination of 6B4 Fab and MA-16N7C2. No significant changes of prothrombin time and activated partial thromboplastin time were observed in any of the groups.

The bleeding time (baseline 2.04±0.59 minutes, interanimal coefficient of variation 29%, n=13) was prolonged only after the injection of 0.3 mg/kg MA-16N7C2, but it returned to normal levels 24 hours later (Figure 2).

Antithrombotic Effect

The frequency of the CFRs (Figure 3) was not significantly different between the different groups before the treatments were started, nor was it changed by the injection of saline (107±7%). A dose of 0.6 mg/kg 6B4-Fab resulted in a significant reduction of the CFRs by 59±15%. A dose of 2 mg/kg abolished the CFRs, which could not be restored by increasing the intimal damage or by increasing the stenosis. MA-16N7C2 significantly inhibited CFRs by 43±8% at a dose of 0.1 mg/kg and abolished the CFRs at 0.3 mg/kg. The combination of 0.6 mg/kg 6B4-Fab and 0.1 mg/kg MA-16N7C2 completely blocked the CFRs.

Platelet Aggregation

The effects of 6B4-Fab and MA-16N7C2 on ex vivo platelet aggregation are shown in Figure 4. 6B4-Fab inhibited the ex vivo ristocetin-induced platelet aggregation in a dose- and time-dependent manner. Aggregation was totally absent 30 minutes after injection of 0.6 mg/kg 6B4 Fab or injection of the combination of 6B4-Fab and MA-16N7C2. Significant inhibitory effects at these doses persisted for >150 minutes and returned to normal values within 24 hours.

ADP- and collagen-induced platelet aggregation was significantly inhibited after the administration of 0.1 or 0.3 mg/kg MA-16N7C2 and also when 0.6 mg/kg 6B4-Fab and 0.1 mg/kg MA-16N7C2 were combined. It is of interest that the combination of 0.6 mg/kg 6B4-Fab fragments and 0.1 mg/kg MA-16N7C2 had a more pronounced effect on the inhibition of ADP-induced platelet aggregation than did a single dose of 0.1 mg/kg MA-16N7C2, the reason for which is unclear at present.

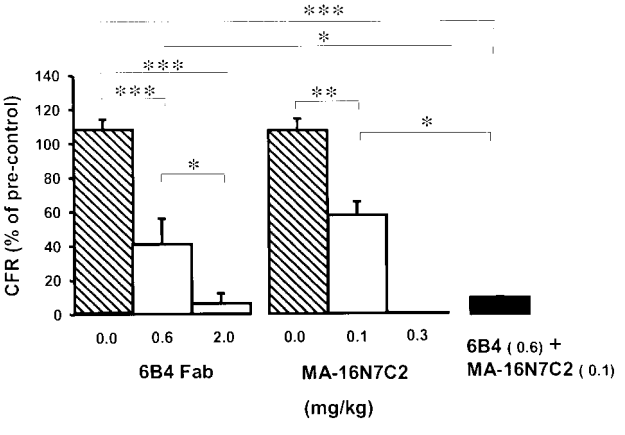

Figure 3. Effects of different doses of 6B4-Fab, MA-16N7C2, or their combination on CFRs in the baboon femoral artery. Saline injection appears as 0.0 mg/kg. The results are expressed as percentage of the pretreatment value. Data represent the mean±SEM. *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

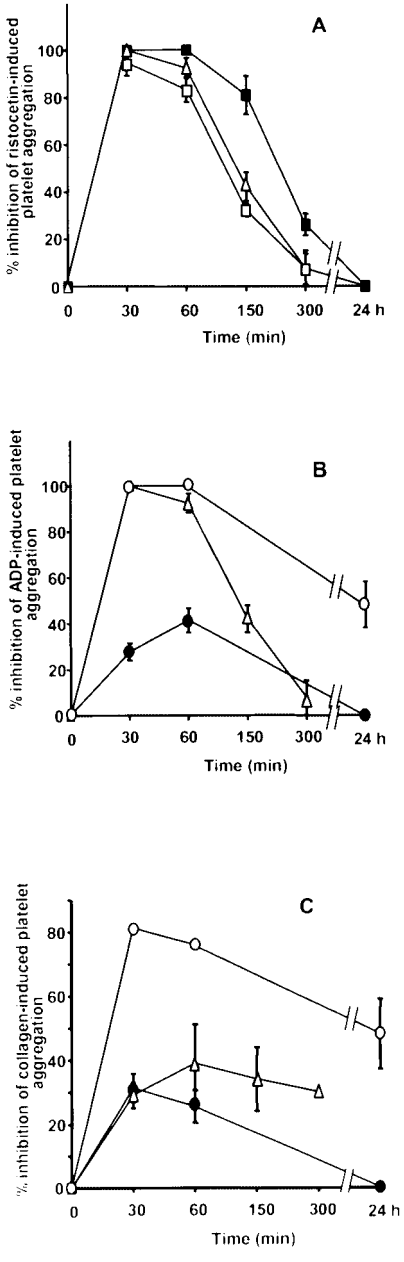

Figure 4. Effects of 6B4-Fab, MA-16N7C2, or their combination on ex vivo ristocetin-induced (A), ADP-induced (B), or collagen-induced (C) platelet aggregation in baboons. Open square indicates 0.6 mg/kg 6B4; solid square, 2.0 mg/kg 6B4; solid circle, 0.1 mg/kg MA-16N7C2; open circle, 0.3 mg/kg MA-16N7C2; and open triangle, 0.6 mg/kg 6B4+0.1 mg/kg MA-16N7C2. Data represent mean±SEM.

Plasma Concentration and Receptor Occupation

The plasma concentrations of 6B4-Fab and MA-16N7C2 are shown in Figure 5. The plasma concentration of both antibodies peaked at 30 minutes after the bolus injection and declined rapidly thereafter. The occupancy of GP Ib and GP IIb/IIIa receptors by 6B4-Fab and MA-16N7C2 also was maximal at 30 minutes; however, it remained elevated for an extended period. GP Ib receptor occupancy was 26%, 69%, and 84% after bolus injections of 0.6, 2.0, or 4.0 mg/kg 6B4-Fab, respectively, and 26% after the combination of 0.6 mg/kg 6B4-Fab and 0.1 mg/kg MA-16N7C2. Occupation decreased almost linearly, reaching baseline levels 24 hours after treatment. Thirty minutes after a bolus injection of 0.3 mg/kg MA-16N7C2, ≈71% of the GP IIb/IIIa receptors were occupied by the antibody. The decrease that followed also fit a linear function best, reaching a value of 40% at 24 hours after treatment. The GP IIb/IIIa receptor occupancy rates were 29% and 30% at 30 minutes after bolus injection of 0.1 mg/kg MA-16N7C2 and after the combination of 0.6 mg/kg 6B4-Fab and 0.1 mg/kg MA-16N7C2, respectively. The occupation remained almost at the same level 24 hours after treatment.

The relationship between GP Ib receptor occupancy, ristocetin-induced agglutination, CFRs, and bleeding time suggests that occupancy of 20% and of 35% of receptors with 6B4-Fab results in 50% reduction of platelet agglutination and CFRs, respectively, whereas up to 80% occupancy does not result in a significantly prolonged bleeding time (Figure 6).

Discussion

The present study evaluated the antithrombotic effects of the Fab fragments of monoclonal anti–GP Ib antibody (6B4) and of the monoclonal anti–GP IIb/IIIa antibody (MA-16N7C2) in injured and stenosed baboon femoral arteries.

Previous studies have shown that 6B4-Fab inhibited ristocetin- and botrocetin-induced vWF-dependent human platelet agglutination. 6B4-Fab inhibited human platelet adhesion to human collagen type I in a perfusion flow chamber with IC_{50} values of 3.5, 1.1, and 0.5 μg/mL at shear rates of 650, 1300, and 2600 s^{-1}, respectively, indicating that this compound is more effective under high-shear conditions.[13] In a baboon model with permanent arteriovenous shunt (700 to 1000 s^{-1}), 6B4-Fab significantly reduced platelet deposition onto collagen.[13] The present study evaluated the antithrombotic efficacy of 6B4-Fab under high-shear conditions by using a modified Folts model in baboons: 6B4-Fab significantly reduced CFRs at a dose of 0.6 mg/kg and completely abolished CFRs at 2.0 mg/kg without causing a marked prolongation of the bleeding time, even when a dose of 4 mg/kg 6B4-Fab was given, suggesting that 6B4-Fab is a powerful antithrombotic agent with, possibly, a low bleeding risk.

Although administration of inhibitory antibodies against vWF resulted in bleeding times that were not prolonged or were only minimally prolonged[20,21] or in bleeding times that resulted in a nearly 7-fold prolongation,[10] the minimal effect of blocking GP Ib on the bleeding time as seen in the present study is correlated with previous findings obtained with antibodies[13,22] or with a recombinant vWF-A1 domain,[11]which resulted in a prolongation of the bleeding time of, at most, 3-fold. Most likely, the rather exceptional combination of an antiplatelet effect without prolongation of the bleeding time, as observed in the present study and in other settings, is due to the shear dependence of the vWF–GP Ib interaction, which would therefore play a lesser role in the lower shear systems and might be more relevant for the bleeding time measurements. The bleeding problems observed in patients with Bernard-Soulier syndrome may be due to either the complete absence of GP Ib, in contrast to the maximum 80% receptor occupancy that was obtained in the present study, or the

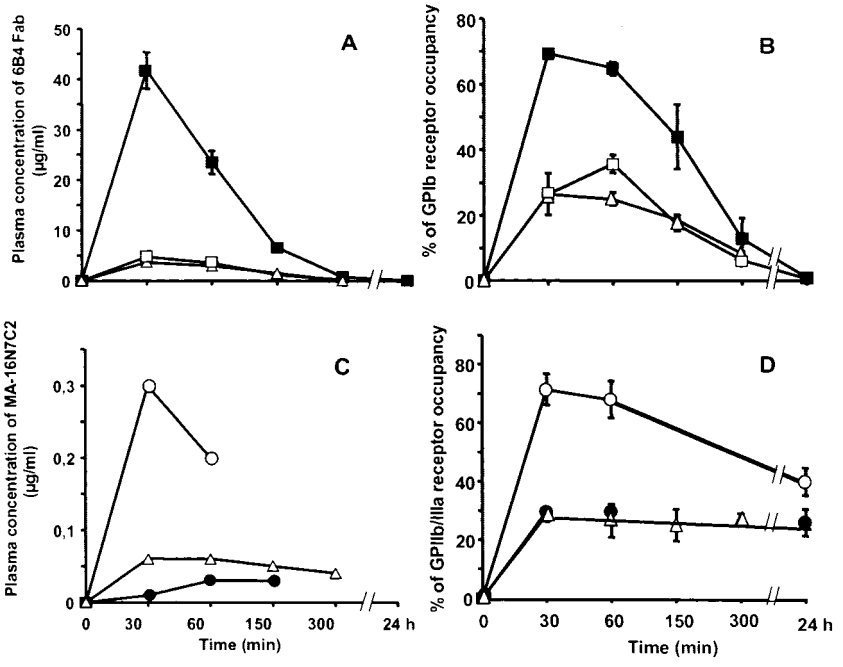

Figure 5. Plasma levels of 6B4-Fab (A) and MA-16N7C2 (C) and of GP Ib (B) and GP IIb/IIIa (D) receptor occupancy by 6B4-Fab and MA-16N7C2, respectively, as function of the time after their administration to baboons. Open square indicates 0.6 mg/kg 6B4; solid square, 2.0 mg/kg 6B4; solid circle, 0.1 mg/kg MA-16N7C2; open circle, 0.3 mg/kg MA-16N7C2; and, open triangle, 0.6 mg/kg 6B4+0.1 mg/kg MA-16N7C2. Data represent the mean±SEM.

combination of GP Ib deficiency, thrombocytopenia, and giant platelets, which may be less readily arrested at the site of injury.

On the other hand, we could not really demonstrate the anticipated higher efficiency of 6B4-Fab in the present high-shear setting compared with the lower shear setting in a previous experiment.[13] This, to a large extent, may be due to the different thrombogenic stimulus that was presented: a collagen-rich glutaraldehyde-fixed bovine pericardium in an extracorporeal arteriovenous shunt in the medium-shear experiments[13] versus a physically damaged and stenosed femoral artery. Part of the explanation may be that in the CFR experiments, ultrahigh-shear levels might occur, which can result in direct GP Ib-vWF–dependent platelet aggregation[23,24] and may be more resistant to inhibition.

6B4-Fab caused a dose- and time-dependent inhibition of ex vivo ristocetin-induced platelet aggregation. A lower receptor occupancy was needed to inhibit ristocetin-induced platelet agglutination than was needed to prevent CFRs,

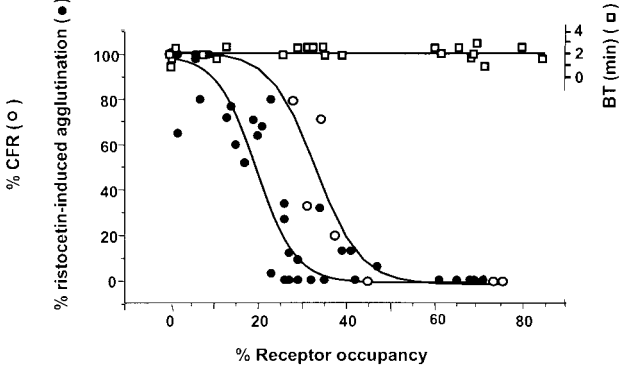

Figure 6. Relationship between percent GP Ib receptor occupancy by 6B4-Fab and percent ristocetin-induced platelet aggregation, percent CFRs, and bleeding time (BT). To construct the CFR curve, receptor occupancy 30 minutes after administration was used.

which is in accordance with the findings of van Zanten et al,[25] who reported that lower available GP Ib numbers were needed to prevent platelet adhesion to collagen under flow-induced compared with ristocetin-induced aggregation. However, the receptor occupancy needed to obtain an effect as observed in the present study may be somewhat underestimated in view of the relative low affinity of 6B4-Fab.

For purposes of comparison, we also used the Folts model to investigate the effect of inhibition of GP IIb/IIIa with MA-16N7C2. A dose of 0.1 mg/kg MA-16N7C2 inhibited CFRs by ≈43%, and CFRs were abolished at a dose of 0.3 mg/kg MA-16N7C2. Thus, inhibition of platelet adhesion and inhibition of platelet aggregation are both viable avenues for preventing arterial thrombosis. However, with the high dose of MA-16N7C2, the bleeding time was significantly prolonged. Therefore, inhibition of aggregation may increase the risk of bleeding, in agreement with studies using other antiplatelet agents.[26,27]

The roles of GP Ib and of GP IIb/IIIa in the formation of a platelet-dependent thrombus are complementary. Indeed, we found that combined inhibition has a more pronounced effect on in vitro platelet accumulation onto collagen in a flow chamber and on the prevention of CFRs in the baboon. With this combination, in addition, a full in vivo antithrombotic effect was obtained without prolongation of the bleeding time.

In conclusion, the present study demonstrates that the inhibition of platelet GP Ib is a powerful intervention in the prevention of platelet thrombus formation in the injured and stenosed baboon femoral artery without prolongation of the bleeding time. The effect on bleeding time is definitely less during inhibition of GP Ib than during inhibition of GP IIb/IIIa. Accordingly, a GP Ib blocker, such as 6B4-Fab, seems to be promising for further development as a compound for the prevention of acute arterial thrombotic syndromes. Moreover, we demonstrated that combining a low

dose of a GP Ib inhibitor with a low dose of a GP IIb/IIIa inhibitor has a potent antithrombotic action, again with minimal effects on the bleeding time. This provides new information that will be beneficial in designing clinical therapeutic approaches.

Acknowledgments

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Inhibition of the von Willebrand (VWF)–collagen interaction by an antihuman VWF monoclonal antibody results in abolition of in vivo arterial platelet thrombus formation in baboons

Dongmei Wu, Karen Vanhoorelbeke, Nancy Cauwenberghs, Muriel Meiring, Hilde Depraetere, Harry F. Kotze, and Hans Deckmyn

The interaction between collagen, von Willebrand factor (VWF), and glycoprotein Ib is the first step in hemostasis and thrombosis especially under high shear conditions. We studied the inhibition of the VWF-collagen interaction by using an antihuman VWF monoclonal antibody 82D6A3 to prevent arterial thrombosis in baboons to develop a new kind of antithrombotic strategy and determine for the first time experimental in vivo data concerning the importance of the collagen-VWF interaction. We used a modified Folts model to study the antithrombotic efficacy of 82D6A3, where cyclic flow reductions (CFRs) were measured in the femoral artery. Administering a dose of 100, 300, and 600 μg/kg resulted in a 58.3%, 100%, and 100% reduction in the CFRs, respectively. When 100 μg/kg 82D6A3 was infused into the baboons, 80% of VWF-A3 domain was occupied, corresponding to 30% to 36% ex vivo inhibition of VWF binding to collagen, with no prolongation of the bleeding time. The bleeding time was also not significantly prolonged when the CFRs were abolished at doses of 300 μg/kg and 600 μg/kg. At these doses 100% of VWF was occupied by the antibody and 100% ex vivo inhibition of the VWF-collagen binding was observed. 82D6A3 has a high affinity for VWF; after 48 hours still 68% VWF (300μg/kg) was occupied with a pharmacologic effect up to 5 hours after administration (80%-100% occupancy). In conclusion, these results clearly indicate that the VWF-collagen interaction is important in vivo in thrombosis under high shear conditions and thus might be a new target for preventing arterial thrombosis. (Blood. 2002;99:3623-3628)

Introduction

In normal hemostasis and thrombosis, platelets adhere to the subendothelium of damaged blood vessels through an interaction with von Willebrand factor (VWF), which forms a bridge between collagen within the damaged vessel wall and the platelet receptor glycoprotein Ib (GPIb/V/IX), an interaction especially important under high shear conditions.[1] This reversible adhesion allows platelets to roll over the damaged area, which is then followed by a firm adhesion through the collagen receptors (GPIa-IIa, GPVI, GPIV, p65, TIIICBP) [2-4] resulting in platelet activation. This leads to the conformational activation of the platelet GPIIb/IIIa receptor, fibrinogen binding, and finally to platelet aggregation.[5]

The VWF subunit is composed of several homologous domains each covering different functions; VWF interacts through its A1 domain mainly with the GPIb/V/IX complex,[6] whereas its A3 domain predominantly interacts with fibrillar collagen fibers.[7] Under normal conditions platelets and VWF do not interact. However, when VWF is bound to collagen at high shear rate, it is believed to undergo a conformational change allowing its binding with the platelet receptor GPIb/IX/V.[8]

One line of search for antiplatelet drugs in the prevention of thrombosis is focusing on the inhibition of the VWF-GPIb axis. Compounds that interact with GPIbα, such as the GPIb-binding snake venom proteins echicetin and crotalin,[9,10] an antiguinea pig GPIb antibody,[11] a recombinant A1 domain fragment (VCL),[12] and recently an antihuman GPIb antibody[13] or compounds that bind to VWF, such as anti-A1-VWF-monoclonal antibodies (mAbs)[14,15] or aurin tricarboxylic acid (ATA),[16] indeed, inhibit thrombus formation in vivo.

Specific blockade of the VWF-collagen interaction in vivo has not yet been demonstrated but based on in vitro data could be a novel strategy for the prevention of thrombus formation in stenosed arteries. We here describe for the first time the antithrombotic effect of a murine antihuman VWF mAb 82D6A3, known to bind to the A3 domain and to inhibit VWF binding to fibrillar collagens type I and III but not to collagen VI.[17]

The antithrombotic efficacy of 82D6A3 was demonstrated in baboons by using a modified Folts model, where cyclic flow reductions (CFRs) due to thrombus formation and its dislodgment are measured in mechanically damaged, severely stenosed femoral arteries.[18]

Materials and methods

Purification of 82D6A3

82D6A3 was raised and purified from ascites by protein A chromatography.[17]

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Surgical preparation

Baboons (*Papio ursinus*) used in this study were of either sex and weighed 12 to 18 kg. All procedures were approved by the Ethics Committee for Animal Experimentation of the University of the Free State and Free State Provincial Administration in accordance with the National Code for Animal Use in Research, Education, Diagnosis and Testing of Drug and Related Substances in South Africa. The experimental procedure followed[19] was that of the original Folts model[18] except that the femoral artery was injured with a spring-loaded forceps. Baboons were anesthetized with ketamine hydrochloride (10 mg/kg, intramuscularly), intubated with a cuffed endotracheal tube, and ventilated by a respirator with oxygen supplemented with 0.5% Fluothane (halothane) to maintain anesthesia. Body temperature was maintained at 37°C with a heating table. A catheter was placed in a femoral vein for drug administration and blood sampling. A segment of a femoral artery was gently dissected free from surrounding tissue and a perivascular ultrasonic flow probe (Transonic Systems, Ithaca, NY) was placed around the distal dissection site. The mean and phasic blood flow were recorded continuously throughout the experiment. Baboons were allowed to stabilize for 30 minutes. The proximal dissection site of the femoral artery was injured by applying 3 occlusions of the artery for 10 seconds with 2-mm interval using a spring-loaded forceps, and a spring-loaded clamp was placed in the middle of the injured site to produce an external stenosis of 65% to 80%. A gradual decline in blood flow due to platelet adhesion and aggregation was observed. When flow was reduced by at least 50%, blood flow was restored by pushing the spring of the clamp to mechanically dislodge the platelet-rich thrombus. This repetitive pattern of decreasing blood flow following mechanical restoration is referred to as cyclic flow reductions (CFRs). Additional endothelial injury and appropriate external stenosis selection was repeated if needed to finally obtain stable CFRs in these baboons. The number of times the thrombus needed to be dislodged determines the number of CFRs.

After a 60-minute control period of reproducible CFRs ($t = -60$ minutes to 0 minute), test agents (saline or 82D6A3) were given via an intravenous bolus injection ($t = 0$) and monitoring was continued up to 60 minutes after drug administration ($t = +60$ minutes). The antithrombotic effect was quantified by comparing the number of CFRs per hour before and after drug administration. If the last flow reduction in the 60-minute recording period was not cyclic, it was considered in the calculations. Blood samples for the different laboratory measurements were drawn at different time points. The doses of 82D6A3 were selected on the basis of preliminary dose-finding studies. Group 1 was a control group where the baboons received saline ($n = 2$). Group 2 received a high dose of 600 μg/kg 82D6A3 ($n = 2$). In group 3, the baboons received an initial dose of 100 μg/kg 82D6A3 and after 60 minutes an additional 200 μg/kg 82D6A3 was given ($n = 3$). In a preliminary study we found that 82D6A3 has a long half-life. This therefore resulted in a final dose of 300 μg/kg. All agents were diluted with saline.

Platelet count, coagulation, and bleeding time

All blood samples were collected on 0.32% (final concentration) trisodium citrate. The platelet count was determined using a Technicon H_2 blood cell analyzer (Bayer Diagnostics, Tarrytown, NY). Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were measured at 37°C using a coagulometer (Clotex II, Hyland, South Africa). The template bleeding time was measured using the Simplate II device (Organon Teknika, Durham, NC). The volar surface of the forearm was shaved and a pressure cuff was applied and inflated at 40 mm Hg. The wound was carefully dabbed every 15 seconds with filter paper. Baseline bleeding time measured in 13 baboons was 2.04 ± 0.59 minutes, with an interanimal coefficient of variation of 29%.

All measurements were performed once at each time point.

Plasma concentration of 82D6A3

Microtiter plates (96-well, Greiner, Frickenhausen, Germany) were coated overnight at 4 °C with 5 μg/mL (in phosphate-buffered saline [PBS], 100 μL/well) goat antimouse IgG whole molecule (Sigma, St Louis, MO). Plates were blocked with 3% milk powder for 2 hours at room temperature (RT). Next dilution series (1:2 in PBS) of the plasma samples (prewarmed for 5 minutes at 37°C) were added for 2 hours at RT and goat antimouse IgG labeled with horseradish peroxidase (HRP) was added for 1 hour at RT. Visualization was obtained with H_2O_2 and ortho-phenylenediamine (OPD, Sigma) and the coloring reaction was stopped with 4 moL/L H_2SO_4. The absorbance was determined at 490 nm. After each incubation step, plates were washed with PBS, 0.1% Tween-20, 3 times after coating and blocking steps and 12 times elsewhere. The plasma concentration of 82D6A3 in each sample was calculated from a standard curve where known amounts of 82D6A3 were added to baboon plasma.

VWF-antigen levels

Determination of the VWF-antigen (Ag) levels was performed essentially as described.[20] Briefly, microtiter plates were coated with a polyclonal anti-VWF-Ig solution (Dako, Glostrup, Denmark), blocked with 3% milk powder and plasma samples, preincubated for 5 minutes at 37°C, and added to the wells at 1:40 to 1:2560 dilutions. Bound VWF was detected with rabbit antihuman VWF HRP antibodies (Dako). VWF-Ag levels were calculated from a standard curve obtained by adding 1:40 to 1:2560 dilutions to the coated wells of a human plasma pool, known to contain 10 μg/mL human VWF.

VWF occupancy

Microtiter plates (96-well) were coated overnight at 4°C with 125 μL/well of a polyclonal anti-VWF-Ig solution (Dako; 1:1000 in PBS). Plates were blocked with 3% milk powder solution for 2 hours at RT. A dilution series (1:2 in PBS) of the plasma samples (prewarmed for 5 minutes at 37°C) was added for 2 hours at RT. Samples containing 100% occupied VWF were obtained by adding a saturating amount of 82D6A3 (6 μg/mL) to each corresponding baboon plasma. Bound 82D6A3 was detected by addition of goat antimouse IgG-HRP (1 hour at RT). Visualization and wash steps were performed as described above. The VWF occupancy of each sample was calculated as follows: (A490-nm sample/A490-nm sample saturated with 82D6A3) · 100.

Determination of the VWF-collagen binding activity

The enzyme-linked immunosorbent assay (ELISA) was performed essentially as described.[20] Briefly, microtiter plates were coated with human collagen type I (Sigma), blocked with 3% milk powder solution and ½ dilution series of baboon plasma (prewarmed for 5 minutes at 37°C) were added. Bound VWF was detected with rabbit antihuman VWF-HRP antibodies. Binding of baboon VWF to collagen in the different plasma samples was compared to the binding of VWF in the respective blood sample taken at time zero (presample), which was set as 100%.

Inhibition of the VWF binding to collagen by 82D6A3

The ELISA was performed as described for the determination of the VWF-collagen binding activity except that serial dilutions of 82D6A3 were either preincubated with constant amounts of VWF in a preblocked plate for 30 minutes or were directly added to the plate containing collagen-bound VWF.

Statistics

Data are expressed as mean ± SD. Student *t* test (paired) or one-factor ANOVA followed by Fisher test was used for statistical evaluation. $P < .05$ was considered as statistically significant.

Results

In vitro interaction of 82D6A3 with baboon VWF

82D6A3, known to inhibit human VWF binding to collagen,[17] cross-reacts with baboon VWF. 82D6A3 has a comparable affinity

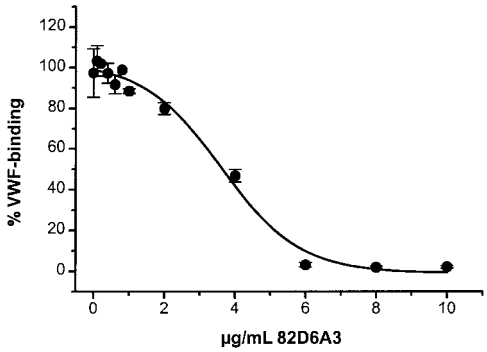

Figure 1. Inhibition of baboon VWF binding to collagen by 82D6A3. Different concentrations of 82D6A3 (20 µL) were incubated with undiluted baboon plasma (220 µL) for 30 minutes before addition to a collagen-coated plate. Bound VWF was detected with rabbit antihuman VWF antibodies. The data are the mean of 4 measurements obtained with the plasma of 2 different baboons

for baboon VWF as for human VWF (median effective concentration [EC_{50}] 200 ng/mL) and inhibited the binding of baboon VWF to collagen with an inhibitory concentration of 50% (IC_{50}) of 3.5 µg/mL (Figure 1).

82D6A3 not only prevented binding of VWF to collagen, but also was able to remove the bound VWF from a collagen surface with somewhat lower efficacy (Figure 2).

Antithrombotic effect

Using a Folts model, the antithrombotic efficacy of 82D6A3 was tested by administering different doses of 82D6A3 to baboons. In Figure 3, a representative tracing of CFR data is given.

Injection of saline did not affect CFRs (107% ± 7%). In a first study, a dose of 600 µg/kg 82D6A3 resulted in a complete disappearance of the CFRs (Figure 4). This dose was expected to be an overdose because according to theoretical calculations, a dose of 600 µg/kg would result in a plasma concentration of 13 µg/mL (45 mL/kg plasma volume), which in in vitro experiments results in a 100% inhibition of the VWF binding to collagen (Figure 1). As a result, in all subsequent in vivo studies lower doses of 82D6A3 were used. Administration of 100 µg/kg 82D6A3 resulted in a significant reduction of the CFRs by 58.3% ± 4.8% (Figure 4). Administration of a total doses of 300 µg/kg completely abolished the CFRs (Figure 4), which could not be restored by increasing intimal damage or increasing stenosis.

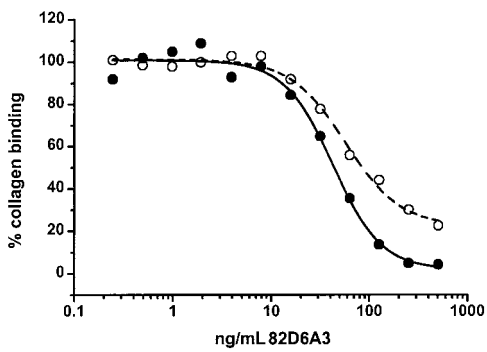

Figure 2. Inhibition of collagen-bound VWF by 82D6A3. Different concentrations of 82D6A3 were either preincubated with diluted human plasma (final dilution of 1:46, ie, 217 ng/mL VWF) before addition to a collagen-coated plate (●) or were added to a collagen-coated plate where VWF (final dilution of 1:46, ie, 217 ng/mL VWF) was already bound for 30 minutes (○). Bound VWF was detected with rabbit antihuman VWF antibodies.

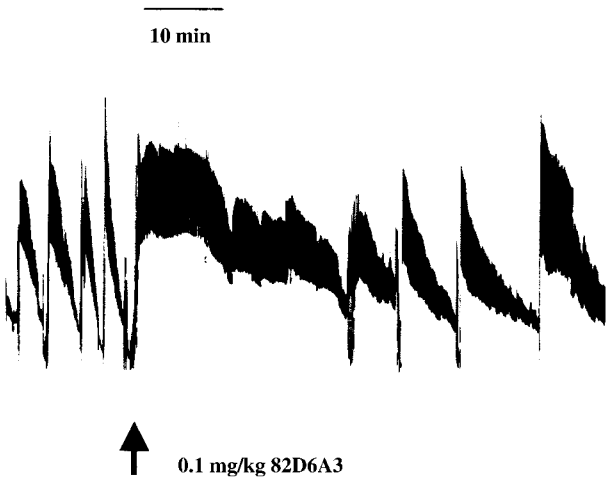

Figure 3. A representative tracing of CFR data. After a 60-minute control period, 100 µg/kg 82D6A3 was administered to the baboon and changes in CFRs were monitored for 60 minutes.

Coagulation, platelet count, and bleeding time

No significant changes in PT and aPTT were observed in any of the animals (data not shown). No major changes in platelet count were observed for the 100 µg/kg (not significant [NS]), 300 µg/kg (NS), and 600 µg/kg 82D6A3 doses (Tables 1 and 2). The bleeding time was not significantly prolonged after injection of 100 µg/kg. After injection of 300 µg/kg the bleeding time was increased 2.7 times at 60 minutes and 2.4 times at 150 minutes; however, these changes were not statistically significant. After injection of 600 µg/kg the bleeding time was increased 1.9, 3.0, 2.8, and 1.7 times after 30, 60, 150, and 300 minutes, respectively.

82D6A3 plasma concentration, VWF-Ag levels, VWF occupancy, and ex vivo VWF-collagen binding

The 82D6A3 plasma levels remained relatively constant during the first 3 hours of the experiment. Then, they decreased to 69%, 23%, and 7.6% after 300 minutes, 24 hours, and 48 hours, respectively, when 300 µg/kg 82D6A3 was administered (Table 1).

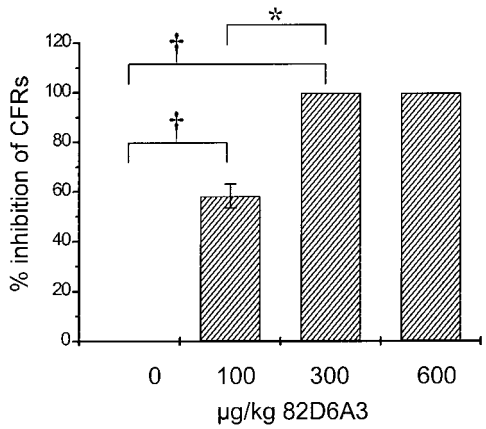

Figure 4. Inhibition of femoral artery CFRs after administration of different doses of 82D6A3 to baboons. Different doses of 82D6A3 were administered to baboons and the CFRs were measured during 60 minutes. Data represent the mean with n = 2 for 0 and 600 µg/kg and mean ± SD with n = 3 for 100 and 300 µg/kg mAb 82D6A3. * = $P < .05$; † = $P < .01$. One-factor ANOVA followed by Fisher test was used for statistical evaluation.

Table 1. Ex vivo analysis of plasma samples after administration of 100 and 300 μg/kg 82D6A3 to baboons

Time	Platelet count (10^3/mm^3)	Bleeding time (min)	VWF-Ag levels (μg/mL)	82D6A3 levels (μg/mL)	VWF occupancy (%)	Collagen binding (%)
100 μg/kg (n = 3)						
0	286 ± 54	2.7 ± 0.4	10.2 ± 1.7	0	2.3 ± 1.3	101 ± 7
30 min	292 ± 65	2.7 ± 0.4	10.2 ± 2.5	0.4 ± 0.07†	80 ± 10.8†	64 ± 7*
60 min	289 ± 49	3.5 ± 2.1	8.9 ± 1.4	0.4 ± 0.1*	80 ± 2.4‡	69 ± 9
300 μg/kg (n = 3)						
0	286 ± 54	2.7 ± 0.4	10.2 ± 1.7	0	2.3 ± 1.3	101 ± 7
30 min	265 ± 41	4.6 ± 0.6	8.8 ± 1.4	2.9 ± 0.3†	102 ± 10.4†	4 ± 1†
60 min	287 ± 53	7.3 ± 2.5	9.1 ± 2.4	2.8 ± 0.3†	99 ± 10.6†	4 ± 1†
150 min	309 ± 83	6.4 ± 3.1	9.7 ± 2.7	2.6 ± 0.1‡	101 ± 7.6†	4 ± 1†
300 min	282 ± 7	3.15 ± 1.2	8.8 ± 0.1	2.0 ± 0.5	94 ± 0.9†	4 ± 1†
24 h	312 ± 46	3.25 ± 0.3	12.8 ± 1.3	0.7 ± 0.2*	74 ± 31	91 ± 18
48 h	306 ± 79	3	13.2 ± 0.8	0.2 ± 0.01*	63 ± 7.8*	93 ± 0

Data are mean data ± SD. At each time point, the plasma samples were measured 3 times in 3 different ELISAs for the 3 animal experiments. For statistical analysis, the mean data were used. Student *t* test (paired) was used for statistical evaluation. *$P < .05$. †$P < .01$. ‡$P < .001$.

Thirty minutes after injection of the different doses of 82D6A3, plasma VWF-Ag levels decreased slightly and increased above baseline at 24 hours. None of these changes were significant (Tables 1 and 2).

At 60 minutes after administration VWF occupancy was 80% for the 100-μg/kg dose and nearly 100% for the 300-μg/kg and 600-μg/kg doses. VWF remained occupied for an extended period; even 48 hours after the injection of 300 μg/kg 82D6A3, still 63% of the VWF-binding sites were occupied by 82D6A3 (Table 1).

Injection of 100 μg/kg 82D6A3 resulted in an ex vivo inhibition of the VWF-collagen binding of 31% (blood sample taken after 1 hour; Table 1). At doses of 300 μg/kg and 600 μg/kg no interaction between baboon VWF and collagen was observed in samples taken up to 5 hours after the administration of the antibody. Twenty-four hours after the injection of the drug the VWF-collagen interaction recovers (Table 1 and 2).

Relation between the ex vivo 82D6A3 plasma levels, VWF occupancy, and collagen binding

The relationship between 82D6A3 plasma levels and VWF occupancy is illustrated in Figure 5. At plasma concentrations between 1 and 2 μg/mL 82D6A3 nearly 100% of circulating VWF was occupied (Figure 5). There was a good relation between the ex vivo VWF occupancy and the VWF binding to collagen. To obtain inhibition of VWF binding to collagen (20%-40%), a VWF occupancy of at least 80% was required, with complete inhibition at 90% to 100% occupancy (Figure 6). These data were confirmed by in vitro experiments, where different concentrations of 82D6A3 were added to baboon plasma (Figure 6). Occupancy levels of up to 60% resulted in no inhibition of the VWF binding to collagen, whereas up to complete inhibition was observed with 80% to 100% VWF-occupancy levels.

Discussion

Platelet adhesion to a damaged vessel wall is the first step in arterial thrombus formation. The tethering of platelets by VWF to the collagen exposed in the damaged vessel wall is especially important under high shear conditions. Antithrombotics that interfere with the GPIb-VWF axis have been studied in animal models and were shown to be effective.[13,14] The present study evaluated for the first time the antithrombotic effects of the inhibition of the VWF-collagen interaction in vivo. For this purpose, we used a monoclonal antihuman VWF antibody 82D6A3, which by binding to the VWF A3-domain, inhibits VWF binding to fibrillar collagens types I and III.[17] We here showed that 82D6A3 cross-reacts with baboon VWF and inhibits baboon VWF binding to collagen type I under static conditions. Moreover, in vitro 82D6A3 is able to remove collagen-bound VWF from its surface, which in view of the presence of collagen-bound VWF in the damaged vessel wall, might be a prerequisite for a proper function in vivo. Because the subendothelium, however, not only includes different types of collagen that may interact differently with VWF, and other matrix proteins that may affect the binding of VWF, and because, in addition, it may contain different VWF forms, such as ultralarge forms unprocessed by the VWF-cleaving plasma protease, no direct extrapolation is possible.

A modified Folts model was used to evaluate the antithrombotic efficacy of 82D6A3 under high shear conditions[18] in baboons. This model allows study of CFRs due to platelet-dependent thrombi forming at the injured, stenotic site of the artery.[21] We demonstrated that inhibition of the VWF-collagen interaction is accompanied by an antithrombotic effect in vivo. Indeed, in all 5 animals receiving doses of 300 μg/kg or higher, a 100% inhibition of the CFRs was

Table 2. Ex vivo analysis of plasma samples after administration of 600 μg/kg 82D6A3 to baboons

	Platelet count (10^3/mm^3)	Bleeding time (min)	VWF-Ag levels (μg/mL)	82D6A3 levels (μg/mL)	VWF occupancy (%)	Collagen binding (%)
0 min	335	1.8	14 ± 1.7	0	6.9 ± 0.1	100 ± 0
30 min	320	3.5	11.5 ± 0.9	4.5 ± 0.5	96 ± 1	4 ± 0.2
60 min	313	5.5	10.8 ± 0.1	4.8 ± 0.7	96 ± 0.2	3.5 ± 0.2
150 min	356	5	11.9 ± 1.8	3.8 ± 0.5	97 ± 4	3.5 ± 0.2
300 min	334	3	10.5	3.8 ± 0.6	97	4
24 h	347	ND	22.9	1.4 ± 0.01	88	45

Data are mean data ± SD. At each time point, the plasma samples were measured 3 times in 3 different ELISAs for the 2 animal experiments. ND indicates not determined.

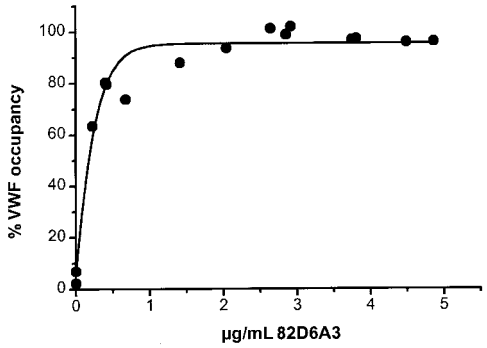

Figure 5. Relationship between the ex vivo VWF occupancy and 82D6A3 plasma levels. All mean data measured at the different time points in the 3 different dosage studies were used (Tables 1 and 2).

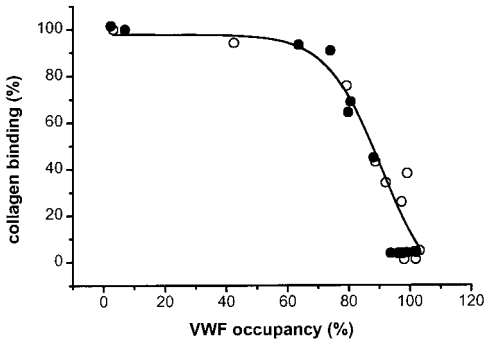

Figure 6. Relationship between the ex vivo and in vitro VWF binding to collagen and the VWF occupancy. The ex vivo data are the mean data measured at the different time points in the 3 different dosage studies shown in Tables 1 and 2 (●) and the in vitro data were obtained by adding known concentrations of mAb 82D6A3 to undiluted baboon plasma and measuring VWF occupancy and collagen binding (○). Data are the mean of 2 determinations in duplicate.

obtained, whereas administration of 100 μg/kg resulted in 58% inhibition.

Administration of the lowest 82D6A3 dose (100 μg/kg) to the baboons revealed that when 80% of the VWF-A3 domain sites were occupied (30%-36% inhibition of VWF binding to collagen), 58% reduction in CFRs was observed with no prolongation of the bleeding time. When a 100% decrease of CFRs was observed after administration of 300 μg/kg 82D6A3 (and also of 600 μg/kg) the bleeding time increased 2.7 times (60 minutes, 300 μg/kg), which was not statistically significant. At these doses, 100% VWF was occupied with 82D6A3 and 100% ex vivo inhibition of the VWF binding to collagen was observed. It is interesting to note that when we studied the effect of 300 μg/kg of an anti-GPIIb/IIIa mAb 16N7C2 in exactly the same animal model, a more than 15-fold increase in bleeding time was observed, which was statistically significant.[19] Thus the therapeutic window of 82D6A3 seems to be broader when compared to the one of an anti-GPIIb/IIIa blocker in our animal model.[19] This suggests that bleeding problems might be less expected when 82D6A3 is used as an antithrombotic agent. The observation that no significant decreases in platelet count and VWF-Ag levels were observed furthermore pointed out that the observed antithrombotic effect is indeed due to the specific inhibition of the VWF-collagen interaction.

82D6A3 has a high affinity for VWF (K_d 0.4 nmol[17]), which is reflected here by the observation that VWF is still occupied by 63% with the antibody 48 hours after its administration. On the other hand, to be functionally active, at least up to 80% VWF occupancy is needed to have an ex vivo and in vitro inhibition of VWF binding to collagen. This level was maintained for up to 5 hours after the administration of 300 and 600 μg/kg 82D6A3. The observation that up to 80% VWF occupancy is needed to be functionally relevant might be explained by the fact that when VWF is occupied for 74%, the ratio VWF monomer/mAb is 8. So too many VWF subunits are devoid of mAb to have some effect in VWF-collagen binding. It has to be noted, however, that at 100% VWF occupancy, the VWF monomer/mAb ratio (both in vitro and ex vivo) is 2.8, which demonstrates that both in vitro and ex vivo not all VWF-A3 domains are accessible for 82D6A3 binding.

Although many in vitro studies demonstrated that the VWF-collagen interaction is vital for sustaining platelet adhesion under high shear conditions, this study is the first experimental proof of the in vivo importance of this interaction. It is of interest to note that for a very long time no bleeding disorders due to an isolated defect in VWF binding to collagen were known. Only recently 2 patients, mother and daughter, were identified with a moderate bleeding disorder due to Ser968Thr mutation in the A3 domain of VWF resulting in a defective binding of VWF to collagen.[22] These recent results together with our data finally unequivocally demonstrate the in vivo relevance of the VWF-collagen interaction.

In conclusion, blockade of VWF-collagen interaction by 82D6A3 reduced platelet thrombus formation in the injured and stenosed baboon femoral arteries in a dose-dependent manner. In view of the lack of effect on the bleeding time, it is worthwhile to develop this approach further to determine its potential to treat acute arterial thrombotic syndromes.

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Inhibition of the von Willebrand (VWF)–collagen interaction by an antihuman VWF monoclonal antibody results in abolition of in vivo arterial platelet thrombus formation in baboons

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Cardiovascular Biology and Cell Signalling

Coronary artery in-stent stenosis persists despite inhibition of the von Willebrand factor - collagen interaction in baboons

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Summary

Revascularization techniques, such as angioplasty and stent implantation, frequently lead to restenosis due to the formation of neointima after platelet activation and the concomittant release of various smooth muscle cell mitogenic and attractant factors. We here investigate whether inhibition of initial platelet adhesion after stent implantation can decrease neointima formation in a clinically relevant baboon model of in-stent stenosis using standard treatment with aspirin, clopidogrel and heparin. Inhibition of platelet adhesion was established by administration of the anti-von Willebrand factor (VWF) monoclonal antibody 82D6A3, which inhibits VWF binding to collagen. Administration of 82D6A3 resulted in a complete inhibition of VWF binding to collagen during the first three days after stent implantation. No thrombocytopenia or prolongation of the bleeding time was observed. Our results show that the formation of neointima was not affected in the group of baboons where primary platelet adhesion was abolished with 82D6A3 when compared to the control group. Vascular injury scores were the same in both groups. Inhibition of platelet adhesion during the first three days after stenting, on top of standard treatment with aspirin, clopidogrel and heparin, had no effect on neo-intima formation in a baboon model of in-stent stenosis. During the last decade, attempts to translate seemingly effective therapies based on smaller animal experimentation to the clinic have consistently failed. This study, using a non-human primate model that more closely resembles the clinical situation, presents a model that may be of further clinical interest for studying the prevention of restenosis.

Keywords

von Willebrand factor, stenosis, baboon model, stent, neointima

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Introduction

Coronary artery disease is a worldwide problem, being one of the leading causes of morbidity and mortality. To treat arterial stenoses, percutaneous transluminal coronary angioplasty (PTCA) and stent deployment has become a widely applied strategy. Implantation of a stent efficiently prevents acute vessel recoil and limits late arterial shrinkage and remodeling, leading to chronic lumen loss after angioplasty. Nevertheless, angioplasty and stent implantation can still lead to a renarrowing of the treated vessel, resulting in a clinically significant restenosis of the targeted area after 6–12 months in a substantial number of treated patients (1). Compared to bare metal stenting, the use of drug-eluting stents reduced the rate of in-stent restenosis significantly (2–5), but concerns are rising regarding a possible increase in the rate of stent thrombosis caused by antiproliferative agents eluting from the stents, possibly due to impaired reendothelialization (6) or altered endothelial gene expression (7).

Restenosis is a multi-factorial complication mediated by several key processes, including platelet activation, which results in the release of mitogenic and chemo-attractant factors that mediate vascular smooth muscle cell (VSMC) migration and proliferation and the formation of neointima. Despite several approaches, targeting different agonists, the 'magic bullet' to pre-

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vent this multi-factorial process has not yet been found. Antithrombotic agents, such as aspirin and clopidogrel, are routinely used during angioplasty procedures to prevent acute thrombus formation (8–11). These inhibit only one out of many amplification loops involved in platelet activation, resulting in only a partial inhibition of acute platelet thrombus formation and no clinical effect on restenosis. Inhibitors of platelet aggregation have been a promising target, but clinical trials using glycoprotein (GP) IIb/IIIa inhibitors such as ReoPro report conflicting results (12–16). Indeed, GPIIb/IIIa antagonists do not interfere with the primary adhesion of blood platelets, and consequently, can not prevent initial platelet activation and the concomitant release of VSMC mitogenic and attractant factors that trigger neointimal hyperplasia. In contrast to those interfering with later steps in platelet dependent haemostasis, antithrombotic agents that effectively block primary platelet adhesion reduce initial platelet activation events, making them attractive candidates for the prevention of neointima formation. The effect of several inhibitors of platelet adhesion on neointima formation after angioplasty has been studied in small laboratory animals, including guinea pig, hamster, rabbit and rat, all with promising results (11, 17–22). Unfortunately however, successful outcome in small animal models for the prevention of restenosis, repeatedly did not allow straightforward extrapolation to human clinical in-stent restenosis (23, 24).

In order to better predict the outcome in patients, we developed a clinically relevant human-like baboon coronary artery model of in-stent stenosis in which we assessed the effect of inhibition of primary platelet adhesion on neointima formation. Standard clinical procedures, closely mimicking the human clinical situation were used, including the standard antithrombotic treatment regimen with aspirin, heparin and clopidogrel. Inhibition of platelet adhesion was established using the monoclonal antibody (moab) 82D6A3, that binds to the VWF A3-domain and inhibits the VWF-collagen interaction resulting in antithrombotic effects (25, 26).

Methods

Animals

Baboons (*Papio ursinus*) of male sex, weighing 18–30 kg, were fed with a standard diet and drinking water was available *ad libitum*. Housing, treatment, surgery and caring of the animals was approved by the Control Committee on Animal Experimentation of the University of the Free State, South Africa.

Production and purification of 82D6A3

Monoclonal antibody 82D6A3 was raised in mice against human VWF (27) and the antibody was produced using the INTEGRA CELLine CL 350 system (Elscolab, Kruibeke, Belgium) using serum-free nutrient medium (TX-HYB, Thromb-X, Leuven, Belgium) as described by the manufacturer. The moab 82D6A3 was purified by Protein A chromatography (GE Healthcare, Uppsala, Sweden) and purity was checked in SDS-PAGE. Endotoxin levels in the antibody preparations were measured using the QCL-1000® Chromogenic LAL Endpoint Assay (BioWhittaker, Walkersville, ME).

Angioplasty and stent implantation

Before surgery, baboons were anaesthetized by intramuscular injection of 10 mg/kg/30 min ketamine (Ketalar®, Pfizer, Brussels, Belgium) and 2 mg/kg/30 min xylazine (Chanazine®, Bayer, Leverkusen, Germany). Electrocardiogram and arterial blood pressure were continuously monitored. A 6F introducer sheath was placed into the femoral artery for blood sampling, whereas drugs were administered intravenous (iv.) via the forearm. Approximately 10 min before angioplasty, 10000 IU heparin (Heparine Rorer, Aventis/sanofi-synthelabo, Brussels, Belgium) and 900 mg aspirin (Aspegic®, Aventis/sanofi-synthelabo) were injected iv. and treatment animals received a bolus of 1 mg/kg 82D6A3, whereas control animals received a comparable volume of PBS. The coronary artery was visualized using a 6F catheter and Ultravist® (Berlex, Montréal, Canada) as a contrast agent. A 18 mm MULTI-LINK VISION™ coronary stent (Guidant, Diegem, Belgium) was mounted on a conventional coronary angioplasty balloon catheter and deployed in a selected arterial segment of either the left anterior descending artery or the right coronary artery, using an inflation pressure of 8 atm for 30 s. Vessel patency after stent implantation was confirmed by coronary angiography after intra-arterial injection of 0.25 mg nitroglycerin. Finally, the arteriotomy was repaired and the dermal layers were closed using standard techniques. After surgical procedure, animals were intramuscularly injected with enrofloxacine (Baytril®, Bayer; 5 mg/kg). Animals received additional boli of 1 mg/kg 82D6A3 (treatment animals) or a comparable volume of PBS (control animals) at 12 h, 24 h and 48 h after stent implantation via the femoral vein. On the day of surgery, blood samples were drawn before the first antibody (or PBS) injection and after the surgical procedure. The next two days, blood samples were drawn via the femoral vein immediately before and after antibody (or PBS) administration. All animals received clopidogrel (Plavix®, Aventis/sanofi-synthelabo, 75 mg/day, peroral) during 28 days, starting from the day before surgery.

After 28 days, the animals underwent a control angiogram and blood samples were drawn before they were sacrificed by iv. injection of an overdose of pentabarbitone (Euthapent, Kryon Laboratories, Johannesburg, South Africa). All experiments and analyses were performed by researchers who were blinded for the treatment regimen of the animals.

Platelet count, coagulation and bleeding time

All blood samples were collected in Vacutainers™ containing 0.105 M citric acid (BD Biosciences, Plymouth, UK). Platelets were counted using a Technicon H3 (Bayer Healthcare, Isando, South Africa). Plasma was prepared by centrifugation for 15 min at 1000 x g. The prothrombin time (PT) and activated partial thromboplastin time (aPTT) were measured at 37°C in a STA® Compact coagulometer (Roche, Mannheim, Germany) using STA®-Neoplastine® and STA®-PTT Automate 5 reagents (Diagnostica Stago, Taverny, France) respectively. Bleeding time in baboons was measured as described (25) on the chest using a Surgicutt® device (ITC, Rodano, Italy).

Blood parameters

Blood samples were collected in Vacutainers™ containing spray coated Li-Heparin and a polymer separator gel (BD Biosciences). Creatinine, alkaline phosphatase, aspartate amino-

transferase (AST), alanine aminotransferase (ALT), total creatinine kinase, creatinine kinase MB iso-enzyme, troponine-I, C-reactive protein (CRP) and lactate dehydrogenase (LDH) were analysed using standard laboratory techniques.

VWF:Ag determination

Antigen levels of VWF in plasma samples were determined using a sandwich ELISA as described (28, 29). The baseline baboon plasma sample (taken before surgery) was used as an internal standard for the VWF:Ag determination in each baboon.

Determination of the VWF-collagen binding activity (VWF:CBA)

Functional binding of VWF to collagen was performed essentially as described (29). The absorbance of the baseline sample was arbitrarily set as 100% VWF:CBA.

Plasma 82D6A3 levels and VWF occupancy

The plasma 82D6A3 levels in plasma and VWF occupancy by 82D6A3 were determined in ELISA as described (25). To calculate VWF occupancy, respective baseline plasma samples supplemented with a saturating amount of 82D6A3 (15 µg/ml) were used as 100% occupancy standards.

Histological preparation of coronary segments

After sacrifice of the animal, the heart was removed and pressure-fixed at 80 mmHg with PBS containing 4% formaldehyde to preserve arterial wall dimensions. The coronary segments containing a stent were carefully dissected and fixed with 4% formaldehyde in PBS for 2–4 h at 4°C. After washing overnight at 4°C in PBS containing 6.8% saccharose, the segments were dehydrated for 8 h at 4°C with 100% ethanol before embedding in hydroxy-ethylmethacrylate using Technovit 8100 (Kulzer Histo-Technik 8100, Heraeus Kulzer, Wehrheim, Germany) following the manufacturer's instructions. Starting from the proximal part of the coronary segment, pairs of 5 µm cross-sections were cut using a carbide tungsten knife with 100 µm intervals and stained with hematoxylin and eosin for morphometric analysis. Where possible, up to ten sections, equally distributed over the stent segments, were blindly selected for analysis.

Morphometric analysis of coronary segments

Blinded morphometric analysis was performed under an Eclipse TE200 microscope (Nikon, Tokyo, Japan) equipped with a Basler 113C RGB color digital camera (Basler, Ahrensburg, Germany). Images were captured and processed using Lucia G software version 4.81 (Laboratory Imaging, Prague, Czech Republic). To determine the degree of neointima formation, the internal elastic lamina (IEL) and the border of the lumen were

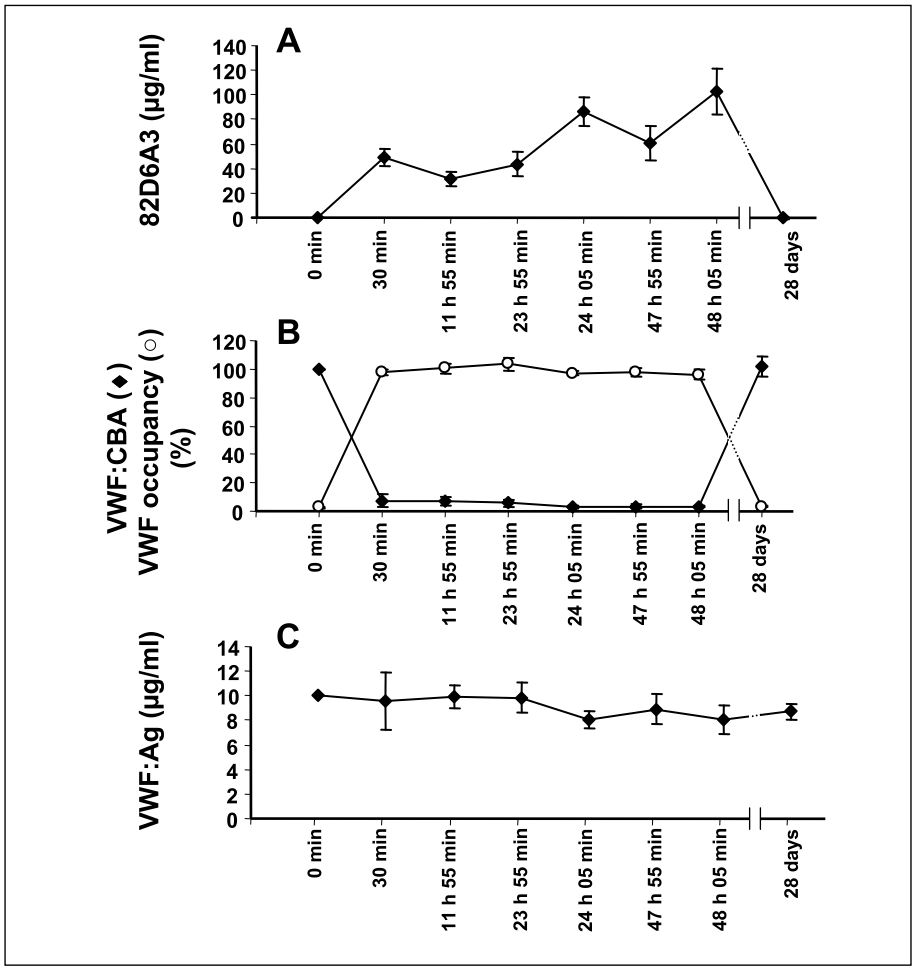

Figure 1: Pharmacokinetics of 82D6A3 after multiple injection in baboons. Baboons were injected with 1 mg/kg 82D6A3 10 min before PTCA and stent implantation and at 12 h, 24 h and 48 h after the first injection. Blood was drawn before the first injection (0 min), after surgery (30 min) and 5 min before and after each of the subsequent 82D6A3 injections. Plasma levels of 82D6A3 (A), VWF:CBA (B, ◆), VWF occupancy (B, ○) and plasma VWF concentrations (C) were determined. Data are represented as mean ± SEM of double measurements of samples taken from five baboons.

Table 1: Ex-vivo plasma analyses, blood platelet count and bleeding time in baboons injected with multiple boli of 82D6A3 or PBS (controls).

	82D6A3				Control			
timepoint	platelets ($x10^3/\mu l$)	bleeding time (min)	PT (s)	aPTT (s)	platelets ($x10^3/\mu l$)	bleeding time (min)	PT (s)	aPTT (s)
0 min	362±30	<1.3	8.8±0.2	26.2±4.1	295±24	<1.3	9.4±0.3	31.4±2.0
30 min	342±30	n.d.	13.7±4.2	>120	277±20	n.d.	9.9±0.3	>120
11 h 55 min	345±38	n.d.	8.7±0.2	32.3±0.9	304±33	n.d.	10.0±0.0	32.0±1.1
23 h 55 min	341±30	n.d.	9.2±0.5	38.2±2.5	276±48	n.d.	9.4±0.2	32.1±0.5
24 h 5 min	339±26	4.9±2.5	9.1±0.5	35.3±1.4	341±50	<1.3	n.d.	n.d.
47 h 55 min	342±27	n.d.	8.5±0.2	32.6±1.2	287±22	n.d.	9.5±0.5	31.5±1.2
48 h 5 min	348±30	3.2±0.8	8.6±0.4	32.0±1.0	n.d.	n.d.	n.d.	n.d.
28 d	282±38	2.1±0.4	9.0±0.8	32.6±0.8	247±24	1.8±0.3	9.6±0.2	32.2±0.8

Data are represented as the mean ± SEM, n = 5; n.d. = not determined.

traced and the areas enclosed by each were calculated. The neointimal area was defined as the area enclosed by the IEL subtracted by the area of the lumen (IEL area – lumen area), whereas the percentage of stenosis was defined as the ratio between the neointimal area and the IEL area ((neointimal area/IEL area)*100). The intima over media ratio (I/M) was calculated by dividing the area of the neointima by the area of the media. The mean injury score for each cross-section was calculated by determining the extent of the injury caused by every individual coil wire in the cross-section according to Schwartz et al. (30). Injury scoring was performed on hematoxylin and eosin stained cross-sections using green light (510–560 nm).

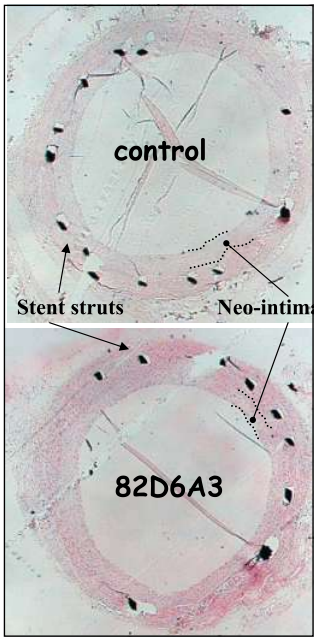

Figure 2: Coronary cross-sections of the stented segments of control and treated baboons. Sections of 5 µm were stained with hematoxylin and eosin for morphometric analysis and show the presence of the stent struts in the media. In-stent neointima formation is clearly visible in both groups.

Statistical analysis

The degree of neointima formation and injury score from control baboons and baboons treated with 82D6A3 were compared by the Student t-test. The differences were considered to be statistically significant when $p<0.05$.

Results

Pharmacokinetics of 82D6A3 in baboons during and after PTCA and stent implantation

The effects of multiple bolus injections of 82D6A3 during and after PTCA were analysed. To achieve an optimal inhibition of platelet adhesion, a complete occupancy of VWF by 82D6A3 is essential (25). The first bolus injection of 1 mg/kg of 82D6A3 resulted in a full occupancy of VWF by 82D6A3 that was sustained for at least 12 h (Fig. 1B). Additional bolus injections of 82D6A3 were given after 12 h, 24 h and 48 h, resulting in increasing plasma concentrations of 82D6A3 and a complete VWF occupancy during the first 48 h after the first injection (Fig. 1A, B). Moreover, in view of the sustained complete occupation for 12 h after the first bolus injection, we assumed that the applied regimen of 82D6A3 administration resulted in a complete VWF occupancy for at least 60 h after PTCA and stent implantation. In accordance with the VWF occupancy data, the ex-vivo binding of VWF to collagen was completely blocked for 48 h after the first antibody administration (Fig. 1B), suggesting a complete inhibition of VWF-mediated platelet adhesion. VWF:Ag levels were not affected by the 82D6A3 treatment and remained constant at physiologic values (10 µg/ml) throughout the experiment (Fig. 1C). VWF:Ag levels and VWF:CBA were normal in control baboons (data not shown).

Inhibition of the VWF-collagen interaction by 82D6A3 did not induce bleeding problems in any of the treated baboons. In agreement with previous studies (25), administration of 82D6A3 did not result in thrombocytopenia and was not accompanied with a significant prolongation of the bleeding time and PT (table 1). Although a prolongation of the aPTT was observed immediately after surgery, it was completely restored 12 h after sur-

gery. The same increase was present in the control baboons, attributing this rise in aPTT to the administration of heparin during PTCA and stent implantation.

Finally, the procedure did not cause liver, cardiac or kidney dysfunction nor inflammation, since alkaline phosphatase, AST, ALT, total creatinine kinase, creatinine kinase MB iso-enzyme, troponine-I, creatinine, CRP and LDH were within normal range throughout the experiments (data not shown).

Morphometric analysis of coronary sections

In order to investigate the effect of complete abolishment of the VWF-collagen binding during the first three days after PTCA and stenting, stented coronary segments were isolated after 28 days for morphometric analysis. Figure 2 shows a representative section isolated from a control baboon and a baboon treated with 82D6A3, 28 days after PTCA and stenting.

The injury score on all selected sections of both control and treated animals was assessed and did not differ significantly between control animals and those receiving 82D6A3 (1.24 ± 0.23 vs. 1.46 ± 0.23; $p>0.1$, Fig. 3A) and resemble injury scores obtained in porcine in-stent restenosis studies (31, 32). The injury scores confirmed that a uniform degree of injury was induced in the coronary arteries by the PTCA and stenting procedure in both groups.

Of all selected sections, the degree of stenosis was morphometrically determined by dividing the area of neointima by the area enclosed by the IEL. This analysis revealed a mean percent stenosis of 31.2 ± 5.9 (n=4) in control animals. However, no significant reduction in neointima formation could be observed in animals treated with 82D6A3 where a mean percent stenosis of 36.2 ± 9.0 (n=4) was observed (Fig. 3B). This observation was confirmed by the I/M ratios, which did not differ significantly between both groups of animals (Fig. 3C).

Discussion

Restenosis is a complex process, involving several physiological processes such as thrombus formation, VSMC proliferation and arterial remodeling. In addition to stent placement, a variety of approaches has been investigated to prevent thrombus and neointima formation, including brachytherapy, VSMC antiproliferative and antithrombotic therapy. However, none of these can exclude that pathological restenosis still occurs in a substantial number of treated patients (1, 33, 34). Despite initial promising results, obtained in a variety of small animal models, extrapolation to human clinical restenosis is far from straightforward (23, 24), indicating the need for more human related animal models.

Therefore, we here developed a clinically relevant baboon model of in-stent stenosis to study whether the inhibition of VWF-binding to collagen can contribute to the reduction of neointima formation after PTCA and stenting using procedures closely mimicking the human clinical situation. Administration of the previously described anti-VWF moab 82D6A3 (25, 26, 35) on top of the standard treatment regimen with aspirin, clopidogrel and heparin, resulted in a complete inhibition of the ex-vivo collagen-VWF interaction during the first 60 hours after surgery, without prolongation of the bleeding time. We previously described that the antibody completely abolishes thrombus formation in a modified Folt's model in baboons, indicating its ability to efficiently block platelet adhesion in this animal. Interestingly however, this study shows no reduction of neointima formation 28 days after stenting when 82D6A3 was used to block platelet adhesion. This finding, most probably reflecting the outcome in man, is in sharp contrast to a variety of studies that reported beneficial effects of inhibitors of platelet adhesion on angioplasty-induced neointima formation (11, 17–22). Though, the present study differs from these in some substantial features which all may account for the observed discrepancy.

First, previous studies were performed in small laboratory rodents, i.e rabbit (17), hamster (18, 20), guinea pig (19) and rat (11, 21, 22) in which the cardiovascular and physiological sys-

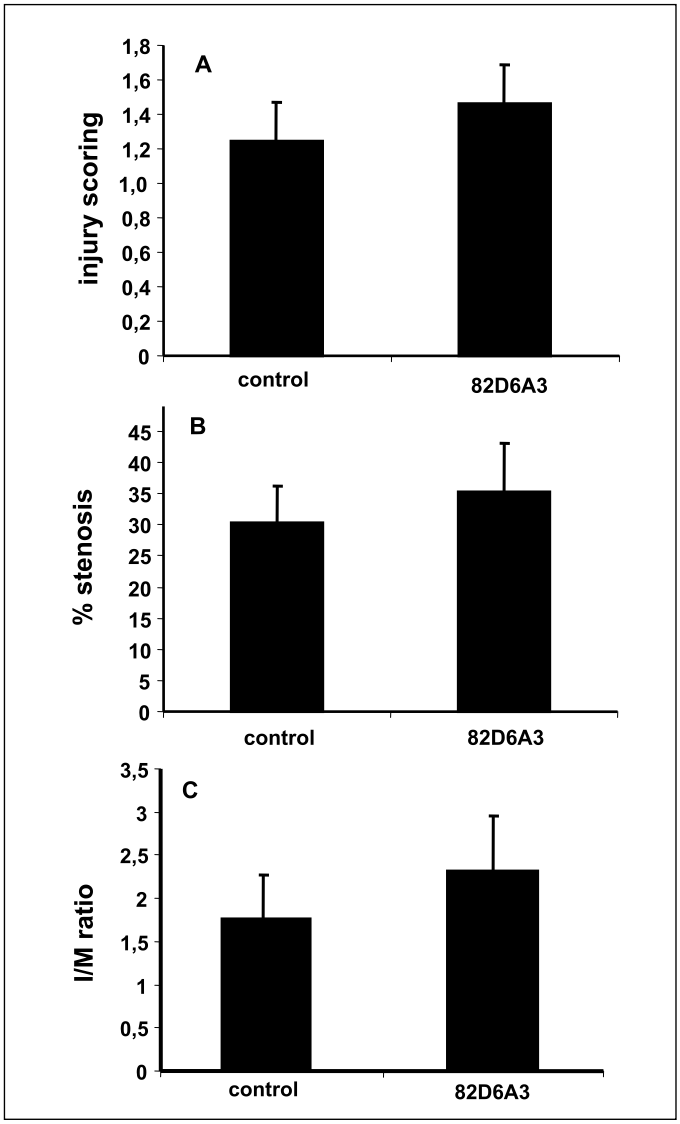

Figure 3: Morphometric analysis of treated arterial sections. A) Both groups of animals showed the same degree of arterial injury induced by the PTCA and stenting procedure. B) No significant difference could be observed in percent in-stent stenosis between the two groups. C) The I/M ratios from both groups of animals were not statistically different. Data are represented as mean ± SEM (n=4).

tems are far from identical to those in man. Second, although angioplasty is the standard technique for revascularization of coronary arteries, which are muscular arteries, arterial injury in previous in-vivo studies was mainly performed in peripheral arteries, namely femoral (11) and carotid arteries (18–22). Clearly, both injury and response to injury may be very different in coronary versus peripheral arteries (36). Third, no stents were implanted in the previous studies. It is well known in man that processes responsible for lumen loss after angioplasty are different from those after angioplasty and stent implantation (1). Finally, in previous studies, the anti-adhesive agent was studied alone, whereas the animals in our study were also treated with aspirin, clopidogrel and heparin, i.e. the routine clinical regimen that is used to minimize the risk of acute thrombosis or other procedural complications (8). Although it would be very interesting to see the possible effect of 82D6A3 alone in our model, such a strategy would not be supported for further clinical studies in humans. Therefore, in an effort to mimick clinical reality as close as possible, moab 82D6A3 was tested on top of the standard clinical treatment. In fact, whereas aspirin inhibits subacute stent thrombosis, it is not effective in the inhibition of restenosis after angioplasty (37) and long term administration of clopidogrel on top of aspirin does not seem to result in increased clinical benefits following angioplasty (38, 39).

Still, it is important to note that also this baboon model does not perfectly mimic human clinical restenosis. Indeed, we used a healthy baboon coronary artery, which is significantly different from the treated pathological human arteries, where intimal (atherosclerotic) lesion already exists and several physiological responses, such as healing, are bound to be abnormal.

Restenosis is a ~6 month phenomenon and in the current setup with a three day inhibition of platelet adhesion and an endpoint analysis after 28 days, we can not exclude possible long-term effects of 82D6A3 on neointima formation. However, both experimental animal models, including baboons, and human autopsy and atherectomy show that the thrombogenicity of the exposed vessel wall drastically decreases after a few days (40–42), suggesting that platelet adhesion (or the inhibition thereof) would not be important in the late (>1 month) development of restenosis. In line with this, studies in small animals indicated that short-term inhibition of initial platelet adhesion results in decreased neointima formation already within two weeks after vascular damage (11, 17–20, 22, 43, 44)

Taken together, our study, using a coronary in-stent stenosis model in baboons that is closely related to the human situation, is the first report investigating the possible antistenotic effect of inhibitors of the VWF-collagen interaction after stent implantation. Our results suggest that, in the current routine clinical setting, additional inhibition of platelet adhesion is not sufficient to reduce neointimal hyperplasia, which could indicate an overestimation of the role of VSMC mitogenic and attractant factors that are released upon platelet activation after PTCA and stenting. Whether inhibition of platelet adhesion affects other biological processes besides neointmial hyperplasia (e.g. alterations in neointimal compostion) remains unclear and may warrant future research. It is very likely that the contribution of the individual elements that mediate restenosis, including platelet activation after platelet adhesion, may not be consistently proportional in every patient or might even differ between lesions in the same patient. In the absence of a single 'magic bullet' therapy that effectively addresses all of these pathogenic elements, the ability to routinely prevent restenosis will likely require accurate clinical identification and understanding of the principal factors governing the biology of a given restenotic lesion.

Finally, in view of the repeated failure of extrapolating beneficial effects of various agents on the prevention of stenosis obtained in small animal models to human clinical restenosis (23, 24), this study points out the importance of evaluating new antistenotic therapies in a clinically relevant model and in the meantime presents a model that may be of further clinical interest for studying the prevention of restenosis.

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ORIGINAL ARTICLE

A human monoclonal antibody inhibiting partially factor VIII activity reduces thrombus growth in baboons

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Summary. *Background:* The inhibitory activity of an anti-factor VIII (FVIII) antibody can be modulated through glycosylation of the antigen binding site, as has recently been described. This offers the opportunity to develop an optimized anticoagulant agent targeting partial FVIII inhibition. *Objectives:* We investigated in non-human primates the antithrombotic activity, pharmacokinetics,and pharmacodynamics of a human monoclonal antibody, Mab-LE2E9Q, inhibiting FVIII activity partially. *Methods:* The ability of Mab-LE2E9Q to prevent thrombosis was evaluated in baboons after administration of 1.25 and 5 mg kg^{-1} antibody or saline as a single intravenous (i.v.) bolus. Thrombus development was recorded in expansion ('venous') and in Dacron® ('arterial') thrombosis chambers incorporated in an extracorporeal arteriovenous shunt implanted between the femoral vessels 1 h, 24 h and 7 days after the administration of Mab-LE2E9Q. *Results:* Mab-LE2E9Q reduced thrombus growth to a similar extend 1 h, 1 day and 1 week after administration of the antibody. *Ex vivo* pharmacodynamic analysis indicated that the evaluation of the residual FVIII activity was strongly dependent on the type of FVIII assay and on the phospholipid concentration in the assay. No significant difference in bleedings was observed between animals treated with Mab-LE2E9Q or with saline. *Conclusions:* Understanding the role of glycosylation in FVIII inhibition by a human monoclonal antibody allowed selection of an antibody inhibiting only moderately FVIII activity while significantly reducing thrombus development in a baboon extracorporeal model. As that antibody did not increase the bleeding tendency, it may represent a novel type of a long-acting antithrombotic agent with an optimal safety/efficacy profile.

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Introduction

Most anticoagulant agents developed so far target enzymes involved in the coagulation cascade. This strategy is associated with well-known risks. Vitamin K antagonists exert their activity not only on procoagulant enzymes but also on inhibitors of the coagulation cascade such as Protein C [1] and require monitoring. The development of synthetic inhibitors of coagulation enzymes has increased the specificity of the inhibition but has generated the possibility of unexpected toxicity, notably for the liver [2]. Targeting the non-enzymatic cofactors of the coagulation cascade therefore appears as a potentially attractive alternative provided that limited inhibition of the activity of the target cofactor can be guaranteed so as to prevent bleeding.

Based on that concept, we have produced a human monoclonal antibody, Mab-LE2E9Q, partially inhibiting factor VIII (FVIII) activity irrespective of the excess of antibody over FVIII. That antibody was generated by introducing a point mutation in the gene of an anti-FVIII antibody, Mab-LE2E9, derived by immortalization of B lymphocytes from a patient with mild hemophilia A, who had developed a strong immune response to FVIII. Mab-LE2E9 inhibits 90% of FVIII activity and prevents FVIII from binding to von Willebrand factor (VWF) [3]. The point mutation introduced in the Mab-LE2E9 gene to generate Mab-LE2E9Q removes a glycosylation site in the variable region of the heavy chain. As a result, Mab-LE2E9Q inhibits only about 40% of FVIII activity and does not prevent FVIII from binding to VWF [4].

The antithrombotic efficacy of Mab-LE2E9Q has been evaluated in mice that carry a mutation in the heparin-binding site of antithrombin III ($At^{m/m}$) and display spontaneous chronic thrombosis at several sites [5]. This model does not require surgical intervention and is representative of thrombosis in patients with severe prothrombotic risk factors. Although it inhibits only about 40% of FVIII activity, administration of Mab-LE2E9Q completely prevented acute thrombosis in $ATIII^{-/-}$ mice [4].

Given the low concentration of FVIII in plasma and the long half-life of an IgG antibody, treatment with the Mab-LE2E9Q antibody could be very convenient, allowing one administration every month. In addition, because Mab-LE2E9Q inhibits FVIII activity only partially, FVIII activity can be normalized very rapidly by administration of FVIII independently of the antibody concentration in plasma [4]. Accordingly, Mab-LE2E9Q is so far the only anticoagulant agent that can be neutralized specifically and without any delay. Mab-LE2E9Q therefore appears as a promising novel type of antithrombotic drug.

Here, we evaluated the ability of Mab-LE2E9Q to reduce thrombus development in a thrombosis model in non-human primates.

Materials and methods

Thrombosis model in baboons

Study design The study was randomized and investigators were blinded towards treatment regimen. Male baboons (*Papio ursinus*) were used. The animals weighed between 7.5 and 19 kg and were disease free for at least 2 months prior to the experiments. All procedures were approved by the Ethics Committee for Animal Experimentation of the University of the Free State in accordance with the National Code for Animal Use in Research, Education, Diagnosis and Testing of Drugs and Related Substances in South Africa.

Baboons were immobilized and handled under anesthesia with ketamine hydrochloride (Anaket-V; Centaur Laboratory, Johannesburg, South Africa). Permanent polytetrafluoroethylene (Teflon) and silicone rubber (Silastic) arteriovenous shunts were implanted in the baboon femoral vessels [6]. Blood flow through the shunts varied between 100 and 120 mL min^{-1}. In each experiment, a thrombogenic device prefilled with saline to avoid a blood-air interface was incorporated as an extension segment into the permanent arteriovenous shunt by means of Teflon connectors (Fig. 1).

Platelet-dependent arterial thrombus was induced using Dacron® (1.26 cm^2; US Catheter Inc., Billerica, MA, USA) inserted into the wall of Silastic tubing (3-mm inside diameter) [7]. An expansion chamber (3.77 cm^2) was used to generate coagulation-dependent venous thrombosis. Blood flowed through the thrombogenic devices at a rate of approximately 120 mL min^{-1}. The initial shear stress was 318 s^{-1} for the Dacron section and 10 s^{-1} for the expansion chamber.

In the control studies, the devices were kept in place for 60 min or until they occluded. After removal of the device, blood flow through the arteriovenous shunt was re-established. The baboons were then treated with a single intravenous (i.v.) bolus of 1.25 or 5 mg kg^{-1} Mab-LE2E9Q or saline. Thrombogenic devices were placed for 60 min 1 h after antibody injection. Additional 60-min studies were carried out at 24 h after the antibody bolus injection. The extracorporeal shunts were then removed. At day 7, the baboons were shunted on the opposite site and 60-min thrombosis experiments were repeated.

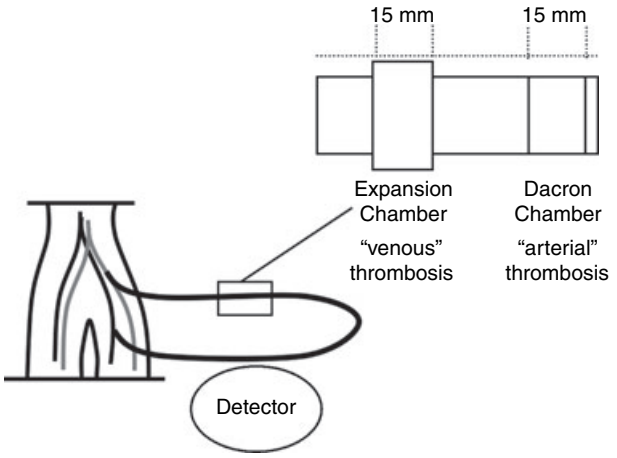

Fig. 1. Experimental protocol for extracorporeal thrombosis. Arteriovenous shunts were implanted in male baboon femoral vessels. Thrombogenic devices prefilled with saline were incorporated as extension segments into the permanent arteriovenous shunt. Platelet-dependent arterial thrombus was induced by inserting Dacron into the wall of Silastic tubing. Coagulation-dependent venous thrombosis was generated in an expansion chamber. The deposition of autologous ^{111}In-labeled platelets was followed with a gamma scintillation camera.

The procedure was carried out simultaneously with one animal treated with 1.25 mg kg^{-1} Mab-LE2E9Q, one with 5 mg kg^{-1} Mab-LE2E9Q and one with control buffer. Randomization of treatment was therefore restricted to sets of three animals. After the thrombosis experiments, animals were followed up for 53 days for clinical and biological parameters.

In the treated group, animals received either 1.25 or 5 mg kg^{-1} Mab-LE2E9Q. These doses of antibody were selected to allow for kinetic analyses and to ensure that Mab-LE2E9Q concentrations in plasma would remain above the concentration required to reach the maximal FVIII inhibition at least up to day 7 when the last thrombosis experiment had to be performed. Because the antibody inhibits FVIII only partially even in large excess over FVIII, the residual FVIII activity was expected to be in the same range for both treated groups up to the end of the thrombosis experiments.

Thrombus imaging Autologous platelets were labeled with ^{111}In-tropolone and reinjected into the animal 1 h before the start of the control experiment [7]. This allowed quantification of thrombus formation within the chambers on day 0 and 1. To provide image acquisition on day 7 the labeling procedure was repeated. Image acquisition of the grafts was done with a gamma scintillation camera fitted with a high-resolution collimator. To determine baseline blood radioactivity, imaging was also performed on an autologous blood sample each time the grafts were imaged. Regions of interest of the graft and expansion segments were selected to determine the deposited and circulation radio-activity in the dynamic image. The number of platelets deposited on the vascular graft material and in the expansion chamber was measured every 3 min.

The highest platelet deposition (maximal platelet binding) occurring during the observation period was determined for each experiment. An alternative model to evaluate platelet deposition was also used to take into account that platelets can detach from the thrombosis chamber, which is followed by further platelet binding. Therefore, the number of platelets bound during each time period of 3 min was calculated as the difference between the number of platelets at the end and at the beginning of the time period. The cumulative platelet deposition was then calculated as the sum of all platelet deposition >0 during each 3-min time period of an experiment (cumulative platelet binding).

Study endpoints

The primary endpoint of the study was the evaluation of the platelet deposition during the observation period. To allow for analysis of the data with a non-parametric test (Mann–Whitney *U*-test), the smallest group had to include at least four animals. Groups of five animals were therefore planned to allow for one potential casualty during the experimental procedure. The secondary endpoints included TB-402 pharmacodynamics and pharmacokinetics.

Mab-LE2E9Q ex vivo pharmacodynamics All animals used in this study were included in the extracorporeal thrombosis study and were treated with a single intravenous bolus of 1.25 ($n = 5$) or 5 mg kg^{-1} Mab-LE2E9Q ($n = 5$) or saline ($n = 5$). Blood samples were taken either directly from the shunt or by venopuncture.

FVIII:C levels were measured in a batch analysis at the end of the study using a chromogenic assay (Coatest^{R} FXa generation assay; Chromogenix-Instrumentation Laboratory SpA, Milano, Italy) according to the manufacturer's instructions. In addition, FVIII concentrations were evaluated using a modification of the Coatest^{R} FXa, in which phospholipids were diluted sixtyfold in tris(hydroxymethyl)-aminoethane (Tris) 50 mM, pH 7.3, 150 mM NaCl, 0.2% bovine albumin [8]. FVIII activity in test sample was determined by comparing FXa generation with that obtained using dilutions of a pool of human plasma. The sensititivity of low- and high-phospholipids assays to Mab-LE2E9Q inhibitory activity was evaluated in an *in vitro* spiking experiment. Various concentrations of Mab-LE2E9Q were added to baboon plasma, supplemented with 25 mM HEPES and incubated at 37 °C for 2 h before measuring the residual FVIII:C activity, using a Coatest^{R} SP FXa generation assay in which phospholipids were either undiluted or diluted sixtyfold, as above. In addition, FVIII:C levels were also measured in a 1-stage clotting assay (Actin FS; Dade Behring, Marburg, Germany), immediately after plasma collection.

Mab-LE2E9Q ex vivo pharmacokinetics Animals used in this study participated in the extracorporeal thrombosis study and the pharmacodynamic study and were treated with a single i.v. bolus of 1.25 ($n = 5$) or 5 mg kg^{-1} Mab-LE2E9Q ($n = 5$) or saline ($n = 4$). In addition, three animals received a single subcutaneous (s.c.) administration of Mab-LE2E9Q. Blood samples were taken at intervals after the administration of a single i.v. or s.c. administration of Mab-LE2E9Q, either directly from the shunt or by venopuncture.

Mab-LE2E9Q concentration was measured in a competitive assay in which the binding of biotinylated FVIII to insolubilized Mab-LE2E9Q was inhibited by Mab-LE2E9Q present in the test sample. One volume of test sample, diluted in Tris 50 mM, pH 7.3, containing 150 mM NaCl (TBS) and 1% bovine serum albumine (BSA) supplemented with 5% baboon plasma, was mixed with 1 volume of biotinylated FVIII. The mixture was added to 96-wells microtitration plates prealably coated with 50 μL Mab-LE2E9Q. Following a 2-h incubation period, plates were washed 3 times with TBS and bound biotinylated FVIII was detected by addition of streptavidine peroxidase followed by o-phenylenediamine. The concentration of Mab-LE2E9Q in the test samples was determined by comparing the inhibition of biotinylated FVIII binding to the inhibition of FVIII binding in mixtures containing known concentrations of Mab-LE2E9Q.

Pharmacokinetic data were fitted to a two-compartment model by weighted non-linear regression analysis or by visual examination. First, data were plotted and fitted to a sum of two exponential terms, $C(t) = Ae^{-\alpha t} + Be^{-\beta t}$, by weighted non-linear regression analysis using GraFit^{R}. Initial estimates of the model parameters were provided by visual examination. When the best fit obtained with GraFit did not provide a good estimation of the terminal elimination phase, data were fitted by visual examination using Excel. Data were plotted and an optimal curve corresponding to $C(t) = Ae^{-\alpha t} + Be^{-\beta t}$ was obtained by selecting different B, α and β parameters. A was calculated as the antibody concentration measured 5 min after i.v. administration minus B.

The drug clearance parameters were calculated from the extrapolated intercepts (A, B) and exponents (α, β) describing the disposition of each administered product, using standard formulas. The following clearance parameters were calculated: initial drug concentration in the blood: $C_0 = A + B$; volume of the central compartment: V_c = dose/($A + B$); total volume of distribution: V_d = dose/B; extrapolated area under the curve: AUC = $A/\alpha + B/\beta$; plasma clearance: Clp = dose/AUC; initial half-life: $t_{1/2\alpha} = \ln 2/\alpha$; secondary half-life: $t_{1/2\gamma} = \ln 2/\beta$, mean residence time: MRT = $V_d \times$ AUC/dose.

The pharmacokinetics parameters were determined for each animal individually. The mean and standard deviation (SD) of the pharmacokinetics parameters were then calculated per dose group.

Laboratory values

Laboratory values were obtained at day 0, 1, 2, 5, 7, 9, 12, 16, 19, 23, 26, 30, 44 and 60. They included hemoglobin level, hematocrit level, platelet count, white blood cell count, FVIII and TB-402 concentration.

Immunogenicity

IgG and IgM antibodies to Mab-LE2E9Q were detected using ELISA. Mab-LE2E9Q (2 μg mL^{-1} in 20 mM glycine, 34 mM NaCl, pH 9.2) was incubated in microtitration plates overnight. After three washes with 140 mM NaCl, 67 mM KCl, 20 mM Na_2HPO_4, 4.4 mM KH_2PO_4, pH 7.4, Tween-80 0.005% (PBS-Tween), baboon PL diluted 1/20 and 1/100 in TBS with 1% BSA 0.005% Tween-80 (Tris-BSA-Tween) was incubated in the wells at room temperature for 2 h. After three washes with PBS-Tween, baboon antibodies bound to Mab-LE2E9Q were detected by addition of goat antibaboon antibodies conjugated to horseradish peroxidase (Biorad; ref. 170-6516) diluted thousandfold in Tris-BSA-Tween followed after three washes with PBS-Tween by addition of o-phenylenediamine. Optical densities were read at 490 nm.

Statistical analysis

For pharmacokinetics analysis, data obtained from animals treated with 1.25 and 5 mg kg^{-1} were analyzed separately. For comparison of hemoglobin levels and platelet deposition in thrombosis chambers, data from the animals treated with both doses were combined and compared with data from untreated animals. Data were analyzed using the Mann–Whitney *U*-test. Significance was defined as $P \leq 0.05$.

Results

Prevention of thrombosis

The ability of Mab-LE2E9Q to prevent thrombosis was evaluated in an extracorporeal thrombosis model in baboons. At the beginning of the experiment, an extracorporeal shunt was inserted between the femoral artery and the femoral vein on one side of the animal. Thrombogenic devices were placed for 60 min 1 h before, 1 h and 24 h after Mab-LE2E9Q or saline injection. The extracorporeal shunts were then removed. At day 7, the baboons were shunted on the opposite site and a thrombosis experiment was repeated. Platelet deposition was recorded as a function of time in the expansion ('venous') thrombosis chamber and in the Dacron^R ('arterial') thrombosis chamber incorporated in the extracorporeal arteriovenous shunt.

In the control group, five animals were treated with saline. In the treated groups, five animals received 5 mg kg^{-1} Mab-LE2E9Q and five others 1.25 mg kg^{-1} antibody. Because the antibody inhibits FVIII only partially even in large excess over FVIII [5], the residual FVIII activity was expected to be in the same range for both treatment groups at least up to day 7 when the last thrombosis experiment was performed. Those assumptions were validated in the pharmacodynamic and pharmacokinetic study (see below).

In the 10 animals treated with Mab-LE2E9Q, the maximal platelet deposition 1 h, 1 day and 7 days after administration of the antibody platelet deposition was lower than in the control animals treated with saline in both the venous and arterial thrombosis chambers (Fig. 2A,B). The difference was statistically significant in the arterial chamber at all time points after Mab-LE2E9Q injection and 24 h after administration in the venous chamber.

A fraction of the platelets bound in the venous or in the arterial thrombosis chamber detached from the thrombus in some experiments. To take this phenomenon into account, a cumulative platelet deposition was calculated by adding up platelet depositions during each time period of 3 min. The cumulative platelet deposition was 1.1 to 2.3 times higher than the maximal platelet deposition in 21 and in 17 out of 60 experiments in the venous and arterial thrombosis chambers, respectively (Fig. 3A,B). The cumulative platelet deposition was significantly lower in treated animals than in controls in both the venous and arterial thrombosis chambers at all time

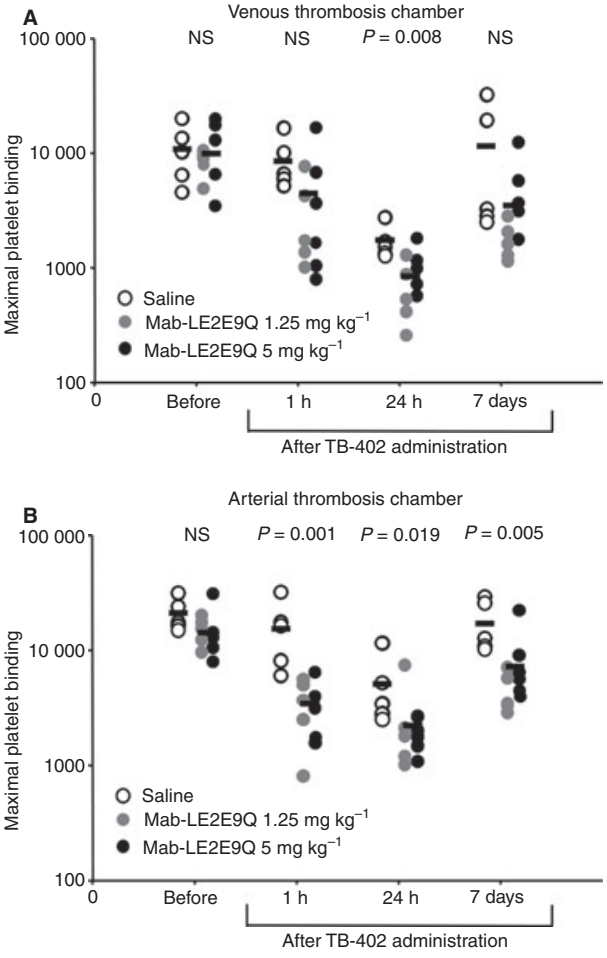

Fig. 2. Reduction of thrombus growth in baboons treated with Mab-LE2E9Q. Thrombogenic devices were placed for 60 min 1 h before and 1 h, 24 h and 7 days after Mab-LE2E9Q or saline injection. Platelet deposition was recorded over 1 h in the venous (A) and arterial (B) thrombosis chambers. The maximal platelet depositions recorded at any time point over the entire observation period are indicated for each animal. The mean platelet deposition per dose group is also indicated. The statistical differences between treated and control groups were calculated using the Mann–Whitney *U*-test (NS, non-statistically significant).

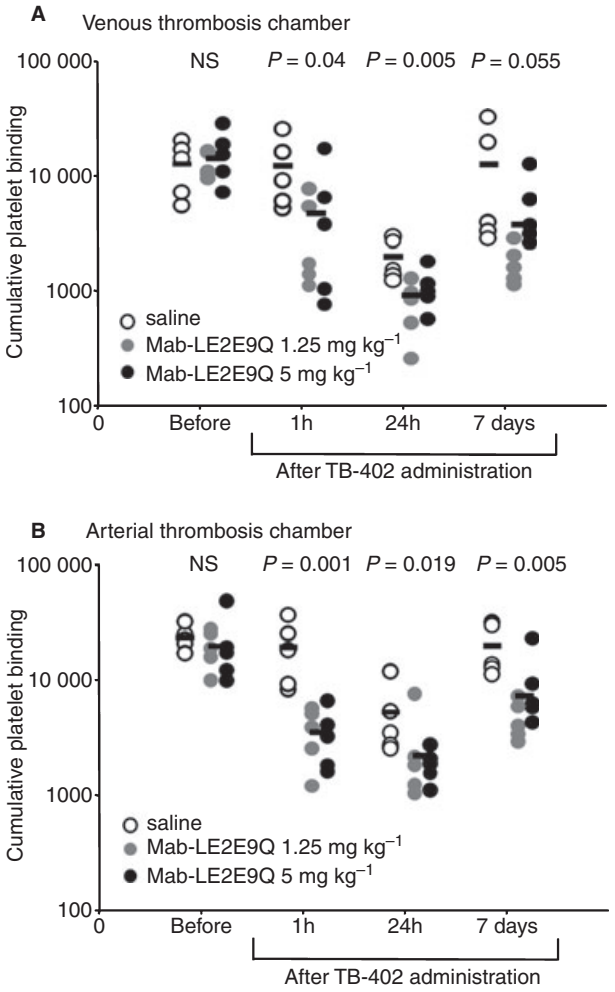

Fig. 3. Reduction of cumulative platelet deposition in baboons treated with Mab-LE2E9Q. Platelet deposition was recorded as in Fig. 2 in the venous (A) and arterial (B) thrombosis chambers. A cumulative platelet deposition was also calculated by summing platelet deposition during each time period of 3 min. The mean cumulative platelet deposition per dose group is also indicated. The statistical differences between treated and control groups were calculated using the Mann–Whitney *U*-test (NS, non-statistically significant).

points after Mab-LE2E9Q administration, except at day 7 in the venous thrombosis chamber ($P = 0.055$).

Pharmacodynamics

Blood samples were taken from baboons included in the thrombosis prevention study at intervals after administration of a single i.v. bolus (1.25 mg kg^{-1} or 5.0 mg kg^{-1}) of Mab-LE2E9Q or saline.

As preliminary experiments had shown that the measurement of residual FVIII activity after incubation of human plasma with Mab-LE2E9Q varies according to the type of FVIII assay used, different types of FVIII assays were used to evaluate FVIII:C in PL of baboons treated with Mab-LE2E9Q. One test, a commercial chromogenic assay, barely detected Mab-LE2E9Q whereas the second assay, a modification of the former one, was rendered sensitive to the inhibitory activity of the antibody by reducing sixtyfold phospholipids concentrations. The second assay had previously been used to detect the effect of a mutation responsible for mild hemophilia A that impaired FVIII interaction with phospholipids [8].

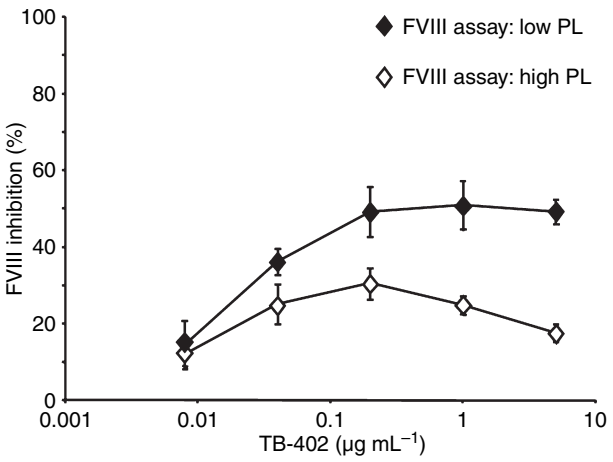

Fig. 4. Sensitivity of high and low phospholipids chromogenic assays to Mab-LE2E9Q inhibitory activity. Baboon plasma spiked with various concentrations of Mab-LE2E9Q and incubated for 2 h at 37 °C. The residual FVIII:C was measured with high- and low-phospholipid chromogenic assays. Results are expressed as mean ± SD of FVIII inhibition by comparison to control samples without Mab-LE2E9Q in two independent experiments.

FVIII:C was measured in baboon plasma spiked with various concentrations of Mab-LE2E9Q and incubated for 2 h at 37 °C. The maximal FVIII inhibitions measured with high and low phospholipids assays were 29% and 50%, respectively (Fig. 4). Noteworthy, no FVIII inhibition was observed when FVIII:C was measured with a one-stage coagulation assay (Actin FS^R, data not shown).

Comparison of FVIII:C measured with the low- and high-phospholipids assays showed a clear decrease in FVIII activity after Mab-LE2E9Q administration (Fig. 5A,B). FVIII levels were normalized by the end of the follow-up period in the group treated with 1.25 mg kg^{-1} whereas FVIII:C was still reduced in the group treated with 5 mg kg^{-1}. In contrast, FVIII:C measured with both assays was similar in animals treated with saline (Fig. 5C). No difference was observed between the different groups when FVIII:C was measured with the one-stage coagulation assay (Fig. 6).

Interestingly, the lack of a clear-cut increase or decrease of FVIII:C measured with the high phospholipids assay and with the one-stage FVIII assay, insensitive to Mab-LE2E9Q, up to 60 days after administration of the antibody indicated that Mab-L2E9Q does not significantly modify the rates of FVIII production and clearance.

Pharmacokinetics

Blood samples were taken for the pharmacodynamic study. The concentrations of Mab-LE2E9Q were evaluated by

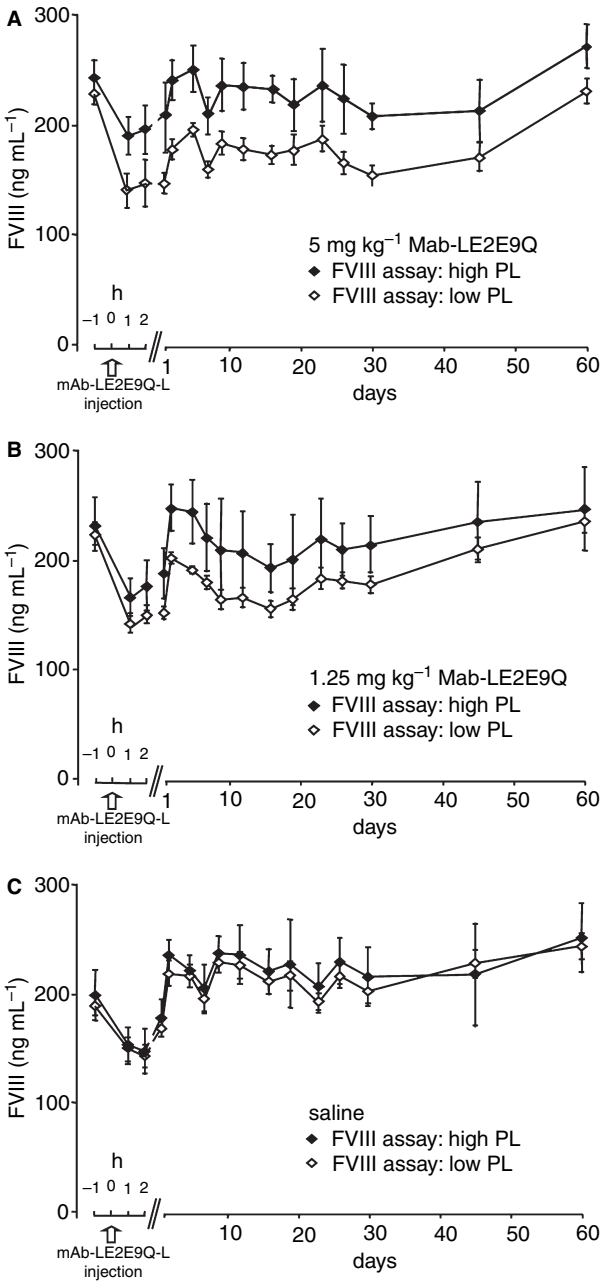

Fig. 5. FVIII levels measured in chromogenic assays in baboons treated with Mab-LE2E9Q. Baboons were treated by one intravenous administration of 5 (A) or 1.25 (B) mg kg^{-1} Mab-LE2E9Q or saline (C) as indicated. Blood samples were harvested starting 1 h before administration. The concentrations of FVIII were measured using chromogenic assays with a high or low phospholipid concentration, which is less sensitive to Mab-LE2E9Q inhibitory activity. Data are shown as mean ± SEM and as a function of time.

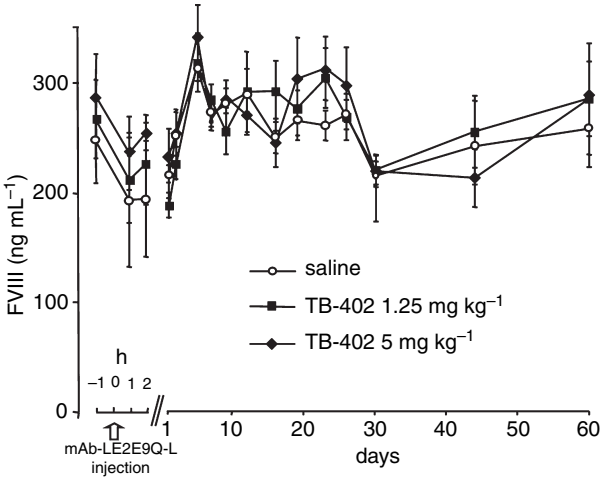

Fig. 6. FVIII levels measured in a 1-stage coagulation assay in baboons treated with Mab-LE2E9Q. Blood samples from baboons treated with Mab-LE2E9Q were harvested as in Fig. 5. The concentrations of FVIII were measured using a 1-stage coagulation FVIII assay, which is insensitive to Mab-LE2E9Q inhibitory activity (Actin FS^{R}). Data are shown as mean ± SEM and as a function of time.

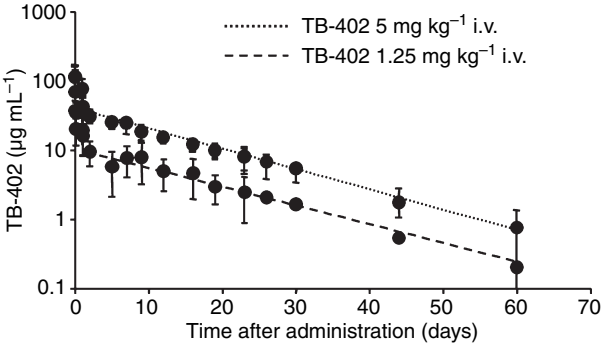

Fig. 7. Plasma clearance curve after a single intravenous injection of Mab-LE2E9Q in baboons. Antibody concentration were measured by ELISA in plasma of baboons treated by one single subcutaneous administration of 1.25 or 5 mg kg^{-1} Mab-LE2E9Q and are expressed as mean ± SD. Data were fitted to a biexponential disposition curve with the parameters of Table 1.

measuring the ability of Mab-LE2E9Q in the plasma samples to inhibit FVIII binding to insolubilized Mab-LE2E9Q. After single i.v. bolus injections of 1.25 mg kg^{-1} Mab-LE2E9Q, a biphasic disappearance curve with rapid initial clearance and slow secondary clearances was observed (Fig. 7). The initial half-life of Mab-LE2E9Q was about 7.2 ± 4 h (mean ± SD) and the secondary half-life was 305 ± 105 h. The volume of distribution was 134 ± 46 mL kg^{-1} (Table 1). Similar bioavailability and terminal disposition phases were observed after s.c. administration of 5 mg kg^{-1} Mab-LE2E9Q.

Clinical and biological observations

The experimental procedure and the administration of Mab-LE2E9Q were well tolerated by all animals. In both groups, most animals presented with small hematomas at the sites of venopuncture and oozing at the sites where the catheters had been inserted. One animal (treatment 5 mg kg^{-1} Mab-LE2E9Q) died 25 days after administration of the antibody. Neither external nor subcutaneous bleedings were observed in this animal. As no necropsy could be performed an internal bleeding problem could not be excluded. However, the

Table 1 Calculated pharmacokinetic parameters of Mab-LE2E9Q after a single intravenous bolus injection in baboons

	Mab-LE2E9Q 1.25 mg kg^{-1}	Mab-LE2E9Q 5 mg kg^{-1}	
C0	39 ± 16	128 ± 46	μg mL^{-1}
A	29 ± 14	88 ± 38	μg mL^{-1}
B	10 ± 3	40 ± 11	μg mL^{-1}
α	0.15 ± 0.12	0.16 ± 0.10	h^{-1}
β	0.0026 ± 0.0012	0.0028 ± 0.0005	h^{-1}
V_c	38 ± 18	46 ± 25	mL kg^{-1}
V_d	134 ± 46	132 ± 37	mL kg^{-1}
$t_{1/2\alpha}$	7.2 ± 4.0	5.5 ± 2.6	h
$t_{1/2\beta}$	305 ± 105	257 ± 49	h
	12.7 ± 4.4	10.7 ± 2	days
AUC	4916 ± 2648	15 611 ± 4990	μg h mL^{-1}
Clp	0.3 ± 0.2	0.3 ± 0.1	mL h^{-1}
MRT	472 ± 168	388 ± 73	h

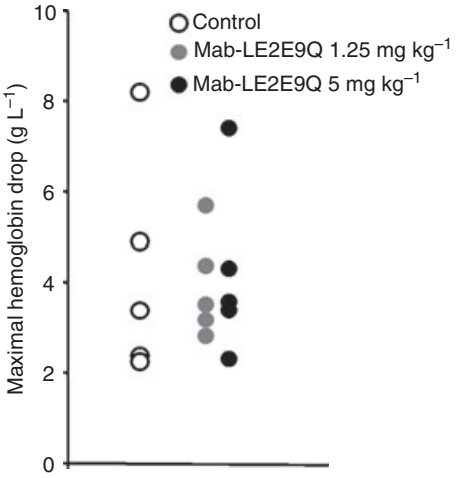

Fig. 8. Hemoglobin drop following Mab-LE2E9Q injection. Baboons were treated with one i.v. administration of 1.25 or 5 mg kg^{-1} antibody. Hemoglobin levels were followed up to 8 weeks after antibody administration. Results are expressed as the most severe hemoglobin drop, as compared with hemoglobin concentrations before treatment, recorded during the observation period. The statistical significance of differences between treated and control groups were tested using the Mann–Whitney *U*-test.

hemoglobin levels in that animal were normal (13.9 g L^{-1}) 2 days before death indicating that that animal did not present with any chronic bleeding problem.

A key parameter for the selection of an anticoagulant agent is the safety with regards to the induction of bleedings. Only a limited drop in hemoglobin level was observed in animals treated with Mab-LE2E9Q. This drop was not significantly different from that observed in control animals treated with saline (Fig. 8).

Immunogenicity

The presence of anti- Mab-LE2E9Q IgG and IgM antibodies in plasma collected before administration of Mab-LE2E9Q and 7, 14, 21, 30 and 60 days after administration of 0, 1.25 or 5 mg kg^{-1} Mab-LE2E9Q was measured in sandwich ELISA. None of the animals developed a detectable humoral response to Mab-LE2E9Q (data not shown).

Discussion

In this study, we sought evidence that Mab-LE2E9Q, a human monoclonal antibody inhibiting FVIII only partially, might constitute a novel approach to anticoagulant therapy, without the risk of overdosing or causing spontaneous bleeding. The data demonstrated that Mab-LE2E9Q significantly reduced thrombus growth in an extracorporeal circulation model in baboons, without inducing excess bleeding. The inhibition of thrombus growth was similar 1 h, 1 day and 1 week after Mab-LE2E9Q, indicating a stable and long-acting inhibition of FVIII when the antibody is in excess over FVIII.

The ability of Mab-LE2E9Q to reduce thrombus development was evaluated in 'venous' and 'arterial' thrombosis chambers inserted in an extracorporeal circulation shunt between the femoral vessels. The venous thrombosis chamber was an extension chamber whereas the arterial chamber was covered by Dacron, a surface that allows platelet adhesion. In both the venous and the arterial thrombosis chambers, thrombus growth was monitored by measuring platelet deposition. The rationale for the selection of that method was that platelets are present in both venous and arterial thrombi, although their contribution to the physiopathological process are lower in the former than in the latter [9,10].

Although treatment with Mab-LE2E9Q did not increase the bleeding tendency, it resulted in a reduction of platelet deposition in both chambers. The reduction of thrombus development in the arterial chamber may appear surprising but is in agreement with the observation that the rate of myocardial infarction is lower when FVIII is reduced [11] and with the well-established role of FVIII in arterial thrombus development in experimental models [12]. However, it cannot be excluded that platelet deposition in one chamber was influenced by the rate of platelet deposition in the other chamber and by the resulting flow alterations.

The major risk of targeting FVIII with an anticoagulant agent is that of causing complete inhibition of FVIII activity. The observation that Mab-LE2E9 V_H *N*-glycosylation determines the maximal inhibitory activity of the antibody [4] offered a unique opportunity to develop an optimal anticoagulant agent targeting FVIII. Indeed, both modulation of the glycosylation and mixing deglycosylated, mutated antibodies with native ones allowed us achieve inhibition ranging from the inhibitory activities of 40% to 90% [4].

Tail clipping experiments in mice treated with Mab-LE2E9Q demonstrated that *in vivo* the antibody only partially neutralizes FVIII activity. Similarly, all *in vitro* assays indicated that the antibody inhibits FVIII activity only partially, even when the antibody is in large excess over FVIII. However, different types of FVIII assays displayed various sensitivities for the detection of the inhibitory activity of Mab-LE2E9Q in baboon

plasma as well as in human plasma [13]. This was unexpected because the type of FVIII assay is not considered as a major cause of variability in the Bethesda assay, possibly because type I inhibitors are usually selected for multicenter studies aiming at standardization of the method.

A potential explanation of this phenomenon is that the binding of a type II inhibitor antibody, such as Mab-LE2E9Q, to FVIII induces a qualitative alteration of the FVIII molecule. It is interesting to compare such alterations with those observed in patients with mild/moderate hemophilia A. Some FVIII variants with reduced stability or impaired thrombin activation show different activities in one-stage vs. two-stages assays [14–18]. This discrepancy has clinical implications as it may lead to misdiagnosed hemophilia A. Similarly, a deletion of one amino acid in the FVIII domain mediating FVIII binding to phospholipids significantly impairs FVIII interaction with phospholipids. However, this alteration is not detected in the conventional FVIII assays but requires assays with reduced phospholipids concentrations or platelets as a source of phospholipids [8]. The last observations prompted the evaluation of the inhibitory activity of Mab-LE2E9Q in different types of FVIII assays.

In this study, two chromogenic assays differing by the concentration of phospholipids and a one-stage coagulation assay were used to measure residual FVIII activity in the plasma of animals treated with Mab-LE2E9Q. The one-stage coagulation assay did not allow us detect a difference in FVIII level between animals treated with Mab-LE2E9Q and control animals. This can be attributed to the poor sensitivity of that assay to the inhibitory activity of Mab-LE2E9Q. in contrast, the comparison of FVIII:C levels measured with the high- and low-phospholipid concentrations allowed us to identify the inhibitory activity of Mab-LE2E9Q. In agreement with the pharmacokinetics data, administration of 1.25 mg resulted in the reduction of FVIII activity up to day 44 whereas FVIII activity was still clearly reduced 60 days after the administration of 5 mg kg^{-1} Mab-LE2E9Q.

The variation of FVIII inhibition by Mab-LE2E9Q as a function of the FVIII assay used to measure residual FVIII:C raises questions about the *in vivo* inhibitory activity of the antibody. The observation that mice treated with Mab-LE2E9Q survive a tail clipping experiment in contrast to $FVIII^{-/-}$ mice indicates that the antibody only partially inactivates FVIII. Similarly, the lack of severe bleedings in baboons treated with Mab-LE2E9Q suggests that FVIII inhibition is only partial. However, the exact level of FVIII inhibition remains unknown. *In vivo* local parameters, such as tissue damage, flow or phospholipids, may not only determine thrombus development but also influence the inhibitory activity of the antibody. Such a mechanism may explain that the antithrombotic activity of Mab-LE2E9Q appears to vary according to the thrombosis model. Thus, in mice with a strong prothrombotic phenotype, Mab-LE2E9Q completely prevented thrombus development [5], whereas in the extracorporeal circulation model in baboons, the antibody only partially reduced thrombus growth.

Interestingly, the use of the assays insensitive to Mab-LE2E9Q (chromogenic assay with high phospholipids concentration and 1-stage coagulation assay) provided information regarding the clearance of FVIII in the presence of the antibody. Thus, when measured with the FVIII assays insensitive to Mab-LE2E9Q, FVIII:C activity remained stable in animals treated with 1.25 mg kg^{-1} or 5 mg kg^{-1} Mab-LE2E9Q, indicating that FVIII clearance was not altered in the presence of the antibody.

Pharmacokinetic analyses indicated that the half-life of Mab-LE2E9Q is about 10 days in baboons, in agreement with the long half-life of IgG. No increase of the clearance of the antibody was observed over the entire follow-up period, in agreement with the lack of immune response to the antibody.

Although the reduction of thrombus development in baboons treated with Mab-LE2E9Q cannot be directly extrapolated to a clinical situation in man, they are in agreement with epidemiological observations that a limited reduction of FVIII activity, such as that observed in carriers of hemophilia A, has a positive impact on vascular disease [11]. These observations may have important implications for the development of efficient, easy and safe strategies for the prevention and treatment of thrombosis. This antibody represents an unique and new strategy to prevent thrombosis and warrants further investigation in other preclinical and clinical studies.

Addendum

M. Jacquemin, R. Lavend'homme, L. VanderElst were responsible for *in vitro* coagulation studies. J.M. Stassen, J.-M. Saint-Remy, P. Badenhorst, H. Pieters, P. Verhamme contributed to the design of the study. M. Meiring, S. Lamprecht, J. Roodt were responsible for thrombosis experiments in baboons. All authors contributed to the revision of the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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IDENTIFICATION OF A COLLAGEN-BINDING PROTEIN FROM *NECATOR AMERICANUS* BY USING A cDNA-EXPRESSION PHAGE DISPLAY LIBRARY

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ABSTRACT: A phage display library was made starting from a cDNA library from the hematophagous human parasite *Necator americanus*. The cDNA library was transferred by polymerase chain reaction (PCR) cloning into phage display vectors (phagemids), using specially designed primers such that proteins would be expressed as fusions with the C-terminal part of the phage coat protein pVI. The vectors used are multicloning site variants of the original pDONG vectors described by Jespers et al. (1995). Electroporation of the ligation mixtures into electrocompetent *Escherichia coli* TG1 cells yielded 3×10^8 pG6A, 1.9×10^8 pG6B, and 1×10^8 pG6C transfectants for *N. americanus*. The final libraries consisted of a mix of equal numbers of insert-containing phages from the A, B, and C libraries. Selection of phages for binding to human collagen was performed. Four rounds of panning on human collagens I and III resulted in a significant enrichment of collagen-binding phages from the *N. americanus* libraries. PCR analysis revealed various insert lengths; however, sequence determination indicated that all phages contained the same protein, albeit with different poly-A tail lengths. The encoded protein itself is a 135-amino acid protein (15 kDa), with no apparent homology to any other known protein. Next the protein was recloned into *E. coli* using the pET-15b-vector. Upon isopropyl-1-thio-β-D-galactopyranoside induction, the recombinant protein, rNecH1, could be recovered by urea treatment from inclusion bodies. The rNecH1 protein binds to different collagens: human I > rat I > human III = calf skin I in a specific, dose-dependent, and saturable manner.

Hookworm infection is one of the leading causes of iron-deficiency anemia in humans (Hotez, 1989; Hotez and Pritchard, 1995). Adult parasites from hookworms, such as *Necator americanus,* attach to the gastrointestinal mucosa, during which intestinal capillaries of the lamina propria are lacerated, and the blood is either digested or leaks at the site of parasite attachment (Kalkofen, 1974). The anemia of hookworm disease results directly from this intestinal blood loss.

Tissue damage and collagen exposure would normally trigger hemostasis, and it was postulated already a century ago (Loeb and Smith, 1904) that hookworms (Cappello et al., 1993; Stanssens et al., 1996), very much like leeches (Munro et al., 1991; Connolly et al., 1992; Depraetere et al., 1999) and other hematophagous creatures (Markwardt et al., 1967; Waxman et al., 1990; Keller et al., 1993; Noeske-Jungblut et al., 1994; Francischetti et al., 2000), secrete anticoagulants to facilitate feeding. Indeed, a series of activities were found in excretory–secretory products of *N. americanus* that either inhibit blood coagulation, platelet aggregation, or that cause fibrinogenolysis (Furmidge et al., 1996). Unlike leeches that are opportunistic feeders on human blood, *N. americanus* feeds exclusively on humans. Furthermore, this parasite seems to be able to escape the human immune system (Hotez and Pritchard, 1995). Therefore, this parasite has evolved to live in humans for, in some patients, decades by secreting nonimmunogenic anti-hemostatic agents.

Study of these parasites thus may not only yield products that interfere with blood clotting that should help us to better understand the hemostatic process and could lead to new antithrombotic products but also may result in the identification of proteins that could be used for the development of vaccines.

So far, anti-platelet agents have been isolated from hematophagous species by using classical separation techniques that, in view of the minute starting quantities, often have been extremely difficult tasks. The present paper reports on an effort to circumvent these problems by taking advantage of the possibilities that phage display technology offers. An efficient methodology to express cDNA libraries on phages has been developed previously (Jespers et al., 1995) and is based on the observation that proteins can be linked covalently in an active and accessible form onto the phage surface by fusion with the C-terminus of the minor phage coat protein pVI. To this end, a set of 3 vectors (pG6A, B and C) is used, each differing from the other by 1 extra nucleotide before the cDNA insertion site. This allows, upon parallel cloning into the 3 vectors, transcription from the 3 reading frames that assures the correct translation of each protein. In this way, an anti-coagulant protein has already been isolated from the dog hookworm (Jespers et al., 1995).

Platelet adhesion and aggregation, the first steps in both the normal hemostatic and thrombotic processes, are caused by the interaction of platelets with collagen exposed upon blood vessel damage. *Necator americanus* produces an inhibitor of the collagen–platelet interaction (Furmidge et al., 1996) that could be either a collagen binder or a binder of one of the several collagen receptors on platelets. In a first attempt, we looked for a collagen binder as the presence of such products was also observed in leeches (Depraetere et al., 1999), where both the direct platelet–collagen interaction as well as the indirect one with von Willebrand factor forming the bridge between platelets and collagen, could be blocked.

To understand fully the molecular basis for the anti-hemostatic properties of *N. americanus,* an *N. americanus*-derived cDNA-expression library displayed on phages was constructed, and we selected, sequenced, and expressed a new collagen-binding protein. This establishes proof-in-principle that this library can be used to study molecular interactions between the parasite and a range of relevant host-derived targets.

MATERIALS AND METHODS

Construction of the phage library

An *N. americanus* cDNA library originally cloned in λ-ZAP-Express (Pritchard et al., 1999) with a diversity of 5.3×10^5 PFU and an insert

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length ranging from 0.3 to 2.0 kb was used as a template for polymerase chain reaction (PCR) amplification of the cDNA inserts. The 5′-primer VIAN3: (5′-CTCGCAACTGCGGCCAAGTCGGCCTGGATCCAAAGAATTCCGGCACGAG-3′) contains an *Sfi*I restriction site and is complementary to the λ-ZAP-Express sequence flanking the cDNA insert, the 3′-primer VIAN2 (5′- AATTCGCGGCCGCTCGAGTTTTTTTTTTTT-3′) contains a *Not*I site and is complementary to the poly-A tail of the cDNA insert.

The PCR products obtained with primers VIAN2 and VIAN3 were digested with *Sfi*I and *Not*I (New England Biolabs, Inc., Beverly, Massachusetts) and ligated in the 3 *Sfi*I–*Not*I-digested pG6 vectors. These vectors are variants of the pDONG vectors described earlier (Jespers et al., 1995); this set of vectors enables expression of the parasite proteins as fusion proteins with the C-terminus of the minor phage coat protein pVI in each of the 3 reading frames. The 3 ligation mixtures (1 for each vector) were digested with *Sal*I (New England Biolabs) to eliminate vector without insert. Before electroporation into *Escherichia coli* TG1 electroporation-competent cells (Stratagene, La Jolla, California) according to standard methods (Sambrook et al., 1989), the ligation mixtures were purified with Prep-A-gene (Bio-Rad, Hercules, California) and concentrated on a Millipore filter (Millipore Corporation, Bedford, Massachusetts).

Transformed cells were plated on LB agar (Gibco BRL, Paisley, Scotland) plates containing 100 μg/ml ampicillin. After overnight growth at 37 C, cells of the 3 electroporations were collected and separate cell stocks (OD_{600} = 20) were prepared in LB medium supplemented with 100 μg/ml ampicillin (Boehringer Mannheim, Germany) and 43% glycerol for storage at −70 C.

PCR with primers VIAN2 and VIAN3 was performed on 8 transformants of each electroporation, and the PCR products were loaded on a 1% agarose (Boehringer Mannheim) gel to analyze the number of insert-containing transformants.

For phage production, a fraction of an overnight preculture of 50 μl celstock in 10 ml LB medium containing 100 μg/ml ampicillin was resuspended in 10 ml 2xTY (16 g/L tryptone [Difco Laboratories, Detroit, Michigan], 10 g/L yeast extract [Difco], 5 g/L NaCl, pH 7) supplemented with 100 μg/ml ampicillin. The log-phase culture was superinfected with M13KO7 helper phage (Amersham Pharmacia Biotech, Uppsala, Sweden) at a multiplicity of infection of approximately 20 helper phage per cell, and infected cells were incubated for 1 hr at 37 C. Cells were centrifuged for 10 min at 750 *g*, resuspended in 150 ml 2xTY containing 100 μg/ml ampicillin, 50 μg/ml kanamycin, 0.3 μM aprotinin, and 1 μM leupeptin (all from Boehringer Mannheim), 5 mM EDTA, and grown overnight at 37 C. Following centrifugation of the overnight culture at 5,000 *g* for 20 min at 4 C, phages in the supernatant were precipitated with 4% PEG 6000, 0.5 M NaCl during 1 hr on ice, and subsequently centrifuged at 2,500 *g* for 20 min. Phages were resuspended in 5 ml sterile water, centrifuged at 750 *g* for 10 min to remove cell debris, filter sterilized on a GyroDisc filter (Orange Scientific, Waterloo, Belgium) with 0.45-μm pore size, and precipitated again on ice for 30 min with 4% PEG 6000, 0.5 M NaCl. The phage pellet obtained by centrifuging 20 min at 2,500 *g* was finally resuspended in phosphate-buffered saline (PBS) containing 5 mM EDTA, 0.3 μM aprotinin, and 1 μM leupeptin to avoid proteolytic removal of the fused parasite proteins by displayed proteinases and 5% glycerol for storage at −20 C.

Finally, the 3 phage preparations were mixed in equal numbers of insert-containing phages to prepare phage stocks for use in subsequent panning experiments.

Affinity selection by panning

Maxisorp tubes (Nunc, Rochester, New York) were coated overnight with 1 ml of a solution of 30 μg/ml human collagen type I or III (Sigma) in PBS and blocked with 4% skimmed milk in PBS for 2 hr. A phage library stock (6×10^{12} insert-containing phages) was added in 1 ml PBS, 2% skimmed milk, 5 mM EDTA, 0.3 μM aprotinin, 1 μM leupeptin, and the tubes were incubated for 2.5 hr at room temperature. Unbound phages were removed by washing 10 times with PBS, 0.1% Tween-20. Bound phages were eluted by incubation with 1 ml 0.1 M glycine-HCl, pH 2.0, for 15 min, and the eluate was immediately neutralized with 250 μl 1 M Tris-HCl, pH 8.0.

Half of the eluted phages was added to log phase *E. coli* TG1 cells and adsorbed without shaking for 30 min at 37 C. Infected cells were spread onto LB agar plates containing 100 μg/ml ampicillin. After overnight incubation at 37 C, cells were resuspended in LB medium supplemented with 100 μg/ml ampicillin until OD_{600} = 40. Twenty-five microliters of these cells were grown in 10 ml 2xTY containing 100 μg/ml ampicillin for phage production. To the remaining cells, the same volume of 75% glycerol was added for the preparation of cell stocks for storage at −70 C.

Phage production, as described before, was performed in 40 ml 2xTY medium, and cells were centrifuged at 5,000 *g* for 20 min at 4 C. The phage-containing supernatant was incubated for 1 hr on ice with 4% PEG 6000, 0.5 M NaCl. The phage pellet, obtained after centrifugation at 2,500 *g* for 20 min, was resuspended in 1 ml sterile water, and remaining cells were removed by centrifugation for 2 min at 14,000 rpm in an eppendorf centrifuge. Phages were again precipitated on ice with 4% PEG 6000, 0.5 M NaCl for 30 min, centrifuged at 14,000 rpm for 4 min at 4 C in a cooled eppendorf centrifuge, and finally dissolved in PBS containing 5 mM EDTA, 0.3 μM aprotinin and 1 μM leupeptin, and 5% glycerol for storage at −20 C. The propagated phages are used in subsequent rounds of panning (3–5 rounds) and tested for binding activity to collagen.

Phage ELISA

Microtiter plates (Greiner, Frickenhausen, Germany) were coated overnight at 4 C with 120 μl of either 30 μg/ml human collagen type I or III in PBS or 50 μg/ml *N. americanus* cuticular collagen and blocked with 4% skimmed milk in PBS for 2 hr at room temperature. Phages (1×10^{12} colony-forming units/ml) diluted in 100 μl PBS, 2% skimmed milk, were added and incubated for 1.5 hr at room temperature. After 6 washes with PBS, 0.1% Tween-20, bound phages were detected by incubating for 1 hr with 95 μl mouse anti-M13-phage coupled to horse radish peroxidase (HRP) (Amersham Pharmacia) (1/5,000 in PBS, 2% skimmed milk). One hundred and sixty microliters of substrate (2.76 mM *ortho*-phenylenediamine dihydrochloride, 8 μl H_2O_2, 10 ml 0.2 M sodium phosphate, and 10 ml 0.1 M citric acid) was added following 9 washes with PBS, 0.1% Tween-20. The reaction was stopped by 40 μl 4 M H_2SO_4, and the absorbance was measured at 490 nm using an EL340 microplate biokinetics reader (Bio-Tek instruments. Inc.,Winooski, Vermont).

Propagation of single colonies

Single colonies from plates of the fourth panning round were grown overnight at 37 C in sterile 96-well plates (Novolab, Geraardsbergen, Belgium) in 200 μl LB supplemented with 100 μg/ml ampicillin ; 200 μl 2xTY was inoculated with 20 μl overnight culture of each clone, and helper phages were added when cells had reached the log phase. Superinfected cells were pelleted and resuspended in 200 μl prewarmed 2xTY supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin; 15 μl of these cells were added to 220 μl prewarmed 2xTY containing 100 μg/ml ampicillin, 50 μg/ml kanamycin, 5 mM EDTA, 0.3 μM aprotinin, and 1 μM leupeptin, and grown overnight at 30 C. After removal of the bacteria, 50 μl of phage-containing supernatant was added to 50 μl PBS, 4% skimmed milk, and incubated for 1.5 hr at room temperature. Detection of binding phages was done as described above.

PCR of the cDNA inserts

PCR amplification of the cDNA inserts in positive phagemid-containing bacteria was performed using the primers from the construction of the phage display library (primer VIAN3 as backward primer and primer VIAN2 as forward primer). PCR was performed on bacteria infected with binding phages or nonbinding phages as a control. PCR products were screened on a 1% agarose gel.

DNA isolation

Single-stranded DNA was prepared from phages grown from positive phagemid-containing bacteria, by phenol–chloroform–isoamylalcohol extraction (Sambrook et al., 1989). Double-stranded DNA from positive phagemid-containing bacteria was prepared using the Qiagen Plasmid Midi kit (Qiagen GmbH, Hilden, Germany), according to manufacturers' protocol.

Sequence analysis of the cDNA inserts

Sequence analysis was performed using the T7 sequencing kit (Amersham Pharmacia Biotech). The universal primer (Amersham Pharmacia Biotech) was used for 3′ end sequencing and internal primers were used for complete sequence determination.

The obtained sequence was compared to known proteins using the FASTA3, FASTAx3t, and BLASTP programs.

Expression of recombinant NecH1

The NecH1 cDNA insert was amplified by PCR with the following primers: BACKH1: 5′-GCAACTGTCATATGTTTTCGGAGAC-CAATTTG-3′ as backward primer FORH1: 5′-AATTCGCGG CTCGAGTCACAAAGATGCAGATATAT-3′ as forward primer. The primers contained *Nde*I and *Xho*I restriction sites, respectively, for cloning into pET15b (Novagen, Inc., Madison, Wisconsin), according to standard procedures (Sambrook et al., 1989), that allows expression of recombinant proteins with a $(His)_6$-tag at the N-terminus of the fusion protein.

DH5α-competent cells (Gibco) were transformed and plated overnight on LB agar plates supplemented with 100 μg/ml ampicillin.

Positive colonies were identified through PCR amplification of NecH1 on bacteria with primers BACKH1 and FORH1.

For expression of the recombinant protein, plasmid DNA of positives clones was introduced in *E. coli* BL21(DE3) cells (Novagen) for isopropyl-1-thio-β-D-galactopyranoside (IPTG)-induced expression. Cells were grown in LB medium containing 100 μg/ml carbenicillin at 37 C with vigorous shaking to an OD_{600} of 0.4, after which production of rNecH1 was induced by addition of IPTG (Acros) to a final concentration of 1 mM. The cells were grown for an additional 3 hr at 28 C, centrifuged at 3,000 *g* for 15 min, and the pellets were stored at −20 C.

Purification of recombinant NecH1

The frozen pellet was thawed and resuspended in 1/10 volume 20 mM phosphate buffer, pH 7.8, sonicated in the presence of 1 mM phenylmethylsulfonylfluoride, and centrifuged. The obtained pellet, containing inclusion bodies, was resuspended and incubated for 1 hr at 37 C in 20 mM phosphate buffer, pH 7.8, containing 6 M urea. After another centrifugation step, the supernatant was loaded onto a Sepharose-immobilized, metal affinity chromatography (IMAC) column (Xpress System ProBond Resin, Invitrogen BV, Groningen, Netherlands), equilibrated with column buffer (20 mM phosphate buffer, pH 7.8, 0.5 M NaCl, 6 M urea). Proteins were eluted in a linear pH gradient (20 mM phosphate buffer, pH 7.8, and 0.1 M citric buffer, pH 4.0, both containing 0.5 M NaCl and 6 M urea). The presence of rNecH1 in the eluted fractions was determined after dilution with an equal volume of PBS, using a collagen-binding assay and detection of the His-tag with India HisProbe–HRP conjugate (Pierce, Rockford, Illinois). Positive fractions were pooled, and urea was removed by dialysis against 20 mM phosphate buffer, pH 7.8, containing progressively decreasing concentrations of urea. A Bio-Rad DC protein assay was used to estimate the protein concentration (relative to a bovine serum albumin standard) according to the manufacturers' instructions.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and blotting

SDS-PAGE was performed to verify the purity of the fractions. A twin gel was run and the proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany). The r-NecH1 was detected after western blotting with HisProbe–HRP conjugates and a chemiluminescent substrate (ECL, western blotting detection reagents, Amersham Pharmacia Biotech).

rNecH1 binding experiments

Column fractions containing rNecH1 were identified as follows. Microtiter plates were coated overnight at 4 C with 100 μl, 30 μg/ml human collagen type I in PBS and blocked with PBS, 3% nonfat dry milk for 2 hr at room temperature; 100 μl of each eluted fraction was incubated for 1.5 hr at room temperature, and detection of bound r-NecH1 was performed with 95 μl India HisProbe–HRP conjugates (1/4,000 in PBS, 0.3% nonfat dry milk) for 1 hr at room temperature.

The binding capacity of rNecH1 to several targets was investigated:

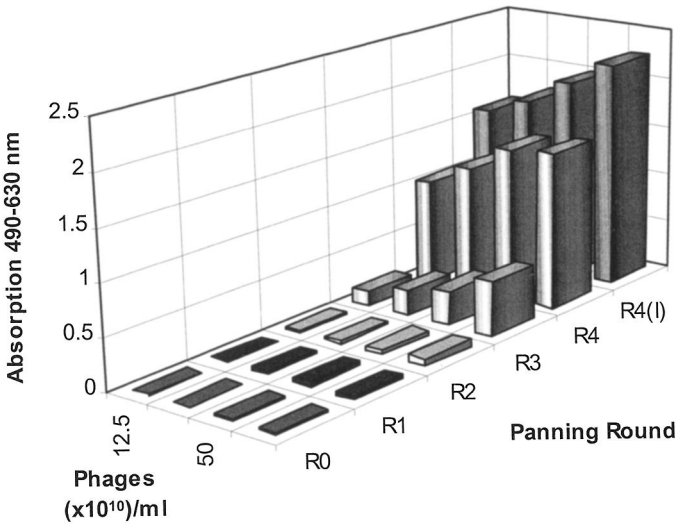

FIGURE 1. Binding of phages, obtained after each of the 4 rounds of selection, to human collagen type III. R0 are phages from the starting libraries. Phages eluted in the 4th round (R4) also bound strongly to human collagen type I (R4[I]).

collagen (human types I, III, and V [Sigma], calfskin type I [Sigma], and rat tail type I [Collaborative Biomedical Products, Bedford, Massachusetts]), fibrinogen, and C1q.

All collagens, except rat tail collagen, were dissolved in 50 mM acetic acid and dialyzed against PBS for 48 hr at 4 C. Rat tail collagen type I was dissolved in 0.02 N acetic acid and used as such. Microtiter plates were coated overnight at 4 C with 100 μl target in PBS and blocked for 2 hr with PBS, 3% nonfat dry milk. Dilution series of rNecH1, in PBS, 0.3% nonfat dry milk, were incubated for 1.5 hr at room temperature and washed 9 times with PBS, 0.002% Tween-80. Detection was performed with India HisProbe–HRP conjugates as indicated above.

RESULTS

Library construction

A pool of PCR inserts, amplified from an *N. americanus* λ-ZAP-Express library (Pritchard et al., 1999) was cloned in 3 different pG6 vectors. These vectors allow cloning of the parasite proteins in 3 different reading frames as fusion proteins with the C-terminus of the phage minor coat protein pVI protein (Jespers et al., 1995).

Based on the PCR results of the inserts of 8 transformants of each electroporation experiment, it was estimated that 88% of the 2.2×10^8 pG6B clones, 38% of the 8×10^8 pG6A, and 63% of the 1.7×10^8 pG6C transformants contained an insert ranging from 0.3 to 1.5 kb.

Phage stocks containing 6×10^{12} insert-containing phages were composed by pooling 2×10^{12} insert-containing phages from each of the 3 individual phage preparations.

Selection of collagen-binding phages

Identical procedures were followed for panning on human collagen type I as for panning on human collagen type III. Phages were subjected to 3 rounds of selection and amplification, and a phage ELISA was performed to monitor an increased selection and amplification of binding phages (Fig. 1). A significant increase in binding phages was obtained after the

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TTT TCG GAG ACC AAT TTG AAA AAA GCC CCC GAG TGC TCC AAG CTG GAG GGG      51
 F   S   E   T   N   L   K   K   A   P   E   C   S   K   L   E   G      17

TTC GCT ATG GCT GGA GTG GAC AAA TTC ACC TTG AAT GAT AGT AGC ATA AGA     102
 F   A   M   A   G   V   D   K   F   T   L   N   D   S   S   I   R      34

GAT CAA CTT TAT AGT GGA ATC TTG CAG TAC GCA ACT CAA TCA CCC GAT ATC     153
 D   Q   L   Y   S   G   I   L   Q   Y   A   T   Q   S   P   D   I      51

AAA TAC GAC TGC AGT GTC GAA TTA GCT GGA GGA TTG GTA CTG GAT GAC CCA     204
 K   Y   D   C   S   V   E   L   A   G   G   L   V   L   D   D   P      68

GCG AAG GAC TTC CGT TTT CTC ATG TCT AAT GGC AGA TTC CCC TTA ATT TCT     255
 A   K   D   F   R   F   L   M   S   N   G   R   F   P   L   I   S      85

GAA ACT GAC GGC AAC TCC ACT GTC GAG GAG GCT ACT CAA GCA GCT TTA AAA     306
 E   T   D   G   N   S   T   V   E   E   A   T   Q   A   A   L   K     102

TTA TGG GAG AAA CAT ATT CCA AAT TTG GGC CCT AGA GAA GCG GTC GGG TGC     357
 L   W   E   K   H   I   P   N   L   G   P   R   E   A   V   G   C     119

AAC TAC GTT TTT GAA GAC GAC AGG CAC AAA TAT ATC TGC ATC TTT GTG TGA     408
 N   Y   V   F   E   D   D   R   H   K   Y   I   C   I   F   V  Ter     135

AAACAAAAAG  418
```

FIGURE 2. NecH1 cDNA sequence and deduced amino acid sequence. Translation starts at the beginning of the sequence and is in frame with the pVI coat protein.

third round of panning. Following an additional panning round to increase the number of binding phages, 95 individual clones were screened for binding to their target; 28% and 33% of the clones were positive for binding to human collagen type I and III, respectively.

PCR on selected clones

The lengths of the cDNA inserts of the binding phages and some of the nonbinding phages were determined on a 1% agarose gel after PCR amplification. All positives clones had an insert of about 600 bp.

Clones selected on human collagen type I could be divided in 5 groups, based on the slightly different insert length; 3 slightly different insert lengths were identified in clones selected on human collagen type III.

Sequence analysis of positive clones

Sequence determination of a part of the 3′-end of 5 of the clones selected on collagen type I or III, showing differences in insert length, indicated that only 1 coding sequence was present and that this coding sequence was the same for both panning experiments. As the poly-A tail of the sequenced clones had a different length, we may assume that the selected clones came from different clones from the original library.

One clone with the largest insert and the shortest poly-A tail was chosen for further sequence analysis. The cDNA insert was 566 bp long and coded for an open reading frame of 135 amino acids in frame with the pVI gene (Fig. 2).

Several databases were screened for a homologue for the identified rNecH1 protein. None of the programs used could identify a protein with a significant homology, indicating that rNecH1 is a newly identified protein.

Expression and characterization of rNecH1

After PCR amplification of the NecH1 cDNA insert and ligation into pET15b, BL21(DE3) cells were transformed with the expression plasmid. Recombinant proteins inserted into the expression vector pET15b are expressed as fusion proteins containing an N-terminal His tag. The rNecH1 was purified by affinity chromatography on a Ni^{2+}-column and eluted at pH 7.

The positive fractions, as determined by the binding assay, were pooled and dialyzed against 20 mM phosphate, pH 7.8. The purity of the positive fractions was confirmed by SDS-PAGE and blotting (Fig. 3).

The rNecH1 protein has a strong affinity for several types of collagen (human types I and III, calfskin type I, and rat tail type I) (Fig. 4) and phages displaying NecH1 also bind to *N. americanus* cuticular collagen (Fig. 5). Denaturing human collagen type I resulted in a substantial loss of binding of rNecH1, whereas human collagen type V, fibrinogen, or C1q were not able to bind rNecH1. Preliminary investigations would appear to indicate that rNecH1 does not interfere with von Willebrand Factor or platelet binding to human collagen type I (not shown).

DISCUSSION

Hookworms are gut-dwelling, blood-feeding nematodes that infect hundreds of millions of people, particularly in the tropics. In 1 part of a program aimed at defining novel drug targets and vaccine candidates for human parasitic nematodes, genes expressed in adults of the human hookworm *N. americanus* were surveyed by the expressed sequence tag approach in which 161 new hookworm genes were identified (Daub et al., 2000). For the majority of these, a function could be assigned by homology to molecules such as proteases, protease inhibitors, a lipid-bind-

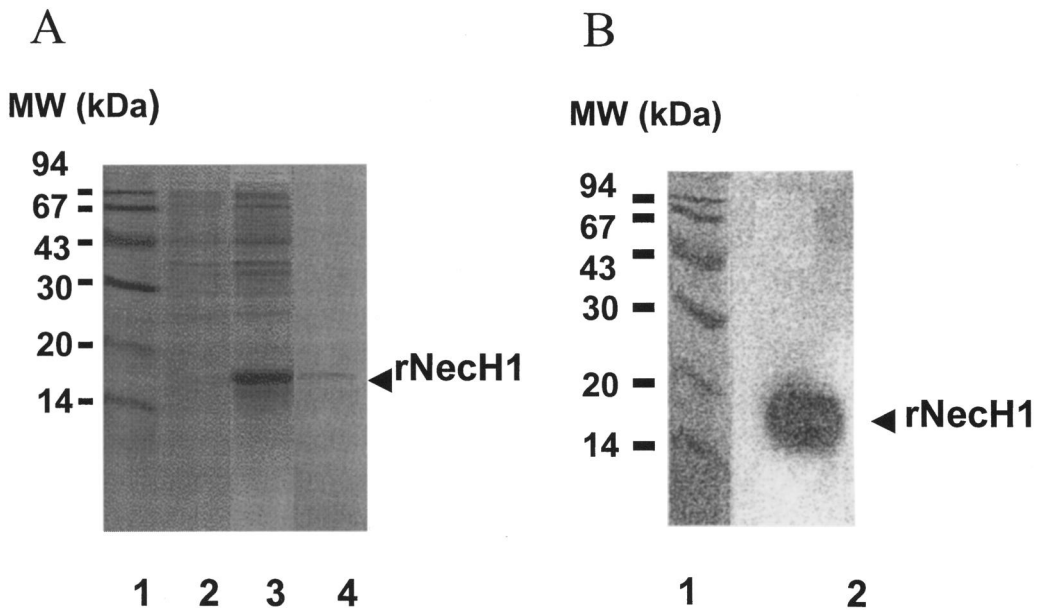

FIGURE 3. Purification of rNecH1. (**A**) SDS-PAGE of molecular weight markers (lane 1), supernatant of sonicated BL21 (DE3) cells before (lane 2) and after (lane 3) IPTG induction and of IMAC-purified rNecH1 (lane 4). (**B**) Western blot of rNecH1 detected with the His probe (lane 2).

ing protein, C-type lectins, an antibacterial factor, and potential potassium channel blocking peptides.

In the present paper, an alternative approach was followed in which proteins are selected based on their binding capacities. This has the advantage of possibly selecting for interesting molecules that may be produced by the hookworm, such as antigens recognized by human anti-parasite antibodies or anti-hemostatics. In contrast to classical chromatography-based purification schemes, where the amount of starting material available frequently is limiting, here, phage display was used as an alternative.

To this end, an *N. americanus* cDNA library was introduced in the gene coding for the minor phage coat protein pVI. In this way, proteins are functionally displayed on filamentous phages through the attachment to the C-terminus of pVI. The utility and feasibility of the method had already been proven by using a cDNA library derived from the parasite *Ancylostoma caninum,* which led to the identification of cDNAs encoding novel members of 2 different families of serine protease inhibitors (Jespers et al., 1995).

Complementary DNA sequences from *N. americanus* were fused in each of the 3 reading frames to the 3′-end of the M13 gene VI expressed by a phagemid vector that ultimately results

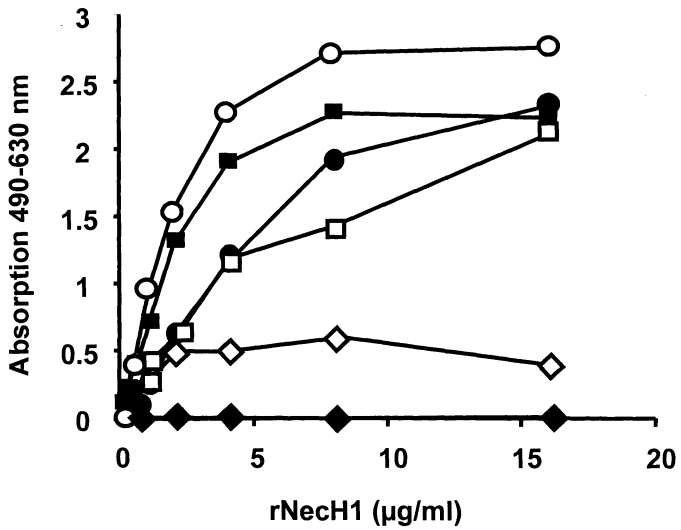

FIGURE 4. Dose-dependent binding of rNecH1 to human collagen type I (○) and III (■), calf skin collagen (●), and rat tail collagen (□). Binding to denatured (0.02 N HOAc) human collagen type I was strongly reduced (◇) and was completely absent for fibrinogen (◆) and human collagen type V (◆).

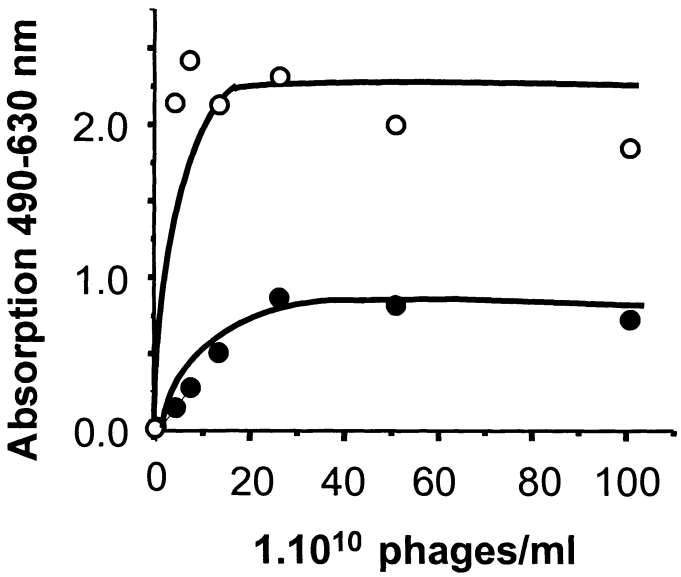

FIGURE 5. Binding of NecH1-phages to human collagen type I (○) and *N. americanus* cuticular collagen (●).

in a monovalent display system. Phages rescued from this cDNA expression library were subjected to panning against human collagen. This led to the identification of a cDNA encoding a hitherto unknown collagen-binding protein, rNecH1, of some 15 kDa, with no apparent homology to other proteins. The authenticity of the cDNA selected was demonstrated by recloning, expressing, and purification of the cDNA-encoded protein and showing that it retained the collagen-binding characteristics with decreasing binding properties: human collagen type I > rat type I > human type III = calf skin type I. On the other hand rNecH1 did not bind to immobilized fibrinogen.

Although it is clear from the sequence data that the full-length protein has not yet been found, the part that was isolated has the collagen-binding capacity looked for. We are at present trying to identify the full-length sequence from an *N. americanus* cDNA library by using rNecH1-based probes on the one hand, and on the other hand we are raising antibodies against rNecH1 to isolate the native protein and to localize the protein histologically.

The initial search for a function of the identified protein so far has not been succesful. In view of the previous findings (Furmidge et al., 1996) that excretion/secretion products from *N. americanus* are able to prevent platelet aggregation induced by collagen, it was first determined whether NecH1 was responsible for this effect. However, rNecH1 neither inhibited platelet aggregation induced by collagen, and mediated by platelet receptors integrin $\alpha_2\beta_1$ and glycoprotein VI, nor Mg^{2+}-dependent, $\alpha_2\beta_1$-mediated platelet adhesion to immobilized collagen. Also, binding of von Willebrand Factor to collagen was not affected. It was, therefore, concluded that NecH1 was not the protein responsible for the antiplatelet effect in the excretion/secretion products. Furthermore, little evidence was seen for binding of rNecH1 to C1q, a subunit of the first component (C1) of the classical complement pathway that has a collagen-like region, indicating an absence of anti-complement activity.

Phages displaying NecH1 did bind to worm cuticular collagen. NecH1, therefore, may be a cuticular antigen. In line with this, preliminary data showed that antibodies in the serum of some infected patients interacted with NecH1 (not shown), indicating that NecH1 is immunogenic and must be, at least to some extent, exposed at some stage. Whether NecH1 can be used for the development of vaccines is at present not clear but will be investigated further.

In summary, the power of using the phage display technology as a cDNA expression system was demonstrated by the selection of new proteins based on binding properties, i.e., a specific, high-affinity collagen-binding protein from *N. americanus* was identified.

Although NecH1 does not have the function predicted at the beginning of the investigation, the ability to produce clean, parasite-derived ligands using this cDNA expression phage display library provides a sound experimental platform for whoever is interested in the further investigation of the interaction between *N. americanus* and its human host at the molecular level.

ACKNOWLEDGMENTS

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Navorsings- en oorsigartikels

Die toepassing van peptiedblootlegging op fage in trombose en hemostase

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**Outeur aan wie korrespondensie gerig kan word.*

Ontvang 6 Julie 1999; aanvaar 31 Augustus 1999

UITTREKSEL

Die omvang en gebruike van die tegnologie van peptiedblootlegging op fage, en die toepassing daarvan op die gebied van trombose en hemostase en van geneesmiddelontwerp word bespreek. Die algemene beginsels van die tegniek word verduidelik.

ABSTRACT

The application of phage display technology in thrombosis and haemostasis

The scope and utility of phage display are reviewed with emphasis on application in the field of thrombosis and haemostasis and drug design. General principles of this technique are described.

INLEIDING

Hemostase is 'n beskermende meganisme wat oormatige bloedverlies beperk wanneer bloedvate beskadig word en help om 'n fisiologiese homeostase te handhaaf. Normale hemostase word bewerkstellig deur 'n reeks komplekse interaksies tussen bloedvate, bloedselle, stollingsfaktore en -inhibeerders, asook die fibrinolitiese stelsel. Trombose, aan die anderkant, word allerweë as die teenbeeld van hemostase beskou. Na beskadiging van die bloedvat-endoteel, sodra die bloed in aanraking met die subendoteel kom, kleef die bloedplaatjies m.b.v. die glikoproteïen (GP) Ib/IX/V-reseptore op hulle membrane aan die subendoteel en word geaktiveer. Geaktiveerde plaatjies stel die reseptor GP IIb/IIIa op hulle oppervlak bloot, waaraan die klewingsproteïene, veral fibrinogeen en von Willebrandfaktor (vWF) bind. Die klewingsproteïene bevat 'n Arg-Gly-Asp-volgorde wat aan GP IIb/IIIa bind. Terselfdertyd word stollingsfaktore geaktiveer om trombien te vorm en word die plaatjieprop versterk om oormatige bloedverlies te verhoed.[1] Endoteelselle in die omgewing van die beskadigde wand skei tromboreguleerders (prostasiklien, stikstofoksied en fibrinolitiese middels) af, wat die grootte van die prop beperk.[2] Die plasminogeenaktiveerders, nl. weefseltipe plasminogeenaktiveerder (tPA) en urokinase-tipe plasminogeenaktiveerder (uPA) is belangrike fibrinolitiese middels wat die pro-ensiem plasminogeen aktiveer om plasmien te vorm. Plasmien verteer die onoplosbare fibrien in die plaatjieprop na oplosbare afbraakprodukte en sodoende beperk dit die grootte van die prop. Plasminogeenaktiveerder-inhibeerders (PAI) is proteïene wat tPA en uPA inhibeer. Oormatige aktivering van plaatjies deur biomateriale en aterosklerotiese letsels, oorkom egter hierdie tromboregulerende meganismes. Wanneer dit gebeur, verander die plaatjies en stollingsfaktore in trombotiese oortreders wat trombo-embolieë veroorsaak en bloedvate kan afsluit. Dit lei tot potensieel fatale siektes, soos akute miokardiale infarksie en beroerte.[3] Die belang van trombo-emboliese komplikasies van arteriële bloedvatsiektes word al hoe meer besef en dit het gelei tot die soektog na meer effektiewe antitrombotiese middels.

Die tegnologie van peptied- of proteïenblootlegging op fage is 'n kragtige en vinnige tegniek om proteïene met spesifieke antitrombotiese funksies te isoleer en/of te ontwerp en word reeds aangewend in die ontwikkeling van nuwe antitrombotiese middels. Dit is egter nodig om die tegniek van peptiedblootlegging op fage te verduidelik voordat die toepassing daarvan in trombose en hemostase bespreek word.

PEPTIEDBLOOTLEGGING OP FAGE

Peptiedblootlegging op fage is 'n tegniek waar 'n peptied of proteïen op die oppervlak van 'n filamenteuse faag blootgelê word. Dit word gedoen deur 'n gekose geen of geenfragment wat vir 'n oppervlakproteïen kodeer in die faaggeen in te bou. Hierdie inbouing beïnvloed nie die noodsaaklike funksies van die oppervlakproteïene nie en lei tot die blootlegging van 'n fusiepeptied of -proteïen op die oppervlak van die faag. Die peptied of proteïen kan dan as 'n ligand, ensiem, immunogeen of enige ander aktiewe deelnemer in 'n biologiese reaksie optree. Hierdie tegniek is eerste deur Smith in 1985[4] gebruik. Hy het 'n versameling of biblioteek van lukraakgekose peptiedvolgordes aan die amino-terminus van die mantelproteïen, glikoproteïen (GP) III, op die oppervlak van 'n faag M13 blootgelê. Wanneer lukraakgekose DNA-volgordes in faaggenome ingebou word, word lukraak peptiede op die oppervlak van die fage blootgelê en dit lei tot 'n groot mengsel van faagklone wat elk 'n verskillende peptiedvolgorde op die oppervlak blootlê. Uit hierdie mengsel van faagklone (faagbiblioteek) word fage wat aan 'n teikenproteïen bind, geselekteer. Die geselekteerde fage kan dan in bakterieë soos *E. coli* vermeerder word. Die aminosuurvolgorde van die peptiede op die geselekteerde fage word afgelei deur die ingevoegde DNA se volgorde m.b.v. bestaande tegnieke te bepaal. Die krag van hierdie tegniek lê daarin dat funksionele aktiwiteit gekoppel kan word aan genetiese inligting. Peptiedblootlegging bring vinnige seleksie van 'n gekose proteïen mee, terwyl die genetiese inligting vinnige en betroubare vermeerdering van fage meebring. Dit lei tot die produksie van groot hoeveelhede moontlike interessante teikens. Die tegnologie word ook gebruik om antiliggaam-epitope suksesvol te bepaal, om

struktuurfunksie-verwantskappe van sellulêre reseptore te bestudeer en om ensiemsubstraat-spesifisiteit na te vors en te verander. Om die tegniek beter te verduidelik, word elke faset daarvan in meer besonderhede bespreek.

Filamenteuse faag

Filamenteuse fage is ideaal geskik vir *in vitro*-seleksie omdat hulle klein genome bevat waarin groot biblioteke van 'n verskeidenheid verskillende gene maklik ingebou kan word. Die enkelstring-DNA-fage (fd of M13) word meestal gebruik. Hierdie fage het 'n deursnee van 6 nm en is 1 000 - 2 000 nm lank. Aan die een punt is daar 5 kopieë van elk van die mantelproteïene GP III en VI (figuur 1). Die hoof-mantelproteïen, GP VIII, kom in 2 800 kopieë op die sye van die faag voor. Beide GP III en VIII stel hulle aminoterminale op die oppervlak van die faag bloot, terwyl GP VI se karboksielkant ekstrasellulêr geleë is. Die faaggenoom bevat sowat 6 500 nukleotiede.

Die lewensiklus van fd en M13 filamenteuse fage begin wanneer hulle *E. coli* infekteer. Die faag bind aan die punt van die F-tipe geslagspilus van die *E. coli* en word in die sel ingeneem. Die F-pilus van die *E. coli* breek dan af sodat 'n *E. coli* slegs deur een faag met 'n spesifieke DNA-samestelling geïnfekteer kan word. Die gevolg is die ontstaan van 'n bakteriële kolonie, wat fage met dieselfde peptiedvolgorde produseer. Na inname word die enkelstring faag-DNA na 'n dubbelstring-vorm omgeskakel. Daarna volg herhaaldelike replisering, transkripsie van faaggene, proteïensintese en uiteindelike vrystelling as fage. Die meganismes wat hierdie prosesse reguleer, is goed bekend maar buite die bestek van hierdie oorsig.[5] Dit is belangrik om daarop te let dat die faag nie die gasheer doodmaak nie, en dat 'n *E. coli* wat deur een faag geïnfekteer is, tussen 100 en 1 000 nuwe fage tydens een generasietydperk produseer.

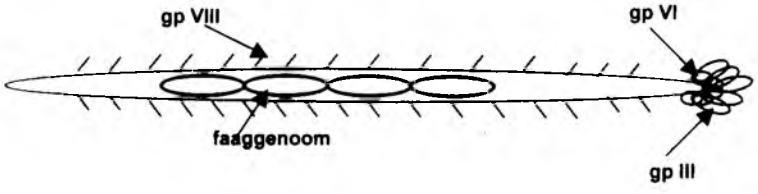
Konstruksie van 'n faagbiblioteek

'n Faagbiblioteek word gebou deur 'n versameling vreemde oligonukleotiede in die M13- of fd-faaggenome in te bou. 'n Tetrasiklien- (of enige ander antibiotikum) weerstandsgeen en restriksie-ensiemsnypunte word eerstens in die faaggenoom ingebou. Die tetrasiklien-weerstandsgeen bring mee dat geïnfekteerde *E. coli* weerstandbiedend is teen tetrasiklien. Vervolgens word 'n stukkie vreemde DNA, wat as 'n vulsel dien, op so 'n wyse m.b.v. restriksie-ensieme in een van die mantelproteïengene (bv. GP III) ingebou dat die leesraam verskuif. Fage wat hierdie DNA-vulsel bevat, sal 'n mutant van GPIII produseer wat verhoed dat die fage *E. coli* kan infekteer. Hierdie vulsel-DNA word dan verwyder en die gaping word met genoegsame oligonukleotiede gevul sodat die leesraam weer herstel word om 'n funksionele GP III te produseer. So byvoorbeeld sal daar vir 'n heksapeptiedbiblioteek, oligonukleotiede ingebou word wat 'n lukraak volgorde $(NNK)_6$ bevat. In hierdie geval beteken dit dat N gelyke hoeveelhede van al vier nukleotiede adenien, guanien, sitosien en timien bevat, terwyl K gelyke hoeveelhede guanien en timien bevat. Deur dit so te doen, word die aantal verskillende trinukleotiede wat gevorm word, verminder van $4 \times 4 \times 4 = 64$ na $4 \times 4 \times 2 = 32$, terwyl geen stopkodons gevorm word nie. Hierdie geneties gemanipuleerde faagvektore word dan gebruik om *E. coli* te infekteer. Die selle produseer fage wat geïsoleer en gesuiwer kan word, en die biblioteek is gereed vir seleksie. Die totale aantal verskillende heksapeptiede wat uit die 20 aminosure gevorm kan word, is $20^6 = 64 \times 10^6$ fage. 'n Praktiese biblioteek bevat sowat 10^9 tot 10^{14} fage wat ongeveer 70% van alle moontlike aminosuur-volgordekombinasies verteenwoordig.

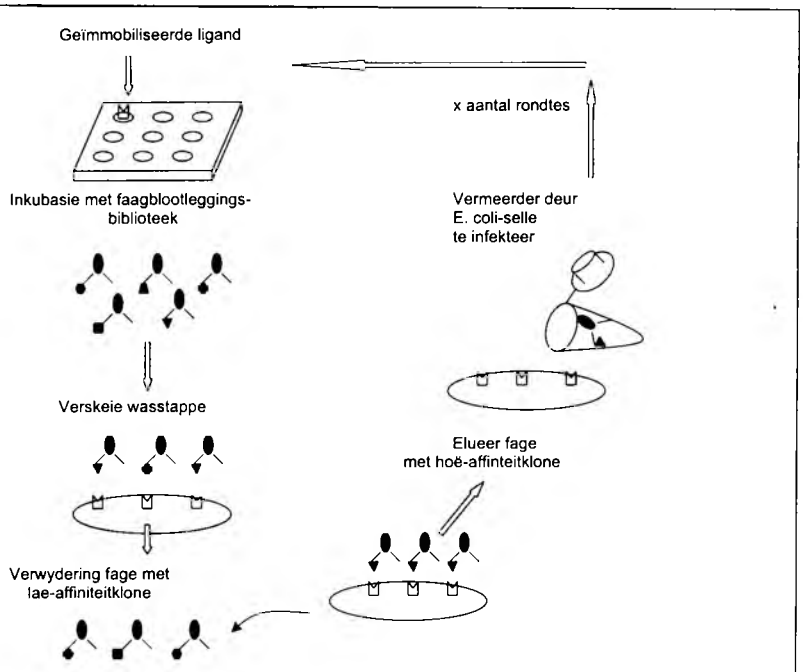
Seleksie en vermeerdering

Seleksie van fage word gedoen deur eerstens 'n teikenproteïen (reseptor, antiliggaam, ligand ens.) aan 'n plastiese immuunbuis of aan magnetiese korrels wat vooraf met kitsmelkpoeier of beesserum-albumien (BSA) bedek is, te immobiliseer (figuur 2). Die kitsmelkpoeier of BSA dien as 'n blokkeermiddel om die bindingsetels wat nie deur die teikenproteïen beset word nie, te beset. Die teikenproteïen word vervolgens met die faagbiblioteek geïnkubeer. Na interaksie word die nie-bindende fage afgewas sodat net fage met peptiede wat aan die teikenproteïen bind, behoue bly. Die bindende fage kan dan nie-spesifiek met 'n sterk suur geëlueer word. Na suur-eluering word die faagoplossing met 'n sterk basis geneutraliseer. Geselekteerde fage kan ook spesifiek geëlueer word. So byvoorbeeld kan antiliggaamgebonde fage met die natuurlike antigeen geëlueer word en reseptorgebonde fage met die ligand. 'n Voorbeeld hiervan is die isolasie van 'n inhibeerder van die trombienreseptor op plaatjies. In hierdie geval word 'n faagbiblioteek met intakte bloedplaatjies geïnkubeer en die bindende fage met die reseptorligand geëlueer. Dit dui daarop dat spesifieke kennis oor die reseptor nie nodig is om binders, of met 'n bietjie geluk, inhibeerders te selekteer nie.[6]

Die bindende fage word dan oornag met F-pilus-bevattende *E. coli* in hulle eksponensiële groeifase geïnkubeer in die teenwoordigheid van 'n antibiotikum (bv. tetrasiklien). Die geïnfekteerde *E. coli* produseer fage wat versamel word deur die *E. coli*-kultuur te sentrifugeer en te suiwer deur 'n aantal poliëtileenglikol-natriumchloried (PEG-NaCl) presipiterings- en hersuspenderingsiklusse. Hierdie geselekteerde fage word dan gebruik vir 'n tweede, derde of selfs vierde rondte van seleksie en vermeerdering. Die aantal bindende fage kan op dié wyse met tussen 1 000 en 100 000 keer per rondte vermeerder word. Die sukses



Figuur 1: 'n Skematiese voorstelling van 'n filamenteuse faag.



***Figuur 2**: 'n Skematiese voorstelling van peptiedblootlegging op fage.*

van die seleksieproses word bepaal deur 'n klein hoeveelheid van die geïnfekteerde bakterieë wat na die laaste seleksierondte versamel is, op agarplate uit te plaat sodat enkelkolonies kan opgroei. Omdat 'n faag slegs een bakterie kan infekteer, sal elke kolonie 'n versameling van identiese fage wees. Enkelkolonies wat opgetel en in mikrotiterplaatputte gekweek word, produseer dus monoklonale fage wat, na 'n sentrifugeringstap, versamel word. Hierdie fage word m.b.v. 'n ensiemgekoppelde immuunbepaling (ELISA)-tegniek getoets vir binding aan die teikenproteïen. Kortliks kom dit daarop neer dat die fage oorgedra word na mikrotiterputte waaraan die teikenproteïen gekoppel is. Na inkubasie word die nie-bindende fage afgewas en 'n antifaag-antiliggaam, gekonjugeer aan peperwortelperoksidase (HRPO), in die putte gevoeg. Na 'n verdere wasstap word 'n kleursubstraat bygevoeg om die bindende faagkolonies te identifiseer.

Volgordebepaling

Die *E. coli*-kolonies wat bindende fage bevat, word gekweek om genoegsame hoeveelhede fage te produseer sodat die faag-DNA geïsoleer kan word. Die DNA-volgorde van die oligonukleotied-invoeging word dan m.b.v. klassieke volgordebepalingstegnieke bepaal.

DIE GEBRUIK VAN PEPTIEDBLOOTLEGGING IN TROMBOSE EN HEMOSTASE

Navorsers op die gebied van trombose en hemostase maak reeds van peptied-, antiliggaam-, cDNA- en proteïenbiblioteke gebruik, enersyds om kandidate te identifiseer wat as antitrombotiese middels ontwikkel kan word, en andersyds om struktuurfunksie-verwantskappe in trombose te bepaal. Wat hierna volg, is slegs 'n kort bespreking van die gebruik van hierdie biblioteke en die inligting wat daaruit verkry is.

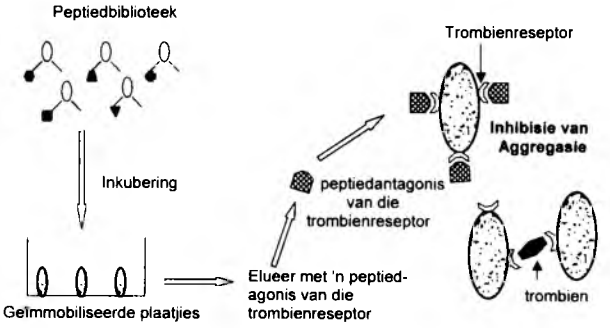
Peptiedbiblioteke

Peptiede wat 7, 10, 15 of 38 lukraakgekose aminosure as liniêre peptiede op die oppervlak van filamenteuse fage blootlê, word algemeen gebruik. 'n Variasie hiervan is om 'n sisteïen aan elk van die amino- en karboksieterminale van die peptiede in te bou sodat 'n sikliese peptied blootgelê word.[7] Daar word algemeen aanvaar dat die bindingseienskappe van 'n sikliese peptied beter is as dié van 'n liniêre peptied.[7]

Die Arg-Gly-Asp-volgorde wat in die klewingsproteïene, fibrinogeen en fibrien voorkom, bind aan die GP IIb/IIIa-reseptor op die plaatjiemembraan en vorm sodoende 'n brug wat plaatjie-aggregasie tot gevolg het.[8] 'n Peptiedbiblioteek waar 'n Arg-Gly-Asp-volgorde tussen twee stelle lukraak tripeptiede ingebou is, is gebruik om 'n effektiewe inhibeerder van plaatjie-aggregasie te isoleer.[9] Dit is gedoen deur die GP IIb/IIIa-reseptor aan die wand van 'n immuunbuis te immobiliseer en met die faagbiblioteek te inkubeer. Peptiede wat aan GP IIb/IIIa bind, is geselekteer en getoets vir inhibisie van plaatjie-aggregasie.

Daar is verskeie groepe wat peptiedbiblioteke gebruik het om die epitoop waarmee monoklonale antiliggame aan hulle teikenproteïene bind, te bepaal. So byvoorbeeld is die epitoop van 'n menslike antiliggaam teen plasminogeenaktiveerder-inhibeerder tipe 1 en α_2-makroglobulien bepaal.[10] In die laasgenoemde geval is daar 'n stel van vyf oorvleuelende fragmente wat oor 54 aminosure strek (van Gly_{4291} tot Cys_{4344}) van die β-ketting van die reseptor, geïsoleer.

Die trombienreseptor op die bloedplaatjiemembraan is 'n integrale membraanproteïen. Trombien bind daaraan en sny sewe aminosure van die C-terminaal af. Die sewe-aminosuurligand wat vorm, is dan in staat om as 'n trombienreseptor-agonis op te tree en die plaatjies via die reseptor te aktiveer. Hierdie eienskap van die reseptor is gebruik om 'n inhibeerder van die reseptor te isoleer.[11] 'n Faagbiblioteek is gemaak met peptiede wat gegrond is op die peptiedvolgorde van die ligand. Hierdie biblioteek is met plaatjies geïnkubeer en die bindende fage m.b.v. die trombienreseptor-agonis geëlueer (figuur 3). Fage wat dus aan die trombienreseptor bind, is geëlueer en getoets vir inhibisie van die trombienreseptor. Die aminosuurvolgorde van die blootgelegde inhiberende peptiede is daarna bepaal en peptiede met dié



Figuur 3: Seleksie van 'n peptiedantagonis van die trombienreseptor.

aminosuurvolgordes is gemaak.

Urokinase-tipe plasminogeen-aktiveerder (uPA) aktiveer fibrinoliese deur plasminogeen na plasmien om te skakel. Sy reseptor op bloedselle, die urokinasereseptor, reguleer die fibrinolitiese sisteem en bemiddel onder meer die metastasesering en indringing van tumorselle. 'n Nuwe teiken vir die terapeutiese behandeling van tumore is dus om die binding van uPA aan sy reseptor te blokkeer.[12] Deur gebruik te maak van 'n 15-aminosuur lukraak peptiedbiblioteek, is twee peptiede wat sterk aan die urokinasereseptor bind, geïsoleer. Dit was interessant dat nie een van die twee peptiede se aminosuurvolgordes ooreengestem het met die bindingsetels van uPA aan die reseptor nie. In hierdie geval is daar dus twee peptiedligande van sel-oppervlakreseptore geïdentifiseer wat kan lei tot die ontwikkeling van inhibeerders van die reseptor.[12]

Plaaslik het ons 'n lukraak sikliese heptapeptiedbiblioteek gebruik om heptapeptiede wat aan α-trombien bind, te isoleer. Hierdie peptiede herken spesifiek α-trombien, maar inhibeer nie die funksie daarvan nie wat daarop dui dat hierdie peptiede aan 'n epitoop bind wat nie die proteolitiese aktiwiteit van trombien beïnvloed nie. Hierdie tipe peptiede kan aan radioaktiewe jodium gebind word en kan moontlik gebruik word om *in vivo*-trombusvorming m.b.v. sintillasiekamera-tegnieke op te spoor.[13] Trombien word in 'n trombus vasgevang[14] en hierdie peptiede kan dan spesifiek aan trombien in die trombus bind waar dit m.b.v. 'n sintillasiekamera opgespoor kan word.

Antiliggaambiblioteke

In hierdie geval word antiliggame of gedeeltes daarvan op die oppervlak van fage blootgelê. Die doel is om veral mens-antiliggame te kloneer wat dan as geneesmiddels ontwikkel kan word of in navorsing gebruik kan word. Soortgelyk aan die B-sel van die immuunstelsel wat antiliggaamgene bevat, en hulle op sy oppervlak blootstel, word antiliggaambiblioteke so gemaak dat mens-antiliggame of gedeeltes daarvan op die oppervlak van fage blootgestel word. Daardie fage wat aan die antigeen bind, word dan geselekteer.

Met behulp van antiliggaambiblioteke kan F_{ab}-fragmente teen komplekse, biologiese antigene geïsoleer word. In 'n poging om antiliggame teen plasmingeen-aktiveerder-inhibeerder-tipe 1 te selekteer, het Lang *et al.*[31] soos volg te werk gegaan: Hulle het 'n konyn met die α-granule van mens-plaatjies geïmmuniseer en daarna die totale RNA uit die milt en beenmurg van die konyne ge-ekstraheer. Die RNA wat vir die κ-ligte en γ-swaarkettings kodeer, is geïsoleer, na cDNA getranskribeer, en in 'n faagmied ingebou om 'n F_{ab}-biblioteek te maak. Ses-en-veertig faagklone wat aan PAI-1 bind, is m.b.v. hierdie biblioteek geïsoleer.

Hierdie antiliggaamfragmente op die fage kan weer van die mantelproteïen vrygestel word.[15] Eerstens kan 'n stopkodon tussen die geen wat kodeer vir die antiliggaamfragment en dié vir die faag-mantelproteïen ingebou word. Wanneer die stopkodon onderdruk word, bly die antiliggaamfragmente aan die mantelproteïen gebind, so nie, word dit vrygestel.[15] Tweedens kan die geen wat kodeer vir die antiliggaamfragment m.b.v. 'n restriksie-ensiem van die mantelproteïengeen gesny word en in 'n ander vektor wat geskik is vir die sekresie van die antiliggaamfragmente, herkloneer word.[16]

cDNA-biblioteke

Die cDNA-biblioteke vind al hoe meer toepassing in trombose- en hemostase-navorsing. Die totale mRNA van 'n sel, orgaan of organisme word voorberei, getrutranskribeer na cDNA en in die faaggeen van GP III of IV ingebou. In die laasgenoemde geval word die vreemde proteïen aan die karboksie-terminale kant van GP VI geheg. Dit is ook die mees praktiese manier om die uitdrukking van 'n cDNA-biblioteek te verseker, aangesien die vreemde cDNA met hulle 5'-kante aan die faag-DNA heg. Die voordeel is dat die 5'-kant nie ribosoombindingsetels het nie en dat daar alreeds translasionele stopseine aan die 3'-kant is. Dit beteken dat die stoppe nie die blootlegging van die vreemde proteïen op die mantelproteïen kan beïnvloed nie.[17] 'n Voorbeeld hiervan is 'n cDNA-biblioteek wat gemaak is van die haakwurm *Ancylostoma caninum*. Hierdie biblioteek is in pDONG6-faagmiedvektore gekloneer en fage wat aan tripsien of faktor Xa bind, is geselekteer. 'n Nuwe tripsieninhibeerder, struktureel verwant aan die Kunitz-tipe serumprotease-inhibeerders, en 'n faktor Xa-inhibeerder is uit hierdie biblioteek geïsoleer.[18]

Proteïenbiblioteke

In die geval van proteïenbiblioteke word 'n hele proteïen op die oppervlak van die filamenteuse faag blootgelê. Hierdie proteïene kan varieer van klein proteïene soos groeihormoon[19] en pankreas-tripsieninhibeerder[20] tot groter proteïene soos alkaliese fosfatase[21] en α-laktamase.[22] Proteïen-struktuurfunksiestudies vind baie hierby baat, veral nadat verskeie groepe al aangetoon het dat 'n groot verskeidenheid proteïene op filamenteuse fage blootgelê kan word sonder 'n verlies aan funksionele aktiwiteit.[19-22] Die tegniek het tot gevolg dat 'n groot aantal mutante proteïene gegenereer kan word en verskaf 'n goeie seleksiestrategie om mutante met die gewenste fenotipe te identifiseer. In die geval van groeihormoon byvoorbeeld, het die seleksie op geïmmobiliseerde reseptore gelei tot die identifikasie van mutante met 'n hoër affiniteit vir sy reseptor as in die geval van die oorspronklike proteïen.[19]

In trombose en hemostase is ensieme soos PAI-1 op fage blootgelê. PAI-1 wat op fage blootgelê word, behou sy vermoë om tPA te inhibeer.[23] 'n Biblioteek van PAI-1-mutante is gebruik om te bepaal watter aminosuurvolgorde van PAI-1 aan trombien bind.[23]

Jespers *et al.*[24] het 'n biblioteek van lukraak variante van staphilokinase (SAK) gemaak en op dié wyse die epitope van 2 monoklonale antiliggame teen staphilokinase (SAK) bepaal. Hierdie variante bevat enkel, dubbel of trippel aminosuur-mutante. Die werkswyse was om die biblioteek negatief te

selekteer vir SAK-variante wat nie aan die monoklonale antiliggame bind nie. Uit hierdie geselekteerde fage is dan variante geselekteer wat aan plasmien bind. Na verskeie seleksierondtes, is die aminosuurvolgorde van die geselekteerde fage bepaal. Die aminosuurvolgorde van daardie mutante wat die hoogste voorkoms het, weerspieël dan die funksionele epitoop van die antiliggaam.

SAMEVATTING

Hierdie oorsig bespreek die tegnieke van peptied- en proteïen-blootlegging op fage, asook variasies daarvan. Toepassings op die gebied van trombose en hemostase word ook gegee. Daar moet egter besef word dat hierdie tegniek ook baie ander toepassings buite die veld van trombose en hemostase het. So byvoorbeeld word die tegniek gebruik om geneesmiddels en gene in geselekteerde weefsels te plaas deur peptiede te selekteer wat vaskulêre selmerkers identifiseer.[25] Dit word ook gebruik in die ontwikkeling van entstowwe.[26] Alhoewel hierdie tegniek nog relatief nuut is, het dit al baie bygedra tot die uitbouing van ons kennis van veral struktuurfunksie-verwantskappe van ensiem-reaksies, inhibisie van *in vivo*-prosesse en die ontwikkeling van geneesmiddels.[6, 18, 27] Dit is baie bemoedigend om waar te neem dat hierdie tegnologie alreeds 'n hoofrol in die gebied van trombose en hemostase begin speel. Daar word verwag dat, soos hierdie tegnieke meer toegepas word, 'n verdere kennisontploffing in die gebied sal plaasvind.

SUMMARY

INTRODUCTION

Haemostasis is a protective mechanism that limits excessive blood loss. Thrombosis, on the other hand, is the antitype of haemostasis. Following blood vessel injury, when the blood comes into contact with the sub-endothelium, the blood platelets adhere to sub-endothelial structures and become activated. Simultaneous activation of the coagulation factors form thrombin. In concert, platelets and thrombin form a platelet plug to prevent excessive blood loss. Endothelial cells in the vicinity of the platelet plug secrete prostacyclin, nitrogen-oxide and fibrinolytic agents that limit the size of the plug. Excessive activation of platelets by atherosclerotic lesions overcome these thromboregulatory mechanisms and the platelets and coagulation factors then become thrombotic offenders that occlude the blood vessel at the site of injury or form thrombo-emboli that can block blood vessels distal to the site of injury. In view of this, more effective anti-thombotic agents are being investigated. For this purpose, phage display technology is ideally suited because it is a powerful and rapid method to isolate or design proteins with specific anti-thrombotic functions.

PHAGE DISPLAY

Phage display is a technique that expresses or "displays" a peptide or protein on the surface of a filamentous phage. This is accomplished by the insertion of a gene or gene fragment in a phage surface protein gene. The insert will result in a fusion peptide/protein on the phage surface, provided that the reading frame is correct and that the insert does not interfere with the essential functions of the surface protein. If the peptide is well exposed on the surface it will be available to act as a ligand, enzyme, and immunogen or otherwise actively participate in a biochemical process. The insertion of random oligonucleotide sequences provides a means of constructing extensive peptide libraries that may be screened to select peptides with specific affinities or activities. The separation of phage particles expressing different peptide inserts on the phage surface protein is accomplished by affinity selection.

THE USE OF PHAGE DISPLAY IN THROMBOSIS AND HAEMOSTASIS

Scientists already use peptide, antibody, cDNA and protein phage libraries to identify and develop effective antithrombotics.

Peptide libraries were used to select peptides that bind to and inhibit the glycoprotein IIb/IIIa-receptor on platelets and an inhibitor of the thrombin receptor on platelets was also isolated. Both these peptides inhibit platelet aggregation. A peptide that inhibits the urokinase receptor that may have anti-tumor activities was also selected. We selected thrombin-binding peptides that can be used to detect *in vivo* thrombus formation with a scintillation camera.

Antibody libraries are formed by displaying antibodies or parts of antibodies on filamentous phages. In this way, antibodies to the plasminogen activator inhibitor-type 1 (PAI-1) were selected from a mixture of antibodies.

In the case of cDNA libraries, the total mRNA of a cell, organ or organism is collected and reverse transcribed to cDNA. The cDNA is then inserted into the phage genome to display foreign proteins on the phage surface. An example is a cDNA library made from the hookworm *Ancylostoma caninum*. Inhibitors of trypsin and factor X_a were selected form this library.

Protein libraries were made where enzymes, such as PAI-1, was displayed on the surface of phages. A library of PAI-1 mutants was then used to determine which sequence in PAI-1 binds to thrombin. The functional epitope of two monoclonal antibodies to staphylokinase were also determined by using a library of random variants of SAK.

Although the technique of phage display is relatively new, its use has already made remarkable contributions in the field of thrombosis and haemostasis. It is not only suitable to select specific binders to proteins but also expand our knowledge of structure function relationships and to develop effective agents for treatment. We expect that the increasing use of phage display will result in an explosion of our knowledge of the mechanisms involved in thrombosis.

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In vitro effect of a thrombin inhibition peptide selected by phage display technology

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Abstract

A repeated selection of phages from a cyclic heptapeptide phage display library resulted in the enrichment of phages that bind to human α-thrombin. One clone of the binding phages that competed with PPACK for binding to thrombin and that had the best binding characteristics was chosen. The amino acid sequence of the peptide displayed on this phage was determined and a peptide with the sequence, Cys-Asn-Arg-Pro-Phe-Ile-Pro-Thr-Cys was synthesised. This peptide, thrombin-inhibiting peptide (TIP), is a full competitive inhibitor of thrombin with an inhibition constant (K_i) of 0.4974 mM. It lengthened the thrombin time and inhibited thrombin-induced platelet activation and the platelet release reaction, both in a dose-dependent manner. It also reduced platelet adhesion onto a human microvascular endothelial matrix in the parallel plate flow chamber under both arterial and venous shear conditions. Thus, we have selected and synthesised a cyclic heptapeptide that competes with PPACK to bind to thrombin and that can be developed as a direct antithrombin.

Keywords: Thrombin antagonists and inhibitors; Thrombin; Peptide library; Recombinant fusion proteins; Bacteriophage M13 genetics; Phage display system

1. Introduction

The technique of phage display is a powerful and quick way to select peptides with specific affinities and activities toward a target protein. Its power lies in the combination of functional activity with genetic information. Although not yet extensively, this technique has been used in the field of thrombosis and haemostasis. Peptide libraries were used to select peptides that bind to and inhibit the thrombin receptor on the platelet membrane and the urokinase receptor [1,2]. Potent anticoagulants have also been derived by targeting the tissue factor/factor VIIa complex with naïve peptide libraries displayed on the M13 phage [3].

In this study, we used a cyclic heptapeptide phage library to select phages that bind to and inhibit α-thrombin. Thrombin was chosen for two reasons. First, it is a multifunctional serine protease that plays a key role in thrombus formation and is an important participant in the sequelae thereof [4]. It is also mainly responsible for maintaining an intricate balance between the interactive elements of the vessel wall, coagulation factors and platelets [4]. Thus, thrombin cleaves fibrinogen and activates factor XIII to form insoluble fibrin. It activates platelets and amplifies its own generation (positive feedback) by activation of factor

Abbreviations: PPACK, D-Phe-Pro-Arg-CH_2Cl_2; TIP, thrombin inhibition peptide; K_i, inhibition constant; TAP, tick anticoagulant peptide; TBS, Tris-buffered saline; *E. coli*, *Escherichia coli*; IPTG, isopropyl β-D-thiogalactoside; X-Gal, 5-bromo-4-chloro-3-indonyl-β-D-galactoside; SM, skimmed milk; HRP, horseradish peroxidase; OPD, *ortho*-phenylenediamine; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; ADP, adenosine diphosphate; FITC, fluorescein isothiocynate; PE, phycoerythrin; HMEC, human dermal microvascular endothelial cells; DNA, dioxyribonucleic acid.

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V and factor VIII [5]. Normally, thrombin generation is under stringent physiological control, because on the surface of intact endothelial cells, thrombin in complex with thrombomodulin activates protein C, which in turn inactivates factors Va and VIIIa, resulting in a decline in thrombin generation (negative feedback). The thrombin concentration in plasma is low due to inactivation by circulating antithrombin III or heparin cofactor II bound to dermatan sulphate at the endothelial cell surface [6]. Second, studies have shown that inhibition of thrombin with recombinant hirudin, or its formation by inhibition of factor Xa with recombinant tick anticoagulant peptide (TAP) effectively inhibits coagulation and arterial thrombosis [7,8].

The central role of thrombin in thrombogenesis makes it an ideal target for antithrombotic intervention [4]. Under pathological conditions, excessive thrombin generation occurs. Since thrombin is formed continuously from prothrombin at the surface of a thrombus, the rate of thrombin inhibition, by a thrombin inhibitor, should at least be as efficient as the rate of thrombin formation. Indirect, as well as direct inhibitors of thrombin represent potentially useful drugs for the treatment of both venous and arterial thrombosis. The clinical effectiveness of the indirect thrombin inhibitors such as heparin, and low-molecular-weight heparin, is hampered by their inability to inhibit clot-bound thrombin, and by the fact that they may cause thrombocytopenia [9]. Direct thrombin inhibitors of thrombin include hirudin, hirulog, etc. They are small peptides that inhibit only thrombin. These compounds are very potent inhibitors, and there are now indications that hirudin may be too potent and may promote bleeding tendencies when the dosage is too high [10]. However, an advantage of these smaller peptides is that they are not immunogenic [11].

There is thus still a need for small, direct thrombin inhibitors, since small peptides are nonimmunogenic and nontoxic and direct thrombin inhibitors inhibit clot-bound thrombin, which is largely involved in thrombosis [8]. In this study, we selected a cyclic heptapeptide named thrombin inhibition peptide (TIP) from a cyclic heptapetide phage library by using phage display technology and tested its effect on thrombin function.

2. Materials and methods

2.1. Selection of thrombin-binding phages

2.1.1. Biotinylation of human α-thrombin

Human α-thrombin (a kind gift from Dr. Pötzsch, Kerckhoff Klinik, Bud Nauhem, Germany) was biotinylated with EZ-Link™ sulfo-NHS-LC-Biotin (Pierce Chemical, Rockford, IL, USA) according to the instructions of the manufacturer. Two molecules of biotin were bound to one molecule of thrombin.

2.1.2. Isolation of thrombin-binding phages

A cyclic 7-mer phage display library was purchased from New England Biolabs (Beverley, MA, USA). Streptavidin magnetic particles (1.5 mg; Boehringer Mannheim, Germany) were first blocked for 1 h with 2% skimmed milk (DIFCO, Detroit, MI, USA) in Tris-buffered Saline (TBS) at room temperature. Phages (2×10^{11}) were added to half of the blocked streptavidin magnetic particles and incubated for 1 h at room temperature. Ten micrograms of the biotinylated thrombin was added to the other half of the magnetic particles, and incubated for 30 min at room temperature. The phage supernatant was then transferred to the thrombin-bound magnetic particles and incubated overnight at room temperature. After incubation, the magnetic particles were washed 10 times with TBS–Tween (0.1% Tween-20 in TBS) to remove the nonbinding phages. Phages bound to thrombin were eluted with 50 μl of 0.1 M glycine, pH 2, for 15 min at room temperature and immediately neutralised with 125 μl of 1 M Tris–HCl, pH 8. The titre of the eluted phages was estimated and half of the eluted fraction was used for amplification. Three additional rounds of panning were performed. During this last three rounds, the thrombin-bound phages were eluted with 8 mg r-hirudin (Hoechst-Marion-Rousell, AGS Frankfurt, Germany).

Phages were amplified after each round of panning by infection of *Escherichia coli* (*E. coli*) ER2537 cells and purified from the supernatant by two polyethylene glycol precipitations. A fraction of the eluted phages of panning round 4 was subjected to serial 10-fold dilution with Luria-Broth (LB) culture media (DIFCO). To 10 μl of each of the phage dilutions, 200 μl of *E. coli* cells in their log phase of growth was added and the mixture was incubated for 5 min at room temperature to allow the phages to infect the *E. coli* cells. Each dilution was spread onto agar plates containing isopropyl β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indonyl-β-D-galactoside (X-Gal) and incubated overnight at 37 °C. Since the library phages were derived from the common cloning vector M13mp19, which carries the LacZα gene, phage plaques with inserts appear blue when plated on media containing X-Gal and IPTG.

2.1.3. Phage ELISA

A 96-well ELISA plate (Maxisorp surface, Nalge Nunc International, Roskilde, Denmark) was coated overnight with 100 μg/ml α-thrombin in 0.1 M $NaHCO_3$ binding buffer at 4 °C in a humidified container. The wells were blocked for 2 h at room temperature with 200 μl 4% skimmed milk (SM) solution. Simultaneously, 96 blue phage colonies were picked from the agar plate and grown overnight at 37 °C in 1 ml LB medium containing 1:100 diluted *E. coli* cells from a preculture. Hundred microliters of this supernatant was added to each well together with 100 μl of 4% SM and incubated at room temperature for 2 h. Bound phages were detected after 1-h incubation with a polyclonal anti-M13 phage horseradish peroxidase (HRP)-

conjugated antibody (Pharmacia, USA) and visualised by adding *ortho*-phenylenediamine (OPD, Sigma, St. Louis, MO, USA). The reaction was stopped with 4 M H_2SO_4 and absorbance determined at 490 nm. Between each incubation step, the plates were washed three to nine times with TBS–Tween (0.1% Tween-20).

The six phage colonies with the best thrombin-binding characteristics were amplified by infecting *E. coli* cells and purified by polyethylene glycol precipitation. A dilution series of these individual phage clones were tested for thrombin binding in an ELISA. The same technique was used as described for the previous ELISA. Again the best binding phages were used for further testing. To determine if these colonies bind to the active site of thrombin, an inhibition ELISA was done where different concentrations of D-Phe-Pro-Arg-CH_2Cl_2 (PPACK, a kind gift from Prof. S.R. Hanson, Emory University, School of Medicine, Atlanta, GA, USA) was added to the thrombin-coated wells. After incubation for 15 min, 5×10^{10} phages in 2% SM were added into each well and incubated for 2 h. Bound phages were again detected after 1-h incubation with the anti-M13 antibody and visualisation done with OPD and absorbance measured at 490 nm.

2.1.4. Thrombin times

Thrombin Times were done where a dilution series of phages of colony A were added to 100 μl of normal human plasma. For the control, phosphate-buffered saline (PBS) was added to the plasma.

2.2. Nucleotide sequencing

Phage DNA of the thrombin-inhibiting phage colony was prepared using the ph.D-C7C phage display peptide library kit (New England Biolabs) and sequencing reaction were performed according to the DY Enamic ET terminator cycle sequencing premix kit (Pharmacia, Buckinghamshire, UK).

2.3. Peptide analysis

2.3.1. Peptide synthesis

A peptide sequence that correlated with the peptide sequence of the best thrombin-binding phage clone was called thrombin inhibition peptide (TIP) and synthesized by Ansynth Service (Roosendaal, The Netherlands).

2.3.2. In vitro enzyme inhibition

Chromogenic substrate hydrolysis was detected using the Bio-Tech EL312e microplate ELISA reader (Biotek instruments, VT, USA) at 405 nm equipped with a microplate mixer in addition to a kinetic module software (Kinetic Calc, Biotek). In several kinetic assays, different fixed TIP concentrations (0–0.956 mM) were incubated with α-thrombin (0.049 pM) for 10 min followed by addition of different chromogenic substrate, chromozym TH (Roche Biochemicals, Germany) concentrations (1.95–125 μM) to start the reaction. The total volume of the reaction was 200 μl. All reagents were diluted in the reaction buffer, HEPES-BSA (50 mM HEPES, 0.05% BSA, pH 7.4). Kinetic parameters, Km and Vm, were determined by least squares nonlinear regression using a single binding site model.

2.3.3. Thrombin times

The thrombin time of a dilution series of the peptide in plasma of healthy volunteers was measured with a Cobas Fibro fibrinometer (Diagnostica Stago, Asnières, France). Reagents were supplied by Dade Behring (Newark, DE, USA).

In vitro platelet aggregation: Blood of healthy volunteers were collected in 3.8% sodium citrate (9:1 v/v). The in vitro aggregation response of platelets at different concentrations of the peptide was measured turbid metrically in a Monitor IV Plus aggregometer (Helena Laboratories, USA). The method is fully described [10]. The aggregation response, measured after 4 min following addition of ADP (20 μM) or collagen (0.05 g/l), was related to the difference in light transmission between platelet-rich and platelet-poor plasma and expressed as a percentage.

2.3.4. Flow cytometry

Fluorescence flow cytometry was used to measure the inhibition of platelet activation and degranulation at different concentrations of the peptide. Samples were analyzed by two-color fluorescence using fluorescein isothiocynate (FITC)- and phycoerythrin (PE)-conjugated antibodies. Whole blood was added to polystyrene tubes containing 5 μl thrombin (1 U/ml, Sigma). Gly-Pro-Arg-Pro (1.25 mM final concentration, Sigma), 2.5 μl, was added immediately to delay fibrin polymerization. Ten microliters of TIP peptide (1000, 250 and 62.5 μg/ml) and HEPES buffer (1

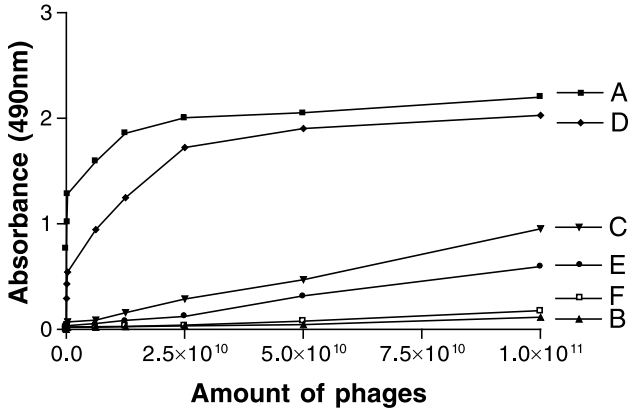

Fig. 1. Binding curves of different concentrations of phages to α-thrombin. An ELISA plate was coated overnight with 100 μg/ml human α-thrombin. A dilution series of phages of the six best binding phage colonies (colonies A to F) were added to the wells of the ELISA plate and incubated. An anti-M13 phage horseradish peroxidase (HRP)-conjugated antibody was added to the wells and the thrombin-binding phages were visualised by adding *ortho*-phenylenediamine (OPD). The reaction was stopped with 4 M H_2SO_4 and absorbance determined at 490 nm.

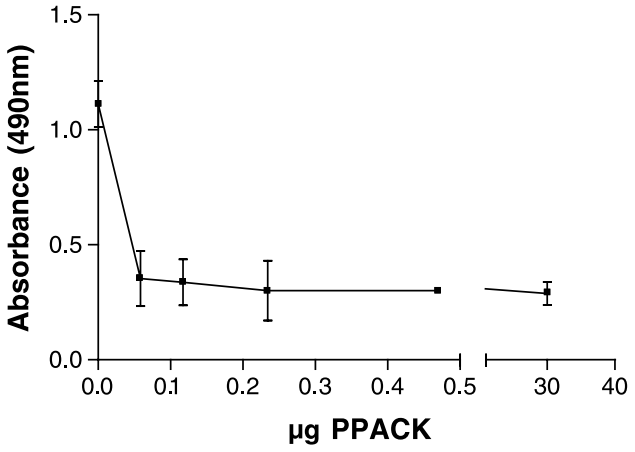

Fig. 2. Prevention of phage colony A from binding to thrombin by PPACK (D-Phe-Pro-Arg-CH_2Cl_2). Different concentrations of PPACK were added to the thrombin-coated wells. After incubation for 15 min, 5×10^{10} phages in 2% skimmed milk (SM) were added into each well and incubated for 2 h. Bound phages were detected after 1-h incubation with the anti-M13 antibody and visualisation done with OPD and absorbance measured at 490 nm.

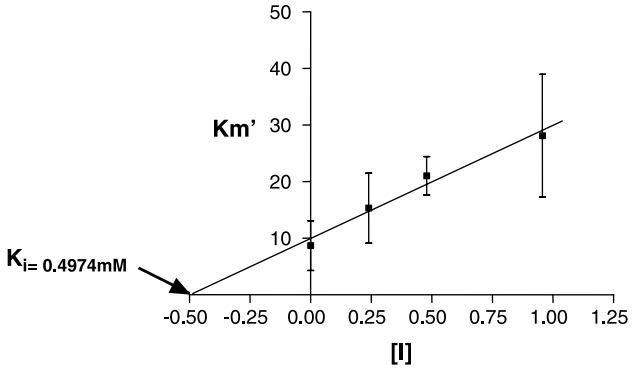

Fig. 4. Kinetics of the inhibition of the α-thrombin-induced chromogenic substrate (chromozym TH) hydrolysis by TIP. The reactions were initiated by different substrate concentrations (0, 1.95, 3.91, 7.81, 15.63, 31.25, 62.5 and 125 µM) to a mixture of 0.049 pM α-thrombin and different TIP concentrations (0, 0.239, 0.478 and 0.956 mM). The Km value was calculated by one-site binding nonlinear regression (r^2>0.990). The K_i value was calculated from the x-intercept, which is $-K_i$. Linear regression of the date yields a K_i of 0.4974 mM. The points of each figure are the mean ± standard deviation of six independent experiments.

mM, pH 7.4) was added to a final volume of 50 µl. The samples were gently mixed and incubated for 10 min at room temperature. This was followed by the addition of 5 µl PE-conjugated CD41 antibodies against the platelet marker GPIIb/IIIa (Beckman Coulter). For measurement of platelet activation and degranulation, 5 µl FITC-conjugated anti-CD62P (P-selectin; Beckman Coulter) antibody or anti-CD63 (Lisosomal glycoprotein, Beckman Coulter) was added. The samples were then incubated at room temperature for 30 min. Finally, each sample was diluted and fixed with 450 µl of 1% *p*-formaldehyde in saline before analysis.

The labelled platelets were analysed using a FAC-Calibur flow cytometer (Becton Dickinson, USA). Forward and side scatter as well as green (FITC) and red (PE) fluorescence signals were acquired with logarithmic amplification. The data from 10 000 cells were acquired and processed. Similarly FITC-labelled isotopic controls were prepared using whole blood. These controls were run with each experiment to determine nonspecific antibody binding. Controls of resting platelets were also used for each antibody to define a

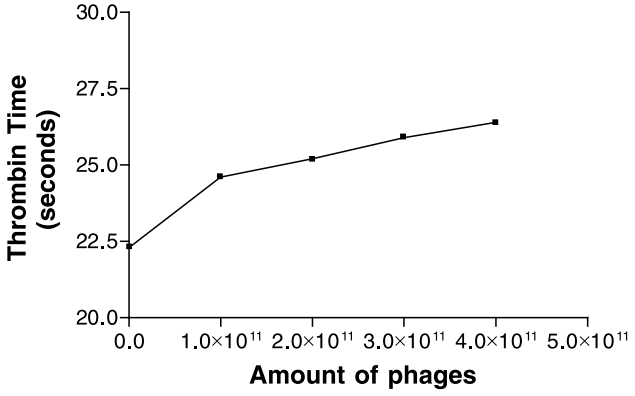

Fig. 3. Effect of a dilution series of phages from phage colony A on the thrombin time. Different concentrations of phages from phage colony A were added to 100 µl of normal human plasma. The phages of this colony also lengthened the thrombin time in a dose-dependent manner.

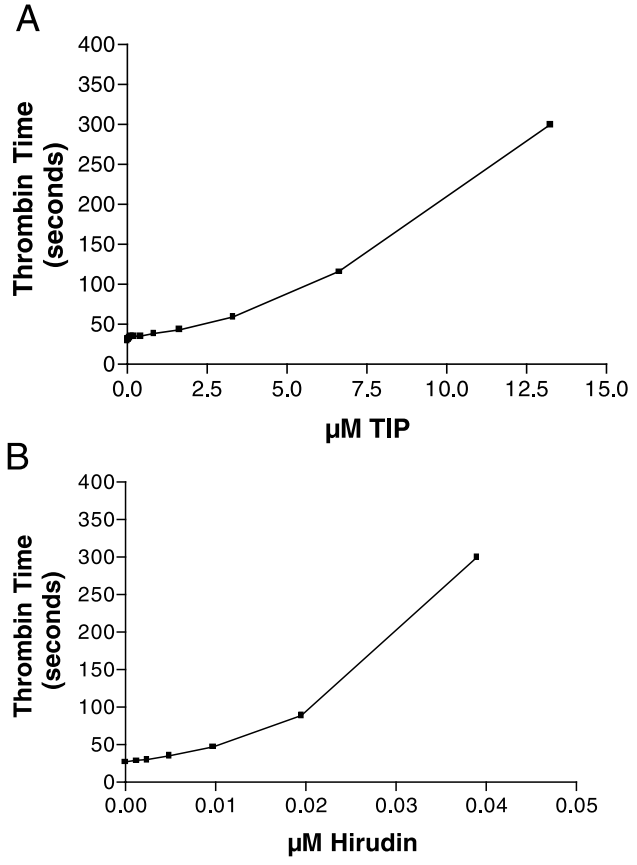

Fig. 5. Thrombin times of a dilution series of the peptide (A) and hirudin (B) in plasma of healthy volunteers.

Table 1
The effect of TIP on the platelet release reaction and platelet activation after stimulation of the platelets with thrombin in the presence of Gly-Pro-Arg-Pro ($n=4$)

	Platelet release reaction (CD62 expression)	Platelet activation (CD63 expression)
Negative control	8.0 ± 3.4	12.8 ± 8.8
Positive control	94.5 ± .3	88.3 ± 5.7
TIP (1000 μg/ml)	26.3 ± 22.7	14.8 ± 6.7
TIP (250 μg/ml)	39.5 ± 28.8	26.5 ± 11.3
TIP (62.5 μg/ml)	74.0 ± 14.5	67.3 ± 18.9

platelet population negative for activation markers. Positive controls of activated, nontreated platelets were included in each assay. Platelets were gated on CD41-positive binding. Platelet activation was calculated from the percentage of CD41- and CD62- or CD41- and CD63-positive platelets, respectively, with and without addition of the thrombin inhibition peptide.

Platelet adhesion with parallel plate flow chambers: The inhibitory effect of TIP was studied at shear rates of 100 and 2600 s^{-1} using parallel plate flow chambers with appropriate slit height (0.4–1 mm) and following a previously described method [11]. Coverslips were coated with either a mixture of types I and III collagen (1 mg/ml, Nycomed Arzeimittel, Norway) or a matrix of human dermal microvascular endothelial cells (HMEC-1). The matrix was prepared by 0.1 N ammonia detachment of cells cultured as described previously [12]. HMEC-1 cell line was a kind gift of Drs. E.W. Ades and T.J. Lawley (Centres for Disease Control and Prevention and Emory University School of Medicine, Atlanta, GA). Cells were cultured and characterized as described previously [1,2]. Perfusions were carried out at 37 °C and by recirculating heparinized blood over the coated coverslips for 5 min. After perfusion, the coverslips were immersed in methanol and stained with May-Grünwald-Giemsa. Platelet adhesion was evaluated with a light microscope connected to an image analyser. An average of 30 fields per coverslip was analysed. Platelet adhesion was expressed as percentage surface coverage by platelets.

3. Results

3.1. Selection of phages that bind to thrombin

Repeated selection of phages from the cyclic heptapeptide phage display library resulted in an enrichment of phages that bind to human α-thrombin. After the fourth round of enrichment (panning), individual colonies were

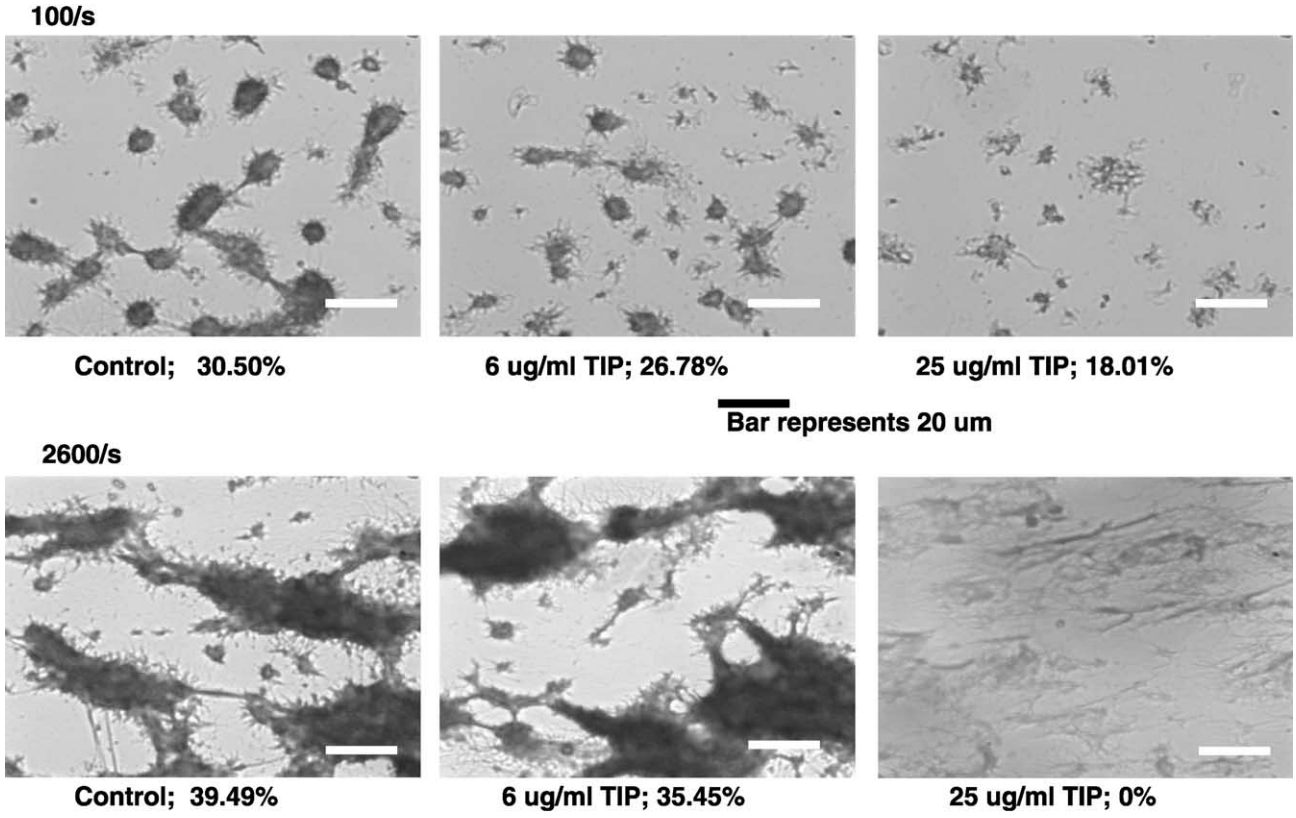

Fig. 6. The inhibitory effect of TIP at shear rates of 100 and 2600 s^{-1} using parallel-plate flow chambers. Coverslips were coated with a matrix of human dermal microvascular endothelial cells (HMEC-1). Heparinized blood was recirculated over the coated coverslips for 5 min at 37 °C. After perfusion, the coverslips were stained and platelet adhesion was expressed as percentage surface coverage by platelets.

tested for its binding affinity to thrombin. The six strongest binding colonies were identified and amplified in *E. coli* for further characterisation. Two of the six colonies bound very strongly and in a dose-dependent manner to thrombin. They did not bind to uncoated wells, indicating that the binding was very specific (Fig. 1). In a competition experiment with PPACK, we found that PPACK prevented, in a dose-dependent manner, phages of one of the colonies to bind to thrombin. The estimated IC_{50} was 0.8 μg/ml at 5×10^{11} phages/ml (Fig. 2). The phages of this colony also lengthened the thrombin time in a dose-dependent manner (Fig. 3). By using specific primers, the sequence of the Cys-flanked heptapeptide insert of the thrombin-inhibitory phage clone was deducted. The amino acid sequence was Cys-Asn-Arg-Pro-Phe-Ile-Pro-Thr-Cys. We had the peptide synthesised and named it thrombin inhibition peptide (TIP).

3.2. Determination of the inhibitory activity of TIP

Since TIP contains the reverse sequence of the well-known thrombin active site binding peptide Phe-Pro-Arg [13,14], it is possible that it acts as a second substrate of which the hydrolysis cannot be detected in the assay. A substrate analogue, which binds reversibly to the enzyme, excluding the substrate, acts as a purely competitive inhibitor. Competitive inhibitors have the effect of increasing the apparent Km of the reaction [15]. This is indeed the case in Fig. 4, where the apparent Km is plotted against different TIP concentrations. The Km value increases with increasing inhibitor concentrations.

TIP lengthened the thrombin time in a dose-dependent manner (Fig. 5A). For purposes of comparison, the lengthening of the thrombin time at different concentrations of r-hirudin is also summarised in Fig. 5B. TIP had no effect on in vitro platelet aggregation in response to ADP or collagen. The data are not shown. Fluorescence flow cytometry, on the other hand, showed that TIP inhibited the release reaction and activation of platelets in a dose-dependent manner when the platelets were stimulated with thrombin (Table 1).

TIP (25 μg/ml) inhibited platelet adhesion onto human microvascular endothelial matrix in the parallel plate flow chamber under both arterial and venous shear conditions, i.e. 2600 and 100 s^{-1} (Fig. 6). Like hirudin and TAP, it did not inhibit platelet adhesion onto the collagen-coated coverslips in the parallel plate flow chamber under similar conditions. The data are not shown.

4. Discussion

In an attempt to identify peptides that may inhibit the function of thrombin, phages from a phage display library were selected on their binding characteristics to human α-thrombin. A heptapeptide phage display library, in which the heptapeptides were flanked by two cysteines, was used. It thus had the potential to form a disulphide linked nanopeptide loop with restricted conformational freedom. We used the cyclic peptide phage library for three reasons. First, peptides that are based on the cyclic sequence are generally more stable than those with a linear sequence. Second, a ligand sequence, where the imposed constraint allows for a productive binding conformation, will bind more tightly than the same linear sequence due to improved binding entropy, and third, cyclic peptides are more resistant to proteolysis than linear peptides [13,16–19]. After four rounds of enrichment (panning), two phage clones that bind the strongest to thrombin (Fig. 1) were tested for binding to thrombin in competition with PPACK. One of these two clones competed with PPACK to bind to thrombin (Fig. 2). This strongly suggested that these clones display the Phe-Pro-Arg sequence on their surfaces. This sequence binds to the active site of thrombin [13]. This phage clone also lengthened the thrombin time in a dose-dependent manner (Fig. 3). Because of their size, only limited number phages can be used in any one assay. In view of this, no further results on inhibition could be obtained by using the phages.

A cyclic peptide, TIP, with the sequence, Cys-Asn-Arg-Pro-Phe-Ile-Pro-Thr-Cys, to correspond with that displayed on the phage surfaces, was synthesised. It is of interest to note that the active site binding sequence, Phe-Pro-Arg, was reversed in TIP. Thrombin-binding peptides with this reverse Phe-Pro-Arg sequence were also selected from peptide-encoded peptide libraries by Vagner et al. [20].

In substrate hydrolysis reactions, TIP acts as a second substrate of which the hydrolysis cannot be detected in the assay. The system behave as if it a full competitive inhibitor (Fig. 4). A plot of Km vs. TIP concentration is a straight line (Fig. 4). The K_i value is calculated at 0.4974 mM. This is much higher than the K_i value for the inhibition of thrombin of other published thrombin inhibitors, such as hirudin, hirunorm IV, argatroban, efegatran and anophelin, which are all in the nanomolar range [21–23]. The K_i value of TIP is also higher than that of the Arg-Gly-Pro-D-Phe peptide in the literature, which also contains the reverse sequence of the active site binding peptide of thrombin, namely Phe-Pro-Arg [20].

TIP lengthens the thrombin time in a dose-dependent manner. The effect was, however, much less pronounced than that of hirudin (Fig. 5). As was expected, TIP had no effect on ADP or collagen-stimulated platelet aggregation, similar to results obtained with hirudin. On the other hand, it inhibited the release reaction and activation of platelets when the platelets were stimulated with the thrombin mimetic peptide, Gly-Pro-Arg-Pro (Table 1).

TIP has antithrombin activity in spite of the fact that the active site binding sequence Phe-Pro-Arg was reversed. This is also shown in the literature that this reverse sequence also inhibits thrombin. However, the peptide Arg-Gly-Pro-D-Phe in the literature inhibits thrombin much stronger [20]. It is known that thrombin cleaves the peptide bond between Arg-Pro in the bifunctional inhibitor D-Phe-Pro-Arg-Pro-

(Gly) 4-hirudin-thrombin complex [19]. Thrombin also hydrolyses fibrinogen by the selective cleavage of 4 Arg-Gly bonds and it cleaved the chromogenic substrate chromozym TH at the carboxyl end of Arg. So it seems that thrombin cleaves peptides at the carboxyl end of an Arg. In this peptide TIP, thrombin may also cleave it at the Arg-Pro bond. If this is the case, one may argue that the advantages of cyclic peptides may be get lost, because the peptide is cleaved and act as a linear peptide. This may also be the reason for the low binding and weak inhibition (high K_i value) of TIP. Another reason may be the fact that TIP is a bigger peptide than PPACK and therefor do not bind as strong because of its allosteric conformation.

TIP also inhibits platelet adhesion onto human microvascular endothelial matrix in the parallel plate flow chamber under both arterial and venous shear conditions. It, however, does not inhibit platelet adhesion to the collagen-coated surface collagen in the flow chamber. Neither PPACK, nor hirudin, has an effect on platelet adhesion to collagen. The reason for this can be that thrombin does not play a role in the binding of platelets to collagen.

TIP has the potential to be developed as an antithrombotic compound. It inhibits thrombin by binding to the active site. It also inhibits platelet activation and the release reaction when platelets were stimulated with a thrombin-mimetic peptide, Gly-Pro-Arg-Pro, and it inhibits platelet adhesion onto a human microvascular endothelial matrix in the parallel plate flow chamber under both arterial and venous shear conditions. The stability and specificity of this peptide in comparison with other serine proteases, such as factor Xa and factor VIIa, will be determined and in vivo studies with this peptide are necessary to compare its effect with that of the other well-known thrombin inhibitors.

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Funksionele beskrywing van ’n faktor VIIa inhiberende peptied, IP-7, geselekteer deur faagblootleggingstegnologie

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UITTREKSEL

Die tegniek van faagblootlegging is gebruik om ’n sikliese heptapeptied te selekteer wat met weefselfaktor (WF) kompeteer vir binding aan stollingsfaktor VIIa. Die aminosuurvolgorde van die peptied is Cys-Ala-Trp-Pro-His-Thr-Pro-Asp-Cys (C-AWPHTPD-C) en dit verleng die protrombientyd (PT) op ’n konsentrasie-afhanklike wyse. Die peptied beperk plaatjieklewing aan beide menslike endoteelsel- en weefselfaktormatrikse in ’n vloeikamermodel onder arteriële vloeitoestande. Die peptied funksioneer as ’n volledig mededingende inhibeerder van faktor VIIa met ’n inhibisiekonstante (K_i) van 123,2 μM. In sy huidige vorm is die peptied waarskynlik nie sterk genoeg om verder as antitrombotiese middel ontwikkel te word nie, maar verskillende strategieë kan gevolg word om die werking daarvan te versterk.

ABSTRACT

Functional characterisation of a factor VIIa inhibiting peptide, IP-7 selected by phage display technology

By using the technique of phage display, we selected a cyclic heptapeptide sequence Cys-Ala-Trp-Pro-His-Thr-Pro-Asp-Cys (C-AWPHTPD-C) that competes with tissue factor for binding to coagulation factor VII. This peptide prolongs the prothrombin time (PT) in a concentration dependent way. It also reduces platelet adhesion to both human endothelial cell and tissue factor matrixes in a flow chamber under arterial flow conditions. Furthermore, it acts as a full competitive inhibitor of factor VIIa with an inhibition constant (K_i) of 123,2 μM. In its current form the peptide is probably not sufficiently potent for development as an antithrombotic agent, but different strategies could be followed to reinforce its performance.

INLEIDING

Bloedstolling begin met die beskadiging van ’n bloedvat en die blootstelling van sirkulerende bloed aan weefselfaktor (WF) in die subendoteel. Stollingsfaktor VII (FVII) wat in die bloed sirkuleer, bind aan WF om ’n FVII/WF-kompleks te vorm. Hierdie kompleks aktiveer faktor X wat op sy beurt protrombien aktiveer om trombien te vorm. Trombien skakel fibrinogeen na fibrien om en aktiveer bloedplaatjies om ’n trombus of stolsel te vorm.[1]

Ten spyte van jare se navorsing is daar slegs twee antistolmiddels wat ruimskoots deur dokters gebruik word, naamlik kumarien en heparien. Kumarien versteur die funksionering van vitamien K-afhanklike proteïene wat beide pro-stollingsfaktore (trombien en faktore X, IX en VII) en anti-stollingsfaktore (proteïene C en S) insluit, terwyl heparien die inhibisie van trombien en faktor Xa deur antitrombien III bevorder. Die nieselektiewe inhibisie van stolling deur beide hierdie antistolmiddels is waarskynlik verantwoordelik vir die onvermoë daarvan om ’n terapeutiese balans tussen trombose en hemostase te handhaaf.[2,3]

Tans word baie aandag bestee aan die ontwikkeling van middels wat faktor X en trombien direk inhibeer. Die feit dat faktor VII hoër op in die stollingsbaan funksioneer,

beteken dat daar teoreties minder inhibeerder nodig is om stolling op hierdie vlak te stuit. Die vorming van die FVIIa/WF-kompleks lei die stollingsproses in en bied ’n goeie teiken vir die ontwikkeling van antistolmiddels. Weefselfaktorbaaninhibeerder (WFBI) is ’n natuurlike inhibeerder van FVIIa/WF en sy werking is van faktor Xa afhanklik.[4] Alhoewel daar nie FVIIa-inhibeerders kommersieel beskikbaar is nie, is dit al voorheen verken.[5,6]

Verbindings wat die binding van WF aan FVIIa blokkeer, sal die aktivering van faktor X verhoed en aldus die vorming van ’n stolsel of trombus bekamp. Ons het ’n studie ontwerp om peptiedinhibeerders van FVIIa te ontwikkel deur van faagblootleggingstegnologie gebruik te maak.

Die tegniek van faagblootlegging maak die sifting van groot getalle faagklone moontlik. Meer as ’n miljard verskillende peptiedvolgordes kan op die manier gesif word en dit gee aan faagblootlegging ’n voordeel bo ander metodes.[7] Ons het besluit om ’n kort oligonukleotiede te selekteer omdat klein peptiede minder immunogenies is.[8]

Verskillende FVIIa-bindende faagkolonies is getoets vir hulle vermoë om FVIIa te inhibeer. Die DNS-volgorde van dié wat die sterkste inhibisie getoon het, is bepaal en ooreenstemmende peptiede gesintetiseer. Op die manier is ’n sikliese heptapeptied met die volgorde C-AWPHTPD-C gesintetiseer en deur middel van ’n protrombientyd (PT) tipeer. Ons het ook die peptied se kinetiese eienskappe ten opsigte van FVIIa-inhibisie bepaal en die effek daarvan op plaatjieklewing aan endoteelselle en WF gemeet.

METODES

1. Seleksie van faktor VIIa-bindende fage

Die faagblootleggingstegniek wat gebruik is, is reeds volledig beskryf.[9] Twee peptiedblootleggingsbiblioteke, ’n sikliese heptameer- en ’n lineêre dodekameerbiblioteek (New England Biolabs, Beverly, MA, VSA) is gebruik. Twintig µg FVIIa (ADI, Greenwich, CT, VSA) opgelos in 1 ml fosfaatgebufferde fisiologiese soutoplossing (PBS) is oornag by 4°C aan ’n immuunbuis (Nunc, IL, VSA) gebind. Fage (2×10^{11}) van elke biblioteek in PBS is tot die buisies toegevoeg en daarna vir 1½ uur by kamertemperatuur geïnkubeer om sodoende aan die faktor VIIa te bind. Die ongebonde fage is afgewas en die FVIIa-bindende fage niespesifiek met ’n sterk suuroplossing (0,2 M glisien by pH 2) geëlueer. Drie seleksierondtes is uitgevoer. Tydens die tweede en derde rondte is die VIIa-bindende fage spesifiek met 10 µg van ’n muis-anti-mens-FVIIa-monoklonale antiliggaam (ADI, Greenwich, CT, VSA) geëlueer. Die FVIIa-bindende fage is na elke rondte vermeerder deur log-fase *Escherichia coli (E.coli)* selle oornag te infekteer en te suiwer deur twee poliëtileenglikol-presipitasies.

Om enkel VIIa-bindende kolonies voor te berei, is seriële tienvoudige verdunnings van ’n fraksie van die geëlueerde fage gemaak en uitgeplaat. Eenhonderd vier-en-veertig (3 X 48) enkel kolonies van elke biblioteek is na die derde seleksierondte uitgekies en oornag by 37°C in E coli-selle gekweek. Die supernatante, wat die geamplifiseerde fage bevat, is met behulp van ’n bindings-ELISA vir binding aan faktor VIIa getoets.

a. Bindings-ELISA

’n 96-putjie ELISA-plaat is oornag met 20 µg/ml FVIIa by 4°C bevestig. Na blokkering met 4% afgeroomde melk (DIFCO, Detroit. MI, VSA) in PBS, is die supernatante (100 µl) van die gekweekte kolonies by die putjies toegevoeg en vir 2 uur by kamertemperatuur geïnkubeer. ’n Peperwortelperoksidase-gekonjugeerde anti-faagantiliggaam (Amersham Pharmacia Biotech, NJ,VSA), is bygevoeg om die FVIIa-bindende fage op te spoor. Ses kolonies van elke

biblioteek het sterk binding getoon en is verder gekweek. Verdunningsreekse van bogenoemde 12 kolonies (ses van elke biblioteek) is vir konsentrasie-afhanklike binding aan FVIIa in 'n verdunnings-ELISA getoets.

b. Inhibisie-ELISA

'n Inhibisie-ELISA is gedoen om te bepaal of WF in staat is om te verhoed dat die twaalf kolonies aan FVIIa bind. Verskillende konsentrasies WF (ADI, Greenwich, CT, VSA) is by die FVIIa-bedekte putjies gevoeg voordat die fage bygevoeg is. Die res van die ELISA is uitgevoer soos bo beskryf.

c. Protrombientyd (PT)

Die PT is gedoen deur 100 µl normale mensplasma by 50 µl van verskillende konsentrasies van die twaalf sterkste FVIIa-bindende fage te voeg en vir 10 min te inkubeer. Die faagkonsentrasies het van 1 x 10^9 tot 2,2 x 10^{12} fage gewissel. Daarna is 200 µl weefselfaktor (Innovin van Dade Behring, Marburg, Duitsland) bygevoeg en die stoltyd met 'n STart®4 stollingsmeter (Diagnostica Stago, Asnieres, Frankryk) gemeet.

2. Nukleotiedvolgordebepaling

DNS is van beide die kolonies wat die PT verleng het, voorberei en die DNS-volgorde is met behulp van die DYEnamic ET Terminator Cycle Sequencing Premix Kitsstel (Amersham Pharmacia Biotec Inc., NJ, VSA) bepaal. 'n Sikliese heptapeptied met dieselfde volgorde as die blootgelegde peptied op die sikliese kolonie is deur ADI, Greenwich, GT, VSA, gesintetiseer. Ons het die peptied IP-7 genoem.

3. Peptiedanalise

a. Protrombientyd (PT)

Die effek wat drie verskillende peptiedkonsentrasies (1,17mM; 0,58 mM; en 0,295 mM) op die PT het, is bepaal. PBS en 'n peptied wat nie aan FVII bind nie is as negatiewe kontrole gebruik. Die PT is gedoen soos bo beskryf.

b. Perfusiestudies met endoteelselle

Die antitrombotiese effek van die peptied IP-7 is met 'n vloeikamer, soos deur Sakaríassen beskryf, getoets.[10] Thermanox plastiese dekglasies (Nunc, IL, VSA) is met menslike endoteelselle bedek wat goedgunstiglik geskenk is deur E.W. Ades en T.J. Lawley van die Centres for Disease Control and Prevention and Emory University School of Medicine, Atlanta, GA, VSA.

Drie verskillende peptiedkonsentrasies (2,34 µM; 4,7 µM; en 9,35 µM) is met 10 ml bloed vir 10 minute by 37°C geïnkubeer. Konstante bloedvloei is met 'n peristaltiese pomp gehandhaaf en die bloed is vir 5 minute oor die dekglasies hersirkuleer. Verskillende skuiftempo's van 1000 s^{-1} en 200 s^{-1} is gebruik. Die dekglasies is daarna verwyder, met HEPES buffer gespoel, in metanol fikseer en volgens die May-Grünwald-Giemsa-metode gekleur. Dertig velde per dekglasie is ontleed en die persentasie plaatjiebedekking is met behulp van 'n Zeiss ligmikroskoop gemeet.[11,12]

c. Perfusiestudies met weefselfaktor

Die dekglasies is met 100 µl weefselfaktor (Innovin van Dade Behring, Marburg, Duitsland) bedek. Die perfusiestudies is uitgevoer soos hierbo beskryf, behalwe dat die skuiftempo's in hierdie geval 200 s^{-1}, 650 s^{-1} en 1300 s^{-1} was. Finale peptiedkonsentrasies van 29,2 µM; 58,5 µM en 117 µM is gebruik.

d. In vitro-ensieminhibisie

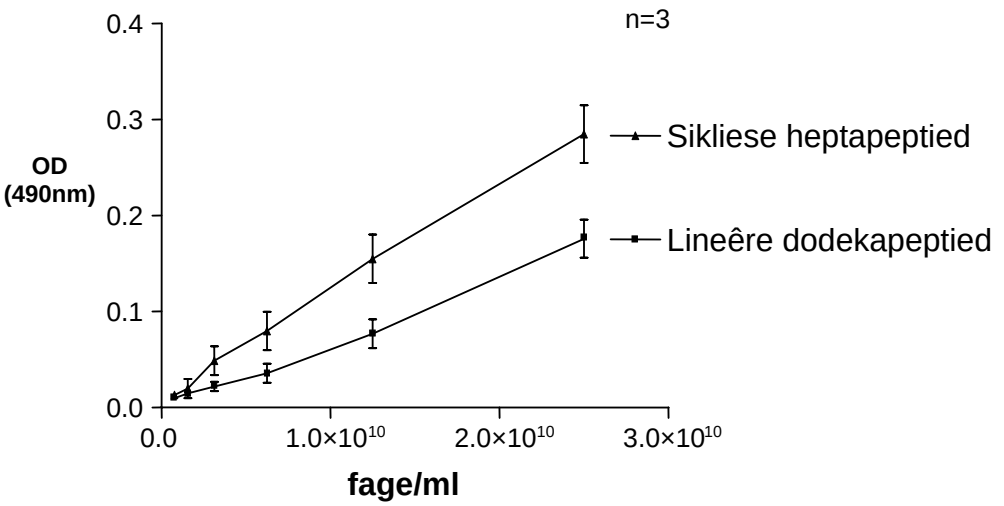
Die ensiemkinetika van die inhibisie van Faktor VIIa met peptied IP-7 is met behulp van chromogeniese substraathidrolise bepaal. Verskeie kinetiese reaksies is uitgevoer met peptiedkonsentrasies wat van 0 tot 0.456 mM gewissel het en waarby verskillende konsentrasies van die chromogeniese substraat van faktor VIIa (Spectrosyme FVIIa van American Diagnostica, VSA) gevoeg is. Die substraatkonsentrasies het gewissel van 0.56 tot 0 mM. 'n Optimale FVIIa konsentrasie van 400 nM is gebruik en Innovin (Dade Behring, Marburg, Duitsland) is as bron van WF gebruik. Die totale reaksievolume was 200 µl en al die reagense is in die reaksiebuffer (0.05 M Tris, 0.1 M NaCl pH 8.4) verdun. Chromogeniese substraathidrolise is vir 40 minute by 'n golflengte van 405 nm gevolg en deur middel van 'n EL312e mikroplaat biokinetiese leser (Bio-tek instruments, Vermont, VSA) gelees.

Die kinetiese parameters K_m en V_m by elke peptiedkonsentrasie is bereken deur gebruik te maak van enkelplekbinding nielineêre regressie. Om die tipe inhibisie van die peptied te bepaal, is 'n Lineweaver-Burk grafiek ($1/V_o$ vs 1/substraat) gestip (figuur 6). Die inhibisiekonstante (K_i -waarde) is bepaal deur die oënskynlike K_m-waardes teenoor die peptiedkonsentrasies te stip (kyk figuur 7). Die K_i–waarde is as die x-as afsnit beskou.

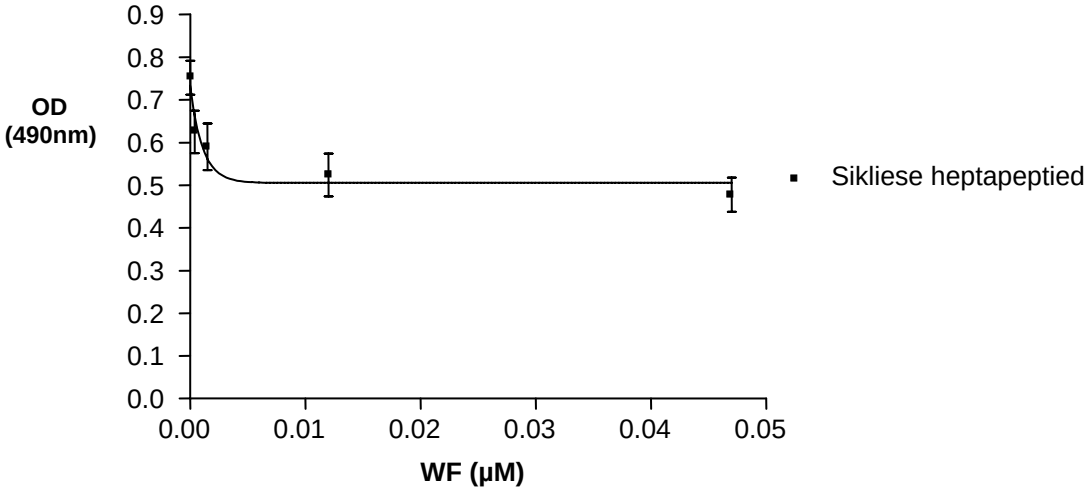
RESULTATE

1. Faagseleksie en DNS volgordebepaling

Die bindings-ELISA het twaalf kolonies geïdentifiseer wat binding aan FVIIa getoon het. Ses van hierdie kolonies was van die dodekapeptiedbiblioteek en ses van die sikliese pentapeptiedbiblioteek afkomstig. 'n Verdunnings-ELISA is uitgevoer op die twaalf kolonies wat FVIIa-bindingsaffiniteit getoon het. 'n Irrelevante faagkolonie is ook getoets vir binding aan FVIIa en is as 'n negatiewe kontrole gebruik. Om die grafiek te vereenvoudig is die bindingsaffiniteit van een kolonie uit elke biblioteek in figuur 1 gestip. Die optiese digtheid het met stygende faagkonsentrasies verhoog, wat daarop dui dat die fage spesifiek aan FVIIa bind. Die faagkolonies van die sikliese heptapeptiedbiblioteek het groter bindingsaffiniteit getoon as dié van die dodekapeptiedbiblioteek (n=3). Die faagkolonie wat as 'n negatiewe kontrole gebruik is, het geen binding aan FVIIa getoon nie. 'n Inhibisie-ELISA is gedoen om te bepaal of WF daartoe in staat is om te voorkom dat die kolonies aan FVIIa bind. Die lineêre kolonies het geen inhibisie getoon nie en slegs een vanaf die sikliese biblioteek het matige inhibisie getoon (figuur 2). Laasgenoemde kolonie sowel as een kolonie vanaf die lineêre biblioteek het die PT verleng (figure 3 en 4). Die negatiewe kontrole het geen effek op die PT gehad nie.

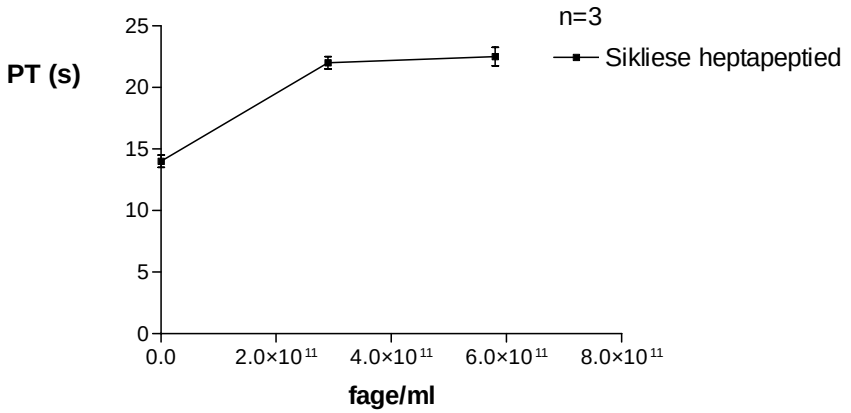


Figuur 1**:** *Verdunnings-ELISA van beide sikliese heptapeptied- en lineêre dodekapeptied-kolonies aan FVIIa. Die OD_{490} vermeerder met stygende faagkonsentrasies. Die sikliese heptapeptied-kolonie bind sterker as die dodekapeptied-kolonie (n=3).*

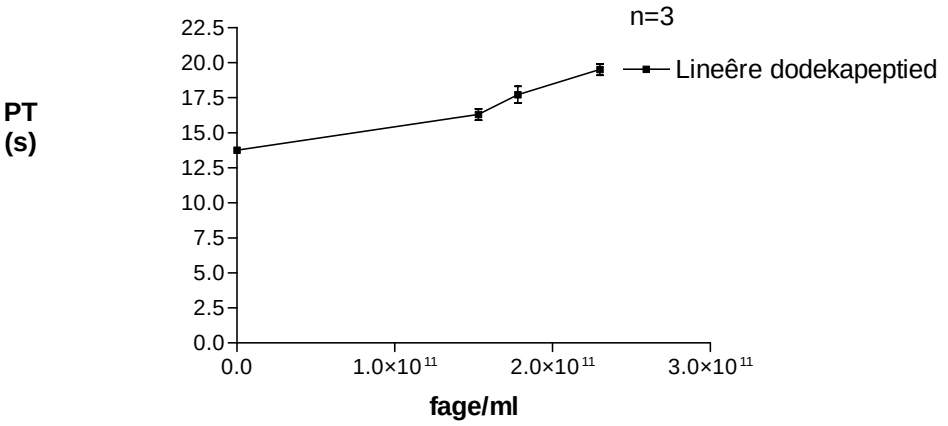


Figuur 2: *Inhibisie-ELISA van die sikliese heptapeptied-kolonie by verskillende WF-konsentrasies. WF-konsentrasies van 0 tot 0.05 µM is by FVIIa-bedekte putjies gevoeg en vir 15 min geïnkubeer. Daar is 5.10^{10} fage van die sikliese heptapeptied-kolonie by die putjies gevoeg en vir 2 uur geïnkubeer waarna die FVIIa-gebonde fage met 'n anti-faagantiliggaam aangetoon is (n=3).*

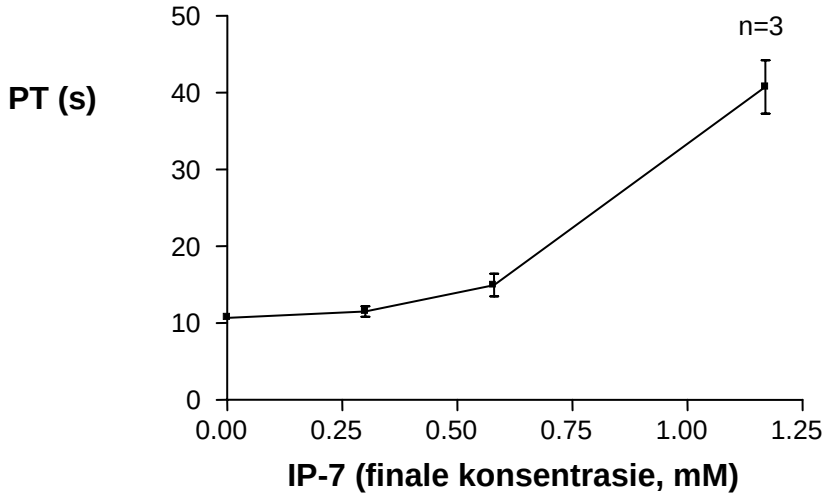
Beide kolonies wat die PT verleng het se DNS-volgorde is bepaal. Dit is interessant om daarop te let dat die volgorde van die kolonie vanuit die sikliese heptameerbiblioteek presies in die middelste deel van die volgorde van die kolonie uit die dodekapeptiedbiblioteek gepas het. Omdat die sikliese kolonie sterker aan FVIIa gebind het, het ons besluit om 'n sikliese peptied met dieselfde volgorde as dié wat op die sikliese kolonie blootgelê is, te sintetiseer. Die volgorde van hierdie peptied is C-AWPHTPD-C. Dit vergelyk nie met die volgorde van 'n reeds gepubliseerde faktor VII inhibeerder PN7051 (C-QY-C) nie.



Figuur 3: *PT's met stygende konsentrasies van die sikliese heptapeptied-kolonie. Stygende konsentrasies van hierdie faagkolonie is vir 10 minute met mensplasma geïnkubeer voor toevoeging van die PT-reagens. Die PT is met 8.3 sekondes by die hoogste faagkoloniekonsentrasie (5.8.10^{11} fage) verleng*



Figuur 4: *PT's met stygende konsentrasies van die dodekapeptied-kolonie. Stygende konsentrasies van hierdie faagkolonie is vir 10 minute met menslike plasma geïnkubeer voordat die PT-reagens toegevoeg is. Hierdie faagkolonie verleng die PT op 'n konsentrasie-afhanklike manier. By die hoogste faagkonsentrasie (2.3.10^{11} fage) word die PT met 5,4 sekondes verleng (n=3).*



Figuur 5: *Verlenging van die PT in mensplasma. Verskillende peptiedkonsentrasies is by normale gepoelde mensplasma gevoeg en vir 10 minute geïnkubeer voor toevoeging van die PT-reagens. By die hoogste peptiedkonsentrasie, 1.17 mM, is die PT met 30 sekondes verleng. (n=3).*

2. Toetse uitgevoer op die peptied, IP-7.

IP-7 het die PT op ’n dosisafhanklike manier verleng, en peptiedkonsentrasies van hoër as 0,58 mM was hiervoor nodig (figuur 5). ’n Irrelevante peptied is as negatiewe kontrole gebruik en het geen effek op die PT gehad nie. Die persentasie inhibisie van plaatjieklewing aan endoteelselbedekte dekglasies word in tabel 1 opgesom. Die peptied het plaatjieklewing op ’n dosisafhanklike manier by beide skuiftempo’s geïnhibeer. Die inhiberende effek was meer uitgesproke by die arteriële vloeitoestande (1000 s^{-1}) omdat die hoogste peptiedkonsentrasie (9.35 μM) plaatjieklewing aan die dekglasies met 74% onderdruk het. By die lae skuiftempo (200 s^{-1}) was die onderdrukking 54% vir dieselfde peptiedkonsentrasie.

TABEL 1: Persentasie inhibisie van plaatjieklewing aan endoteelselbedekte dekglasies by skuiftempo’s van 200 s^{-1} en 1000 s^{-1} (n=3)

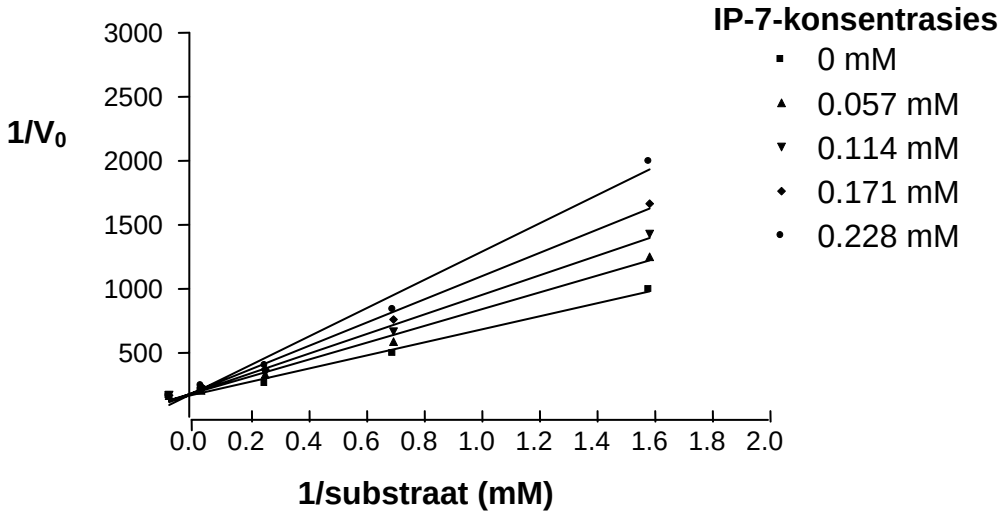
	Persentasie inhibisie by ’n skuiftempo van 200 s^{-1}	**Persentasie inhibisie by ’n skuiftempo van 1000 s^{-1}**
Kontrole (0 μM IP-7)	0%	0%
2.34 μM IP-7	19%	22%
4.70 μM IP-7	55%	47%
9.35 μM IP-7	54%	74%

Die peptied het ook die klewing van plaatjies aan WF geïnhibeer. Die persentasie inhibisie van plaatjieklewing aan die dekglasies word in tabel 2 opgesom. Die peptied het ’n meer uitgesproke inhibisie van plaatjieklewing by die hoër skuiftempo veroorsaak. By ’n skuiftempo van 1300 s^{-1} is plaatjieklewing aan die WF-bedekte dekglasies op ’n dosisafhanklike manier onderdruk.

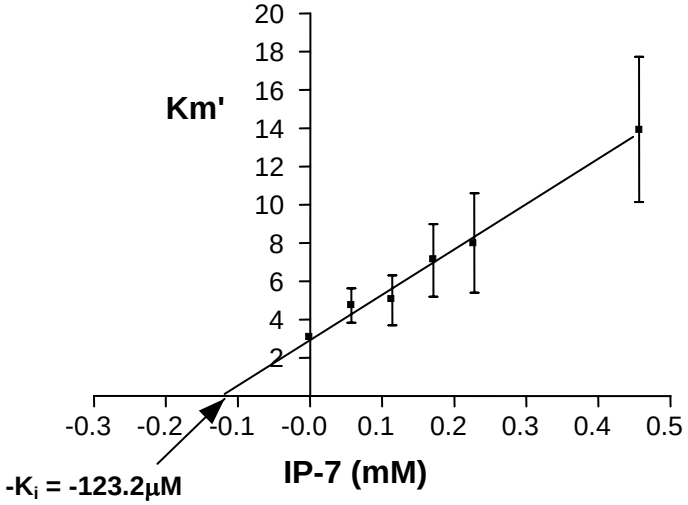
TABEL 2: Persentasie inhibisie van plaatjieklewing aan weefselfaktorbedekte dekglasies by skuiftempo's van 200 s^{-1}, 650 s^{-1} and 1300 s^{-1} (n=2)

	Persentasie inhibisie by 'n skuiftempo van 200 s^{-}	**Persentasie inhibisie by 'n skuiftempo van 650 s^{-1}**	**Persentasie inhibisie by 'n skuiftempo van 1300 s^{-1}**
Kontrole (0 µm IP-7)	0%	0%	0%
29.2 µM IP-7	0%	0%	53%
58.5 µM IP-7	33%	59%	87%
117 µM IP-7	Nie bepaal nie	Nie bepaal nie	85%

Met die kinetiese studies het die maksimum omloopsnelheid (V_m-waardes) by die verskillende peptiedkonsentrasies onveranderd gebly terwyl die Michaelis-Menten konstante (K_m-waarde) verhoog het met stygende konsentrasies. Dit kon op mededingende inhibisie dui en om dit te bevestig het ons 'n Lineweaver-Burk grafiek gestip (figuur 6). Hiermee is mededingende inhibisie bevestig omdat die helling van die grafiek toegeneem het met stygende peptiedkonsentrasies. Die inhibisiekonstante (K_i-waarde) is bepaal as die x-as afsnit by 123, 2 µM soos aangetoon in figuur 7.



***Figuur 6**: Lineweaver-Burk-stip wat die effek van mededingende inhibisie aantoon. Die helling van die grafiek neem toe met stygende peptiedkonsentrasies, wat dui op mededingende inhibisie van FVIIa.*



Figuur 7: *Die K_m-waarde van die reaksies met verskeie IP-7 konsentrasies is gestip teenoor die betrokke peptiedkonsentrasies. Met behulp van hierdie grafiek kon die K_i bepaal word deur die X-afsnit van 'n lineêre regressie te meet. (n=4)*

BESPREKING

Met hierdie ondersoek het ons die tegniek van faagblootlegging gebruik om peptiede wat FVIIa inhibeer, te selekteer. Daar is op peptiede besluit omdat kleiner molekules minder immunogenies as groter molekules is.[8] Ons het gepoog om peptiede wat aan die katalitiese domein van FVIIa bind, te selekteer deur die FVIIa-bindende fage in die laaste 2 seleksierondtes met 'n muis monoklonale antiliggaam wat aan die aktiewe setel van FVIIa bind, te elueer.[13]

Die feit dat die faagkolonies van die sikliese heptapeptiedbiblioteek sterker aan FVIIa as dié van die lineêre een bind, is nie onverwags nie omdat dit bekend is dat sikliese peptiede hegter as lineêre peptiede aan hulle onderskeie ligande bind vanweë verbeterde bindingsentropie.[14] Verder het WF die binding van een van die kolonies van die sikliese peptiedbiblioteek verhoed, wat daarop dui dat hierdie spesifieke sikliese peptied aan dieselfde bindingsetel as WF bind. Die effek is egter beperk omdat WF nie die binding van die fage volledig voorkom het nie (kyk figuur 2). 'n Sikliese peptied met dieselfde aminosuurvolgorde as dié van die sikliese heptapeptiedkolonie, is gesintetiseer. Geen ooreenstemming tussen die volgorde van die gesintetiseerde peptied, IP-7, en dié van WF, FX of WFBI is gevind nie. Daar was twee redes waarom ons besluit het om die sikliese peptied te sintetiseer, naamlik omdat dit sterker aan FVIIa gebind het, en dit bekend is dat sikliese peptiede meer stabiel is as dié met 'n lineêre volgorde en meer bestand is teen proteolise.[15,16]

IP-7 het plaatjieklewing op 'n dosisafhanklike manier by beide skuiftempo's inhibeer, maar die inhibisie was meer uitgesproke by arteriële vloeitoestande (skuiftempo van 1000 s^{-1}). Die rede hiervoor is dat plaatjies meer by arteriële trombose betrokke is en fibrien by veneuse trombose.[17] Dieselfde resultate is ook tydens die perfusiestudies met WF-bedekte dekglasies gevind. Uit die literatuur blyk dit dat soortgelyke resultate verkry is met 'n ander TF/FVIIa-inhibeerder, PN7051.[18] Lg. peptied het 50% inhibisie met 'n konsentrasie van 500 µM met die protrombien tyd getoon in vergelyking met IP-7 wat 58% inhibisie met 58,5 µM met die

protrombientyd getoon het. IP-7 blyk dus 'n sterker onderdrukkende effek te hê op trombusvorming as PN7051.[18]

Dit is belangrik om daarop te let dat die perfusiestudies met gehepariniseerde bloed uitgevoer is. Dit is bekend dat lae molekulêre gewig heparien (LMWH) nie alle stollingsaktiwiteit blokkeer nie en 'n mate van trombienvorming toelaat sodat fibrienneerlegging op die oppervlakte plaasvind.[17] Fibriennetwerke kan duidelik waargeneem word op die WF-matriks by lae skuiftempo's.

Die inhibisiekonstante (K_i-waarde) is as 123,2 µM bepaal, wat beteken dat IP-7 nie 'n sterk FVIIa-inhibeerder is in vergelyking met van die ander FVIIa-inhibeerders nie. Byvoorbeeld, WFBI inhibeer die aktivering van FXa met 'n K_i van 30 nM, en Kunitz domeinvariante van WFBI inhibeer die FVIIa/WF-kompleks met 'n K_i van 1,9 nM.[7] 'n Ander peptiedinhibeerder, A-183, wat aan die eksosetel van die proteasedomein van FVIIa bind, inhibeer FX aktivering met 'n K_i van 200 pM.[6,19]

IP-7 verleng die PT op 'n dosisafhanklike manier. In vergelyking met ander inhibeerders van WF-afhanklike stolling, het 'n peptiedkonsentrasie van 1,17nM die PT drievoudig verleng. PN7051 verleng ook die stoltyd op 'n dosisafhanklike manier met 'n IC_{50} van 1.3 mM.[18] Die rede vir die verlenging van die PT kan toegeskryf word aan die binding van die peptied aan FVIIa om die vorming van die FVIIa/WF-kompleks te verhinder en sodoende die aktivering van FX te verhoed. In 'n studie waar die effek van twee verskillende peptiedinhibeerders van die eksosetel van FVIIa met PT gemeet is, het die verlenging van die PT by konsentrasies van groter as 100 nM plaasgevind. IP-7 is dus nie so potent as eksosetel inhibeerders van FVIIa nie[6], wat waarskynlik aan die kort lengte van die peptied toegeskryf kan word. Dit is in die literatuur aangetoon dat langer peptiede meer aktief is as kort peptiede. 'n 27-aminosuur peptied het byvoorbeeld 'n sewevoudige hoër aktiwiteit as 'n 15-aminosuur peptied getoon.[6] Die kort sikliese peptied PN7051 (Cys-Gln-Tyr-Cys) het ook 'n baie laer aktiwiteit as langer peptiede getoon.[6,18]

Verdere studies is nodig om die aktiwiteit van IP-7 te verhoog en sluit onder meer in die koppeling van twee of selfs drie van die peptiede, of die kombinering van die peptied met ander faktor VIIa of WF inhiberende peptiede.[6] Aangesien die faagblootleggingstegnologie so 'n kragtige instrument in die ontwikkeling van geneesmiddels is, kan dit ook aangewend word in die ontwikkeling van antistolmiddels wat die FVIIa/WF-kompleks inhibeer. [7]

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VERKORTE CURRICULA VITAE

Muriel Meiring is medeprofessor in die Departement Hematologie aan die Universiteit van die Vrystaat. Sy behaal haar PhD graad in Hematologie in 1996 waarna sy ’n beurs van die Vlaamse regering ontvang het om haar post-doktorale studie in 1997 in België te doen. In 1998 sluit sy samewerkingsooreenkomste met navorsingsgroepe in België en Hongarye met wie sy nog steeds saamwerk. Sy is outeur van verskeie navorsingsartikels en publiseer in Afrikaans en Engels. Sy het die THUTHUKA-toekenning vir Vroue in Navorsing ontvang en doen ook kontraknavorsing vir verskeie farmaseutiese maatskappye in die ontwikkeling van nuwe antistolmiddels.

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DRUG DEVELOPMENT RESEARCH 70:199–205 (2009)

DDR

Research Article

Development of an Inhibitory Antibody Fragment to Human Tissue Factor Using Phage Display Technology

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Strategy, Management and Health Policy				
Enabling Technology, Genomics, Proteomics	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT Tissue factor is involved in the etiology of thrombotic diseases initiating the thrombosis associated with the inflammation that occurs during infection. The prevention of blood coagulation and inflammation is of primary importance in a number of pathological situations. A single-chain variable antibody fragment of molecular weight of 26 kD that inhibits the action of human tissue factor was selected by phage display technology, purified and tested for its tissue factor inhibitory effect, purified on a protein A column, and its purity evaluated on SDS-PAGE. The effects of the antibody fragment on prothrombin times, Factor Xa production, and thrombin generation were assessed with increasing fragment concentrations, using chromogenic and fluorometric substrates. The antibody fragment dose-dependently prolonged the prothrombin time ($IC_{50} = 0.5\ \mu M$) and delayed the lag phase before the thrombin generation burst and the peak thrombin concentration in the thrombin generation assay. The effect on thrombin generation was more pronounced in thrombophilic plasma than in normal plasma. Antibody-based tissue factor inhibitors therefore may provide an effective treatment for thrombotic disease without serious bleeding complications. Drug Dev Res 70:199–205, 2009. © 2009 Wiley-Liss, Inc.

Key words: phage display; tissue factor; anticoagulants

INTRODUCTION

Recent developments in the field of thrombosis and hemostasis have highlighted the crucial role of the tissue factor/Factor VIIa complex in the initiation of coagulation processes. Coagulation is triggered by exposure of zymogen Factor VII (FVII) to its membrane-bound cofactor, tissue factor (TF), to form the TF/FVII complex [Mann et al., 2003]. The TF/FVII complex is converted to the enzymatically active TF/FVIIa complex by Factor Xa (FXa) or autocatalytically by TF/FVIIa. The TF/FVIIa complex can then activate Factor IX (FIX) to form FIXa, FX to FXa, and FVII to FVIIa. FX can also be activated to FXa by the complexing of FIXa with its cofactor FVIIIa, and FIX can be activated to FIXa by FXIa. FXa in complex with its cofactor Factor V (FV) activates the conversion of prothrombin to thrombin. Thrombin cleaves fibrinogen to fibrin, ultimately resulting in the formation of a fibrin clot. Thrombin further amplifies the coagulation process by activating cofactors, e.g., FV and FVIII and zymogens such as FXI. Moreover, thrombin activates platelets leading to platelet aggregation, which is necessary for the formation of a hemostatic plug [Mann et al., 2003].

A major challenge in the field of cardiovascular therapeutics is to develop antithrombotic agents to

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prevent uncontrolled thrombosis, but with less bleeding complications than with the existing widespread clinically used drugs (coumarins and heparins). Heparins enhance antithromin III-mediated inhibition of thrombin and factor Xa, whereas coumarins impair the function of the vitamin K-dependent proteins including procoagulants (thrombin, FXa, FIXa, and FVIIa) and anticoagulants (activated protein C and protein S). Although unfractionated heparins and coumarins are of great clinical value, both require careful dosing and frequent monitoring. Significant progress has been made by introducing low molecular weight heparin and pentasaccharides, but there is still a need for improved anticoagulants with a broad therapeutic window [Hirsh and Weitz, 1999].

The TF/FVIIa complex is an interesting target in thrombosis-related disease because TF/FVIIa-directed inhibitors might achieve anticoagulant efficacy without significantly interfering with normal hemostasis [Frédérick et al., 2005]. Intravascular "blood-borne" TF exhibits procoagulant activity and can be incorporated into platelet thrombi and contribute to thrombosis. Therefore, specific anti-TF antibodies may cause less bleeding as they inhibit intravascular TF at concentrations that are far below those necessary to block the high amounts of the hemostatic extravascular TF [Giesen et al., 1999].

Antibodies and antibody fragments represent the fastest growing segment of the biopharmaceutical market [Nicolaides et al., 2006]. This is mainly due to their drug safety profiles and because it could elicit clinical benefit by antagonizing a specific antigen without the common side effects that are prevalent with small chemical entities due to their nonspecific effect on homeostatic biochemical pathways.

Phage display is a powerful and rapid means to select antibody fragments with specific affinities and activities toward a target protein. Its power lies in the combination of functional activity and genetic information, although this technique has been used to a limited degree in the field of thrombosis and hemostasis. Antibody libraries were used to select antibodies that bind to the platelet receptor GPIIb/IIIa [Schwarz et al., 2006] and human vascular endothelial growth factor [Lin et al., 2008]. We used two single-chain variable fraction antibody libraries to select an inhibitory antibody fragment to human TF using phage display; we selected a single-chain variable fraction (scFv) antibody fragment that inhibits the in vitro function of TF, which therefore inhibits coagulation.

METHODS AND MATERIALS

Selection of Tissue Factor Binding Phages

Human single fold scFv libraries I and J (Tomlinson I+J; http://www.geneservice.co.uk/products/proteomic/scFv_tomlinsonIJ.jsp) were kindly provided by Professor Greg Winter (MRC Centre for Protein Engineering, Cambridge, UK). Four Immune tubes were coated overnight with 2 ml of each of two TF preparations. Two tubes were coated with the prothrombin time reagent Thromborel (Dade Behring, GmbH, Marburg Germany) and two tubes with Innovin (Dade-Behring, South Africa), respectively. After 3 washes with phosphate-buffered saline (PBS), the immune tubes were blocked with 4 ml of PBS containing 2% skimmed milk powder (SM) (Difco Laboratories, Becton Dickinson, MD) for 2 h at room temperature. The tubes were washed 3 times with PBS after the blocking solution was removed. Then 1 ml of the primary I and J libraries (1×10^{12} phages) was added to each of the Thromborel and Innovin-coated tubes together with 3 ml of 4% SM solution and rotated for 1 h at room temperature. After another 1-h incubation at room temperature, the unspecific binding phages were washed away with 0.1% Tween PBS 10 times (20 times for the second and third round). Bound phages were eluted by 500 µl trypsin-PBS (50 µl of 10 mg/ml trypsin stock solution added to 450 µl PBS) and rotated for 10 min at room temperature.

Escherichia coli TG1 cells (optical density [OD] 600 = 0.4) cultured in 2 × TY medium (16 g tryptone, 10 g yeast extract, and 5 g NaCl in 1 L distilled water) was infected by the eluted phages at 37°C for 30 min. A portion of the infected *E. coli* TG1 was serially diluted into 10^0, 10^1, 10^2, 10^3, and 10^4 dilutions. Ten µl of each dilution was spotted separately on TYE plates (15 g Bacto-Agar, 8 g NaCl, 10 g tryptone, 5 g yeast extract in1 L distilled water including 100 µg/ml ampicilin, and 1% glucose). These plates were incubated at 37°C overnight to titer the eluted phages by counting the clones on the TYE plates. At the same time, the rest of infected *E. coli* TG1 cells were all spread out on large TYE plates (including 100 µg/ml ampicilin and 1% glucose) and also incubated overnight at 37°C. The next day, bacteria on the large plates were scrapped and added into 100 µl 2 × TY (including 100 µg/ml ampicilin and 1% glucose) for amplification. Phages amplified in *E. coli* TG1 were rescued as follows: Helper phages (5×10^{11} KM13) were added to 10 ml of the amplified *E. coli* TG1 cells and incubated for 30 min at 37°C. After centrifugation for 10 min at 3000 *g*, the supernatant was discarded and cells were resuspended in 50 ml 2 × TY medium containing 100 µg/ml ampicilin, 50 µg/ml kanamycin, and 0.1% glucose. These cells were amplified overnight at 30°C, centrifuged at 3000 *g* for 15 min and the supernatant added to 10 ml of polyethylene glycol (PEG) for 1 h on ice. After another centrifugation, the rescued phages were diluted in 2 ml of PBS and stored at 4°C for the next selection round in which 10^{12} rescued phages

were added into 2% SM solution to start the next selection round. Four selection rounds were performed. During the last selection round, the TF binding phages were specifically eluted with an excess amount of Factor VII to select inhibitory clones.

Preparation of scFv Phage for Monoclonal Enzyme-Linked Immunosorbent Assay (ELISA)

From the last selection round, 400 single clones from each library were randomly picked out from the TYE plates and cultured in different wells containing 100 µl 2 × TY medium (including 100 µg/ml ampicillin and 1% glucose) of four 96-well microculture plates. After culturing overnight at 37°C, a small inoculum (2 µl) was transferred to other plates with 200 µl 2 × TY (including ampicilin and glucose) in each well. The original plates were stored at 4°C temporarily. The transferred plates were shaken at 37°C for 2 h, and the phages were rescued in *E. coli* TG1 by adding 10^9 helper phages to each well and shaken for 1 h at 37°C before spinning the plate at 1800 *g* for 10 min. Pellets were suspended in 200 µl 2 × TY (including 100 µg/ml ampicilin and 50 µg/ml kanamycin) and shaken overnight at 30°C. The next day, culture plates were spun down at 1800 *g* for 10 min, and 100 µl of the supernatant containing scFv-phage was used in a monoclonal phage ELISA.

Monoclonal phage ELISA

Eight ELISA plates (Nunc. Maxisorp) were coated overnight at 4°C with 50 µl of the TF preparation (4 plates with Innovin and 4 plates with Thromborel). The plates were washed 3 times with PBS and blocked with 2% SM (200 µl per well) for 2 h at room temperature. After 3 washing steps with PBS, 50 µl of the scFv supernatant of each well of the cell culture plates were added to each of the wells of the coated ELISA plates together with 50 µl of 4% SM. After incubation for 1 h at room temperature, plates were washed 3 times with PBS-0.1% Tween-20. Subsequently, 100 µl of a horseradish peroxidase-anti-M13 antibody were added to each well in a 1:5000 dilution on PBS-2%SM (100 µl per well) and incubated for another hour at room temperature. After 3 more washes with PBS-0.1% Tween-20, 100 µl substrate solution (100 µg/ml TMB in 100 mM sodium acetate, pH 6.0, with 30% hydrogen peroxide directly before use) was added to each well. The reaction was stopped by adding 50 µl 2 M H_2SO_4. Absorbance at OD_{450} and OD_{650} was determined and the 14 colonies with the highest $OD_{450–650}$ values were amplified (see next section).

Amplification of Single Colonies

The 14 strongest tissue factor binding phages were amplified by adding 50 µl of the cell stock of the culture plates to 50 ml 2 × TY (with 1% glucose) and grown at 37°C for 2 h. Ten ml of these cultures was then added to a centrifuge tube and 5×10^{10} helper phages were added and incubated for 30 min at 37°C. After centrifugation at 300 *g* for 10 min, the pellets were resuspended in 50 ml 2 × TY containing 100 µg/ml ampicilin, 50 µg/ml kanamycin and 0.1% glucose and shaken at 30°C overnight. The phages from the single colonies were rescued the next day by centrifuging the overnight culture at 3000 *g* for 15 min. The supernatants were added to 10 ml PEG/NaCl solution (20% polyethylene glycol [PEG] 6000, 2.5 M NaCl) for 1 h at 4°C to precipitate the phages. After another centrifugation of 3000 *g* for 30 min, the phage pellets were dissolved in 2 ml PBS and the phage concentration determined as follows. Phage concentration (phages/ml) = OD_{260} × dilution × 2.214×10^{11}. The phages from the 14 strongest binding colonies were stored in PBS with 15% glycerol at −70°C for further testing.

TF Activity Assay

The Actichrome TF assay kit from American Diagnostica (USA) was used to determine the TF inhibitory activity of the14 strongest TF binding phages. An aliquot (450 µl) of the amplified phages of each of the 14 colonies was precipitated on PEG/NaCl and the pellet dissolved in 200 µl assay buffer of the kit. The phage concentration of each colony was determined as previously described. An amount of 5×10^{10} phages of each colony was put into each of 2 wells on an ELISA plate. A phage that did not bind or inhibit TF was use as a control. TF (15 pM) was added to the phages and incubated for 10 min at room temperature followed by 25 µl of Factor VII and 25 µl of Factor X. Following incubation for 15 min at 37°C, 25 µl of the substrate, Spectrozym Xa was added to each well and the absorbance measured at 405 nm every 20 min for a period of 80 min. The 6 colonies with the lowest TF activity, e.g., the six strongest inhibitors of the reaction were diluted 1:2 for 4 times and the kinetic reaction repeated to determine concentration-dependent inhibition of TF by these colonies.

Prothrombin Times

Prothrombin times (PT) were conducted on the three strongest inhibitory phage colonies selected via the TF activity assay; 5×10^{10} phages were incubated with the PT reagent, Thromborel for 10 min, after normal plasma was added and the time measured until the clot formed using a STAR-4 coagulation instrument (Diagnostica Stago, Asnières sur Seine, France). The strongest TF inhibitory clone was selected for the production of soluble scFv antibody fragments.

Production of Soluble scFv Antibody Fragments

The selected phage clone, JT C5 was used to infect HB2151, a nonsuppressor *E. coli* strain that is then induced to provide soluble expression of antibody fragments. An aliquot (3 ml) of overnight activated *E. coli* HB2151 (infected by the selected scFv phage) culture was transferred to 300 ml 2 × TY (0.1% glucose and 100 μ/ml ampicilin) and shaken at 250 rpm at 37°C for ±3 h until the $OD_{600} = 0.9$. Isopropyl β-D-thiogalactoside (IPTG, final concentration 1 mM) was then added and shaking continued overnight at 30°C. After centrifugation at 1800 *g* for 10 min, the supernatant containing the soluble antibody fragments was stored at 4°C.

Purification of JT C5 scFv Antibody Fragments

JT C5 scFv antibody fragments were purified from the cell culture medium by using a Protein A column. Five ml of Protein A Sepharose Fast Flow (Pierce, IL) was packed into a column. After rinsing with 10 ml of binding buffer (0.1 M phosphate, 0.15 M NaCl, pH = 7.2), the cell culture medium was diluted 1:1 with binding buffer and applied to the column. After the medium was run through, the column was washed with a further 15 ml of binding buffer. The antibody fragments were eluted in 1-ml fractions with an acidic elution buffer (0.1 M glycine, pH 2–3). The protein content of each fraction was measured at OD_{280} and the fractions with the highest protein content were pooled. Purified scFv was evaluated by 12% SDS-PAGE and Western blotting using a mouse anti-cMyc antibody (Pierce). The purified antibody fragment was dialyzed with Tris-buffered saline (TBS) and the concentration determined via absorbance at 280 nm.

Functional Characterization of the Tissue Factor Inhibiting Antibody Fragment

The selected antibody fragment JT C5 was tested for TF inhibitory activity using the Actichrome TF activity assay. Prothrombin times were conducted with different concentrations of the antibody fragment and the effect of the antibody fragment was also tested on thrombin generation in normal plasma as well as plasma from a thrombophilic patient.

TF Activity Assay

Antibody fragment concentrations of 625ng, 313ng, 156ng and 78ng were added in duplicate to an ELISA plate. TF(15 pM) and assay buffer to a volume of 75 μl were added to the antibody fragment and incubated for 10 min at RT. Factor VII (25 μl) was added to each well followed by Factor X (25 μl factor). After incubation for 15 min at 37°C, 25 μl of the substrate, Spectrozym Xa were added to each well and the absorbance at 405 nm measured every 15 min for 90 min.

Prothrombin Times

Prothrombin times were evaluated using antibody fragment concentrations of 1.5 μM, 0.75 μM and 0.375 μM. The antibody fragments were incubated with the PT reagent, Thromborel, and buffer (TBS) for 10 min. Normal plasma was then added and the time measured until clot formation using a STAR-4 coagulation instrument (Diagnostica Stago).

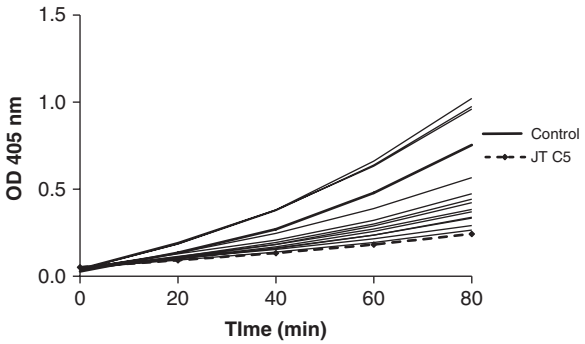

Fig. 1. Tissue factor activity of the 14 strongest tissue factor binding phage colonies. 5×10^{10} phages of each colony were added to each of 2 wells on an ELISA plate. TF (15 pM) was added to the phages and incubated for 10 min at room temperature. Factor VII (25 μl) was added to each well followed by Factor X (25 μl). After incubation for 15 min at 37°C, 25 μl of the substrate, Spectrozym Xa was added to each well and the absorbance at 405 nm measured every 20 min for a period of 80 min.

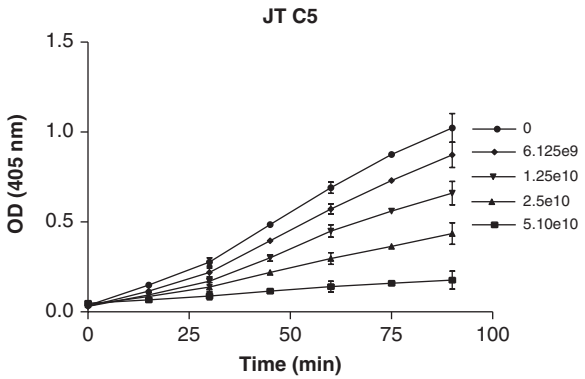

Fig. 2. Concentration-dependent tissue factor activity of the strongest inhibiting phage clone, JT C5. Phage concentrations ranging from 6.125×10^9–5×10^{10} were added to each of 2 wells on an ELISA plate. TF (15 pM) was added to the phages and incubated for 10 min at RT. Factor VII (25 μl) was added to each well followed by Factor X (25 μl). After incubation of 15 min at 37°C, 25 μl of the substrate, Spectrozym Xa were added to each well and the absorbance at 405 nm measured every 15 min for a period of 90 min ($n = 3$).

Thrombin Generation Assay

The TECHNOTHROMBIN TGA kit (Technoclone, Surrey, UK) was used to determine thrombin generation with time in platelet-poor plasma upon activation of the clotting cascade by TF that was incubated with different concentrations of the anti-TF antibody fragment. The TGA reagent C that contains approximately 5 pM of TF was incubated with different JT C5 scFv concentrations (13, 6.25, and 3.125 μM) and TBS for 10 min at 37°C. Normal pooled plasma and plasma from a thrombophilic patient with low free protein S level of 16% were then added in parallel to the reactions (40 μl per reaction). Fluorogenic TGA substrate (50 μl/reaction) containing 1 mM Z-G-C-R-AMC and 15 mM $CaCl_2$ was added to start each reaction that were conducted in duplicate. Fluorescence was measured at 360 nm/460 nm (excitation/emission) using the SYNERGY 2 Kinetic reader (BIOTEK) at 1-min intervals for 2 h.

RESULTS

FT Activity of the Strongest Tissue Factor-Binding Phage Colonies

Figure 1 shows the effect of the 14 strongest TF binding phages on the tissue factor activity assay. Approximately 6 phage clones inhibited the TF activity strongly. Colony JT C5 had the strongest inhibitory

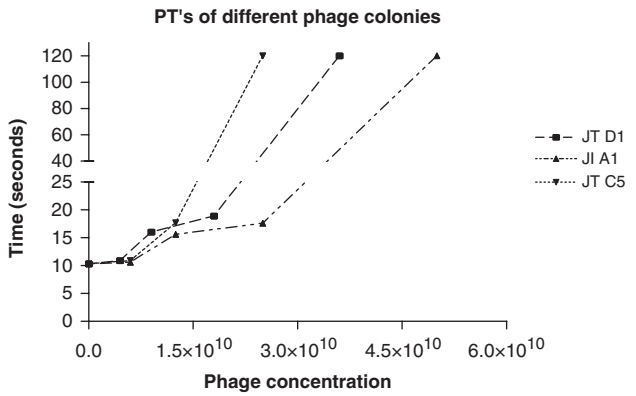

Fig. 3. Prothrombin times of the 3 strongest tissue factor inhibiting phage clones. 5×10^{10} phages were incubated with the PT reagent, Thromborel for 10 min, after which normal plasma was added and the time measures until the clot was formed ($n = 3$).

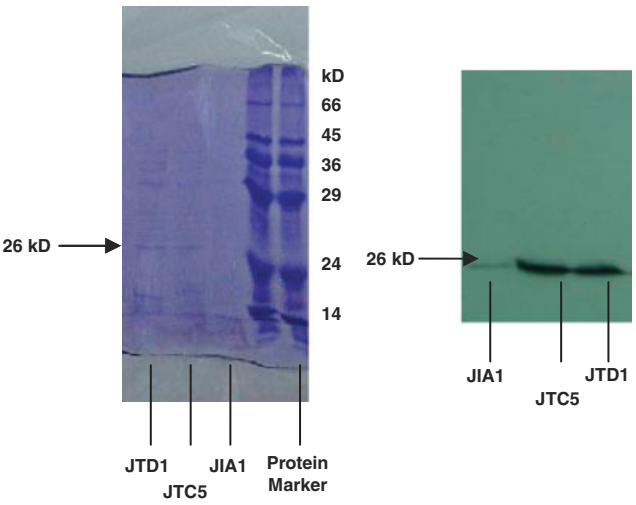

Fig. 4. SDS-PAGE and Western blotting of the antibody of the three strongest inhibiting phage clones. The purified scFv was evaluated by a 12% SDS-PAGE and Western blotting, using a mouse anti-cMyc antibody.

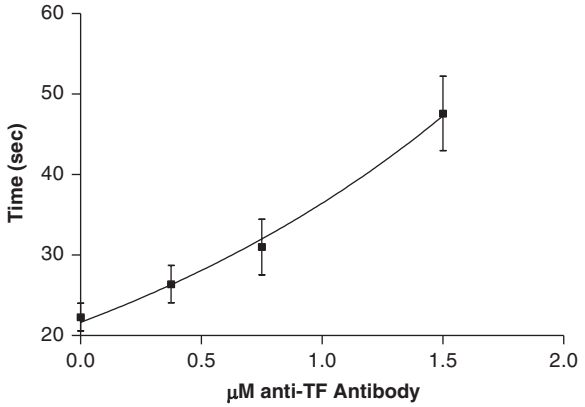

Fig. 5. Prothrombin times of the purified C5 scFv antibody fragment. Prothrombin times were conducted at antibody fragment concentrations of 1.5, 0.75, and 0.375 μM. The antibody fragments were incubated with the PT reagent, Thromborel, and buffer (TBS) for 10 min, after which normal plasma was added and the time measured until clot formation ($n = 3$).

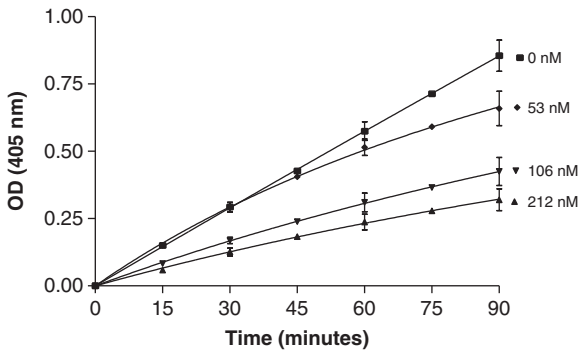

Fig. 6. Concentration-dependent inhibition of tissue factor activity by the purified JT C5 scFv antibody fragment. Antibody fragment concentrations of 212, 106, and 53 nM were added in duplicate to the wells of an ELISA plate. TF (15 pM) and assay buffer to a volume of 75 μl were added to the antibody fragment and incubated for 10 min at room temperature. Factor VII (25 μl) was added to each well followed by Factor X (25 μl). After incubation for 15 min at 37°C, 25 μl of the substrate, Spectrozym Xa were added to each well and the absorbance at 405 nm measured every 15 min for a period of 90 min ($n = 3$).

effect. There were three colonies that even increased the TF activity. The inhibitory effect of JT C5 is shown in Figure 2, where different concentrations of this phage were tested. At a concentration of 5×10^{10} almost no Factor Xa activity was measured. The other five inhibitory phages (JI B1, JI E3, JI A3, JI A1, and JT D1) were also tested for concentration-dependent TF inhibition, but the results are not shown here as the inhibitory effects were not as strong as that of JT C5.

Prothrombin Times of the Three Strongest Tissue Factor Inhibiting Phage Clones

The effect of the three strongest inhibitory phage clones (JT C5, JI A1, and JT D1) on the thrombin times of normal plasma is shown in Figure 3. Again the JT C5 phage colony showed the strongest inhibitory effect.

Evaluation of the Strongest Inhibiting scFv Antibody Fractions by SDS-PAGE and Western Blotting

SDS-PAGE and Western blotting (Fig. 4) showed that the scFv antibody fragments of the 3 strongest inhibiting phage clones were expressed in the *E. coli* culture medium. The expression of scFv JTC5 and JTD1 was better than that of JTA1.

Functional Characterization of the TF Inhibiting Antibody Fragment

Purified JT C5 scFv increased the prothrombin time dose-dependently (Fig. 5) with 1.5 μM JT C5 scFv increasing PT twofold. Factor Xa activity was also inhibited by the JT C5 scFv ($IC_{50} = 100$ nM; Fig. 6). Thrombin generation of normal plasma and of thrombophilic plasma was decreased with increasing concentrations of the JT C5 scFv. The effect of JT C5

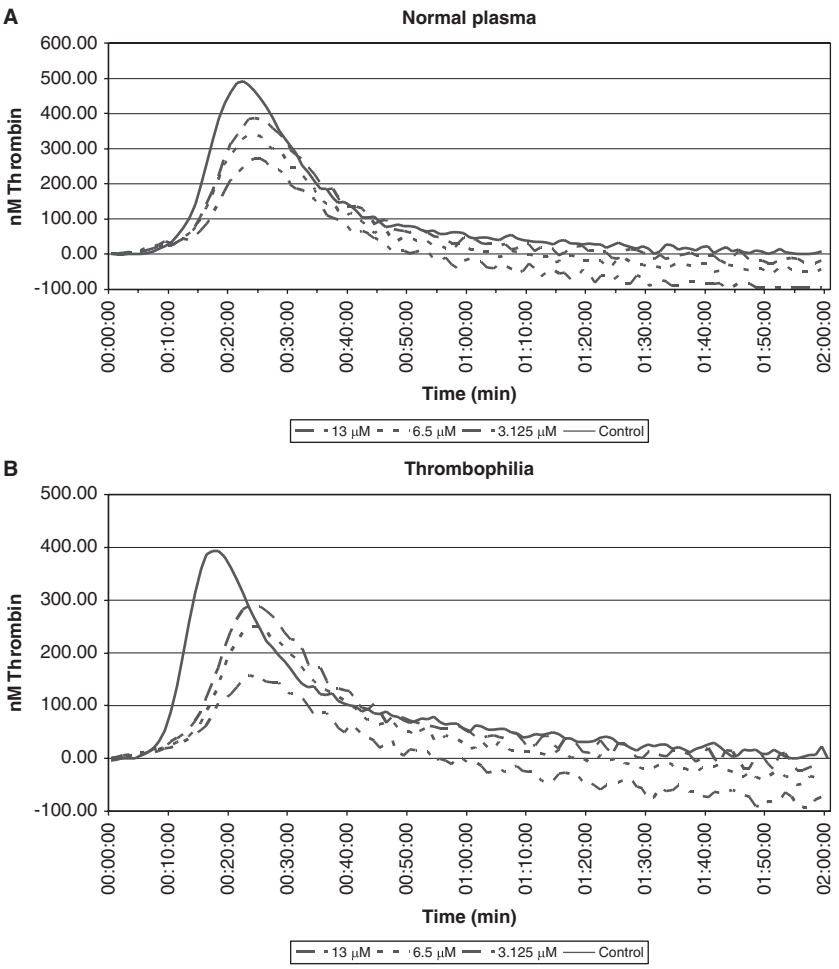

Fig. 7. Thrombin generation assays of different C5 scFv antibody fragment concentrations with normal plasma (A) and thrombophilic plasma (B). The TGA reagent C from the Technothrombin TGA kit (Technoclone, Surrey, UK) that contains about 5 pM TF, was incubated with different C5 scFv concentrations (13, 6.25, and 3.125 μM) and TBS for 10 min at 37°C. Normal pooled plasma and plasma from a thrombophilic patient were then added in parallel to the reactions (40 μl per reaction). The fluorogenic TGA substrate (50 μl per reaction) was added to start each reaction. Fluorescence was measured at 360 nm/460 nm (excitation/emission) at 1-min intervals for 2 h.

was more pronounced in thrombophilic plasma, since the lag phase before the thrombin generation burst was also increased, which was not the case with normal plasma (Fig. 7).

DISCUSSION

To our knowledge, JT C5 is the first human single-chain variable antibody fragment selected that bound to and inhibited the action of human TF using phage display technology. JT C5 was identified using four rounds of selection. Most phage display applications use 3–4 selection rounds [Schwarz et al., 2006; Lin et al., 2008]. Some 800 TF binding phages were tested in the monoclonal phage ELISA for TF binding larger than other efforts [Zhang et al., 2007; Schwarz et al., 2008; Lin et al., 2008].

Expression of JT C5 scFv was evaluated using SDS-PAGE and Western blotting. The scFv has a molecular weight of 26 kD and was purified on a protein A column, since it gives a better yield than a protein L column. The yield, however, is insufficient for in vivo testing with only about 1 mg of scFv from 1 L of *E. coli* culture. Larger *E. coli* cultures as well as larger columns are required to ensure a better yield of the purified scFv fragment. Purified JT C5 scFv increased prothrombin time in a concentration-dependent manner with a two-fold prolongation at 1.5 μM (39 ng/ml). This is 1,000 times stronger inhibition than the rat anti-mouse monoclonal antibody, 1H1 [Kirchhofer et al., 2005]. It also slowed Factor Xa production concentration-dependently in the TF activity assay with an IC_{50} value of 100 nM, 10 times stronger than that observed for the monoclonal anti-TF antibody, TF4A12 [Peng et al., 2007]. It is also a stronger inhibitor than other orally available TF/FVIIa complex inhibitors [Miura et al., 2007].

The thrombin generation assay shows a reduction in the peak thrombin concentration as well as an increase in the lag phase before thrombin is generated. The effect was more pronounced in thrombophilic plasma than in normal plasma, indicating that TF inhibitors may achieve anti-coagulant activity in thromboplilic plasma without interfering with hemostasis in normal plasma. The reduction in peak thrombin generation by JT C5 scFv was about 30 times less than the inhibitory effect of direct thrombin inhibitors such as Ximelagatran [Boström et al., 2004].

The limitation of the current scFv phage display technology is the production of sufficient scFv fragments. A larger production system is needed to enable further testing in in vivo studies.

In summary, we selected a single chain variable antibody fragment that inhibits tissue factor activity in a concentration-dependent manner by using phage display technology. The effect on thrombin generation is more pronounced in thrombophilic plasma than in normal plasma. TF inhibitors may provide an effective treatment for thrombotic disease without serious bleeding complications. Additionally, phage display is a powerful tool to select novel antithrombotics.

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Evaluation of *in vitro* refolding vs cold shock expression: Production of a low yielding single chain variable fragment

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ABSTRACT

The development of therapeutic antibodies in their various forms has been a constant challenge since the development of the first monoclonal antibodies in 1975. This is especially true for the development of therapeutic single chain variable (scFv) fragments in *Escherichia coli*. In a previous study the selection of a tissue factor inhibiting single chain variable fragment (TFI-scFv) isolated from the Thomlinson I + J phage libraries was described. Although the initial findings were promising, additional characterization of the antibody fragment and subsequent application was hampered due low protein yield. This study reports on: i) the improved expression of a previously low yielding TFI-scFv in the cytoplasm of *E. coli* BL21 (DE3) through modifications to the expression systems in conjunction with codon optimization ii) evaluation of two commercial methods of protein recovery: *in vitro* refolding and the utilization of cold shock expression systems in conjunction with *E. coli SHuffle*. Results showed that TFI-scFv could be expressed at higher levels in the cytoplasm of *E. coli* than previously achieved in the periplasm. Both the *in vitro* refolding and cold shock strategies were capable of producing functional TFI-scFv with varying degrees of success. These procedures could be applied to improve the production of other problematic low yielding scFv isolated from phage display repositories in order to facilitate their characterization.

1. Introduction

The development of antibodies as therapeutic agents has always been an attractive prospect due to their high specificity, affinity to a wide variety of molecules and stability [1]. Furthermore, the use of antibody sub-fragments is especially attractive due to the relatively small size (27 kDa), low immunogenicity and relative ease of selection by means of phage display [2]. Previously we reported on the utilization of phage display technology to select a scFv from the Tomlinson I + J Human Single Fold Phage Libraries that functionally inhibits human tissue factor [3]. Tissue factor is constitutively expressed in vascularized organs [4]. The distribution of tissue factor in vital organs provide a protective hemostatic barrier in order to minimize blood loss during injury [5]. Despite this critical role in hemostasis, excessive or aberrant expression of tissue factor has been linked to inflammatory and thrombotic disorders, which are major contributors to global disease and mortality [6,7]. As a result, the inhibition of TF holds great potential for the development of novel anti-thrombotic agents.

Despite phage display's exceptional ability to generate antibodies against a variety of targets, the utilization of the scFv remains limited due to complications that arise during protein production. Expression in *Escherichia coli* systems are often preferred for production of therapeutics, for laboratory as well as industrial scales, due to low cost and simplicity of cultivation. However, the successful production of functional protein is often hampered due to codon bias and incorrect folding of the target protein [8–12]. Thus, a constant challenge in the field of protein engineering is to overcome the shortcomings of the native molecular mechanisms through alternative expression strategies [13].

Due to the high frequency of scFv aggregation, the regeneration of functional scFv from insoluble aggregates through *in vitro* refolding techniques has become standard practice [14–16]. Various refolding techniques have been developed to refold scFv, which include direct

Abbreviations: Codon Adaptation Index, CAI; Dimethyl sulfoxide, DMSO; E.coli Codon Usage Analyzer, ECUA; *Escherichia coli*, *E.coli*; Guanidinium-HCl, GdnHCl; Heavy chain variable domains, V_H; Immobilized Metal Affinity Chromatography, IMAC; Isopropyl β-D-1-thiogalactopyranoside, IPTG; Light chain variable domains, V_L; Medical Research Council, MRC; Oxidized glutathione, GSSG; Prothrombin Time, PT; Reduced glutathione, GSH; Single chain variable fragment, scFv; Tissue factor, TF; Tissue Factor Inhibitor scFv, TFI-scFv; Tris Buffered Saline, TBS

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dilution, dialysis, diafiltration, chromatographic methods, matrix-assisted refolding, and chemical-assisted refolding with varying levels of success [10,17–20]. The major disadvantage to commonly used *in vitro* refolding techniques is that they are often complex and require intensive optimization of multiple operational steps in order to obtain functional protein [20]. Target protein recovery yields are also highly variable due to the protein specific nature of *in vitro* refolding techniques [21].

As an alternative approach, cold shock expression is a popular method used to overcome protein aggregation. It utilizes the native response of *E. coli* to sudden reductions in temperature to overcome protein aggregation [22]. Sudden temperature fluctuations strongly influence gene regulation in mesophilic bacteria such as *E. coli* [23–25] Additionally, a number of chaperones are up-regulated with increased activity at lower temperatures which in turn facilitates appropriate protein folding [26]. These expression mechanisms have also been successfully utilized to facilitate the solubilization of proteins containing multiple disulfide bonds [27]. In conjunction with cold shock, the utilization of fusion partner expression systems has also been shown to facilitate the production of soluble recombinant proteins in *E. coli* [28]. Fusion expression systems make use of a highly soluble partner that is fused to the N-terminus of the target protein in order to improve the solubility of the target protein [29].

An additional complication to consider is the presence of inter domain stabilizing disulfide bonds. All antibodies have highly conserved disulfide bonds and scFv contains one intra-domain chain disulfide bond for each of the variable regions [30]. The reducing conditions of the *E. coli* cytoplasm hampers the formation of disulfide bonds that are necessary for stable tertiary structure formation [31,32]. It has been shown that, modification of the expression host itself can promote disulfide bond formation in the cytoplasm. The enhanced oxidizing cytoplasmic conditions in combination with improved isomerization capability have been shown to contribute to improve target protein solubilization through improved disulfide bond formation [33].

In this study we report on the (i) improved expression of a previously low yielding TFI-scFv against human tissue factor by means of codon optimization, (ii) we evaluate the effectiveness of two commonly utilized strategies for the generation of functional scFv namely: the directed regeneration of inclusion bodies through *in vitro* refolding and the expression of functional TFI-scFv using a commercial (TAKARA) cold shock pCOLD DNA II and pCOLD Trigger Factor expression vectors in conjunction with *E. coli* BL21 (DE3) and *E. coli SHuffle* strains.

2. Materials and methods

2.1. Bacterial cell lines and plasmids

E. coli strain TG1 (MRC Centre for Protein Engineering, Cambridge, UK) was used for phagemid production. Cloning host, *E. coli* Top10 (Invitrogen, USA) was used for the *in vitro* amplification of pSMART (Lucigen, USA) cloning vectors. All scFvs constructs were expressed using either pET22 (Novagen, Germany), pCOLD DNA II or pCOLD TF expression vectors (TAKARA Bio Inc, Japan) utilizing *E. coli* BL21 (DE3) (Invitrogen, USA) or *E. coli SHuffle*® (New England Biolabs, USA).

2.2. Rare codon analysis

Rare codons were identified using *E. coli* Codon Usage Analyzer v2.1 (*E*CUA) (http://www.faculty.ucr.edu/). The frequency and distribution of codons was analyzed using Graphical codon usage analyzer v2.0 utilizing codon usage table for *E. coli* [34]. The Codon Adaptation Index (CAI) value was calculated using the CAIcal server [35]. Distribution of rare codons within the native TFI-scFv (JTC5-scFv) was visualized using web-based application Protter: Interactive protein feature visualization and integration with experimental proteomic data v1.0 [36].

2.3. Homology modelling of TFI-scFv

DNA sequence of TFI-scFv was translated to the final amino acid sequence using online translations tool ExPASy: translate from Swiss Institute of Bioinformatics. The amino acid sequence was used to model a three-dimensional structure of TFI-scFv. Homology modelling of TFI-scFv was performed using Yet Another Scientific Artificial Reality Application (YASARA) version 16.4.6.L.64 with the following set parameters; Modelling speed: Slow, Number of PSI-BLAST iterations in template search (PsiBLASTs): 3, Maximum allowed (PSI-) BLAST E-value to consider template (EValue Max): 0.5, Maximum number of templates to be used (Templates Total): 5, Maximum number of templates with same sequence (Templates SameSeq): 1, Maximum oligomerization state (OligoState): 4 (tetrameric), Maximum number of alignment variations per template: (Alignments): 5, Maximum number of conformations tried per loop (LoopSamples): 50 and Maximum number of residues added to the termini (TermExtension): 10 [37].

2.4. Construction of TFI-scFv expression vectors

The codon-optimized gene was synthesized for expression in *E. coli* by GeneART (Regensburg, Germany). The codon-optimized TFI-scFv sequence was synthesized to incorporate 5′ *NdeI* and 3′ *XhoI* restriction sites for downstream cloning. The optimized scFv gene was amplified using sense primer 5′-GAAGGAAGGCCGTCAAGG-3′, anti-sense primer: 5′-CTCGAGATTACGTTTAATTTCAACTTTGG-3′ and sub cloned into *NdeI/XhoI* linearized expression vector pET-22 b (+), pCOLD DNA II, and pCOLD TF expression vectors. The new constructs were designated as pET22 TFI-scFv (Supplementary Fig. 1), pCOLD DNA II TFI-scFv, and pCOLD TrF TFI-scFv (Supplementary Fig. 2) respectively. Constructs were verified by Sanger DNA sequencing.

2.5. Sanger DNA sequencing of the scFv gene fragment

All DNA sequencing reactions were performed using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) in conjunction with the 3130xl Genetic Analyzer HITACHI (Applied Biosystems, USA) according to manufacturer's specifications. The original pIT2 plasmid containing the TFI-scFv gene was sequenced using LBR (sense) 5′-CAGGAAACAGCTATGAC-3′ and pHEN (anti-sense) 5′-CTATGCGGCCCCATTCA-’3 primers. The pET22-TFI expression vector was sequenced using generic T7 Promoter Primer: 5′TAATACG ACTCACTATAGGG 3′ and T7 Terminator Primer: 5′GCTAGTTATTGCT CAGCGG 3’ (Integrated DNA Technologies, Belgium. The pCOLD DNA II TFI-scFv expression vector construct was sequenced using sense primer 5′-ACGCCATATCGCCGAAAGG-3′ and universal anti-sense primer 5′-GGCAGGGATCTTAGATTCTG-3’. The pCOLD TF expression vector constructs were sequenced using 5′-CCACTTTCAACGAGCTGATGA-3′ in conjunction with universal anti-sense primer 5′-GGCAGGGATCTTAGA TTCTG-3’ (Integrated DNA Technologies, Belgium).

2.6. In vitro refolding of TFI-scFv inclusion bodies

2.6.1. Expression of pET22 TFI-scFv in E. coli BL21 (DE3)

E. coli BL21 (DE3) cells transformed with pET22 TFI-scFv in parallel with a control harboring empty pET22b (+) plasmid were incubated in LB-media (5 g L^{-1} NaCl, 5 g L^{-1} yeast extract, 10 g L^{-1} Bacto-tryptone) containing 100 μg mL^{-1} ampicillin for 16 h at 37 °C, 160 rpm. The overnight cultures were used at 1:100 dilution to inoculate a 5-L Sartorius bioreactor filled with 4 L LB media containing 100 μg mL^{-1} ampicillin, controlled by BIOSTAT B Plus universal controller unit (Sartorius, Germany). The cultures were incubated at 37 °C, 300 rpm agitation and 2 L per minute (lpm) aeration up to mid-log phase (OD_{600} = 0.6) and induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cultures were harvested by centrifugation at 15,000 g for 10 min at 4 °C, the supernatant discarded, and cell pellet stored at

−20 °C. Harvested cells were disrupted using the one-shot homogenizer (Constant Systems, UK) at 30,000 KPSI. Soluble and insoluble fractions were separated by centrifugation at 15,000 g for 10 min at 4 °C. The soluble fraction was further fractionated using ultra-centrifugation at 100,000 g for 20 min at 4 °C. All fractions were evaluated by 10% SDS-PAGE as described by Laemmli (1970) and stained as described by Fairbanks and co-workers (1971) [38,39].

2.6.2. Isolation of inclusion bodies

Cell pellets were thawed on ice and washed twice using disruption buffer (50 mM Tris buffer containing 500 mM NaCl, 10 mM EDTA, 5 mM DTT and 2% Triton X-100 at pH 7.5). Cells were disrupted using the one-shot homogenizer (Constant Systems, UK) at 30,000 KPSI. Soluble and insoluble fractions were separated by centrifugation at 15,000 g for 10 min at 4 °C. The insoluble fraction was again washed twice using disruption buffer and a third time using a chelating buffer (50 mM Tris and 10 mM EDTA at pH 7.5). The pellets were suspended in a minimal volume of dimethyl sulfoxide (DMSO) and kept at room temperature for 30 min. Pellets were solubilized in denaturing buffer (50 mM Tris at pH 7.5 containing 6 M guanidinium-HCl (GdnHCl) and 5 mM dithiothreitol) at room temperature for 3 h with slow magnetic stirring. The soluble and insoluble fractions were further separated using ultra-centrifugation at 100,000 g for 20 min at 4 °C in order to remove residual membrane fraction. Protein concentrations of the solubilized fractions were determined using the Q-Bit Illuminometer and Q-Bit Protein assay (ThermoFisher Scientific, USA) according to manufacturer's specifications.

2.6.3. Purification of TFI-scFv using IMAC under denaturing conditions

TFI-scFv inclusion bodies were purified under denaturing conditions using a 5-mL HisTrap FF column (GE Healthcare, USA) in conjunction with the ÄKTAprime plus (Amersham Biosciences, UK) chromatography system according to manufacturer's specification. The system was equilibrated with 100 mL binding buffer at 5 mL min^{-1} (50 mM Tris containing 6 M Gdn-HCl, 0.5 M NaCl and 20 mM imidazole at pH 7.5). The denatured sample was diluted (1:10) with binding buffer and loaded at 1 mL min^{-1}. The column was then washed with 50 mL binding buffer at 1 mL min^{-1}. Elution was performed in binding buffer using a 100 mL linear Imidazole gradient (20–300 mM) at 1 mL min^{-1}. Protein concentration of the solubilized fractions was determined using the Q-Bit illuminometer and Q-Bit Protein assay. Purified TFI-scFv was resolved by means of 10% SDS-PAGE as described previously.

2.6.4. Screening of refolding conditions

Refolding conditions were screened using the Pierce Protein Refolding Kit (Thermo scientific, USA) according to manufacturer's specifications. Due to the presence of intra-domain disulfide bonds, redox conditions were investigated using oxidized (GSSG) and reduced (GSH) glutathione (Supplementary Material: Table 1). Batch-wise refolding reactions were performed by a 20-fold dilution of the denatured TFI-scFv in each of the 1 mL screening reactions. The final refolding reactions containing 500 μg mL^{-1} TFI-scFv were performed overnight at 4 °C. Buffer exchange was performed by overnight dialysis at 4 °C against 3 L TBS (50 mM Tris containing 150 mM NaCl, pH 7.5) using 10,000 MWCO SnakeSkin pleated dialysis tubing (Thermo scientific, USA) as described by Stanton and co-workers [40]. The soluble fraction was isolated by centrifugation at 15,000 g for 10 min at 4 °C and evaluated by 10% SDS-PAGE. Densitometric analysis was performed using ImageJ v 1.51 d (http://imagej.nih.gov/ij/) image analysis software.

2.7. Cold shock expression

2.7.1. Expression of pCOLD DNA II TFI-scFv and pCOLD TrF TFI-scFv in E. coli BL21 (DE3) and E. coli Shuffle

Chemically competent *E. coli* BL21 (DE3) and *E. coli SHuffle* cultures were transformed using pCOLD DNA II TFI-scFv, pCOLD TrF TFI-scFv, pCOLD DNA II (Empty Control), and pCOLD TrF (Empty Control). The transformants were incubated as described in section 2.4.1 with slight modification. Once mid-log phase (OD_{600} = 0.6) was reached, cold shock was initiated. The incubation chamber was submerged in ice slurry to enable rapid temperature drop required for cold shock. Once the cultures reached 4 °C the bioreactor was removed from the ice-slurry and incubated for an additional 30 min at 15 °C. Expression was induced by adding 1 mM IPTG (final concentration) and 24 h incubation at 15 °C with 300 rpm agitation. The following day cultures were harvested by centrifugation at 15,000 g for 10 min at 4 °C stored at −20 °C. Harvested cells were disrupted using the One-Shot Homogenizer (Constant Systems, UK) at 30,000 KPSI. The soluble- and insoluble fractions were separated by means of centrifugation at 15,000 g for 10 min at 4 °C. The membrane fraction was then pelleted by means of ultra-centrifugation at 100,000 g for 90 min at 4 °C. All fractions were evaluated by 10% SDS-PAGE and densitometry as previously described.

2.8. Purification of TFI-scFv by immobilized metal affinity chromatography

Fractions containing the soluble TFI-scFv and pCOLD TF and fusion construct Trigger factor -TFI-scFv (TrF:TFI-scFv) were purified using a 5-mL HisTrap FF column (GE Healthcare, USA) in conjunction with the ÄKTAprime plus (Amersham Biosciences, UK) chromatography system according to manufacturer's specifications. The column and system were initially equilibrated with 100 mL binding buffer (50 mM Tris, 150 mM NaCl containing 20 mM Imidazole at pH 7.5) at 5 mL min^{-1}. The TFI-scFv and TrF-TFI-scFv sample was diluted (1:10) with binding buffer and loaded at 1 mL min^{-1}. The column was then washed with 10 column volumes binding buffer at 1 mL min^{-1}. Target protein was eluted from the column using a 100-mL linear imidazole gradient (20–500 mM) at 1 mL min^{-1}. The TrF:TFI-scFv construct contains a thrombin cleavage (Leu-Pro-Val-Arg-Gly-Ser) site nested between the trigger factor and the insert (Supplementary Fig. 2). Target TFI-scFv was cleaved from the TrF-TFI-scFv fusion construct using the Thrombin Cleancleave™ Kit (Sigma-Aldrich, USA) per manufacturer's instructions. Briefly, 1 mL batch reactions were performed by adding 1 mg of fusion construct to agarose immobilized thrombin resin. Reactions were incubated for 8 h at room temperature and then stored overnight at 4 °C. The following day the cleavage reactions were centrifugation at 500*g* for 5 min and the supernatant collected and pooled together. The cleaved trigger factor and TFI-scFv were separated by means of a secondary purification using a 5-mL HisTrap FF column in conjunction with the ÄKTAprime as described previously. The purified TFI-scFv

Table 1
Isolation and purification of denatured TFI-scFv.

Total biomass (wet weight in g)	[a] Isolated Inclusion bodies	[a]Purity	[a]Calculated TFI-scFv in inclusion body fraction	[b]Total Purified Denatured TFI-scFv	[b]Purity	[b]Retention
5.5 g L^{-1}	54 mg L^{-1}	> 80%	37 mg L^{-1}	4.25 mg L^{-1}	> 95%	11.%

The *in vitro*refolding of TFI-scFv.
[a] Prior to IMAC.
[b] Following IMAC.

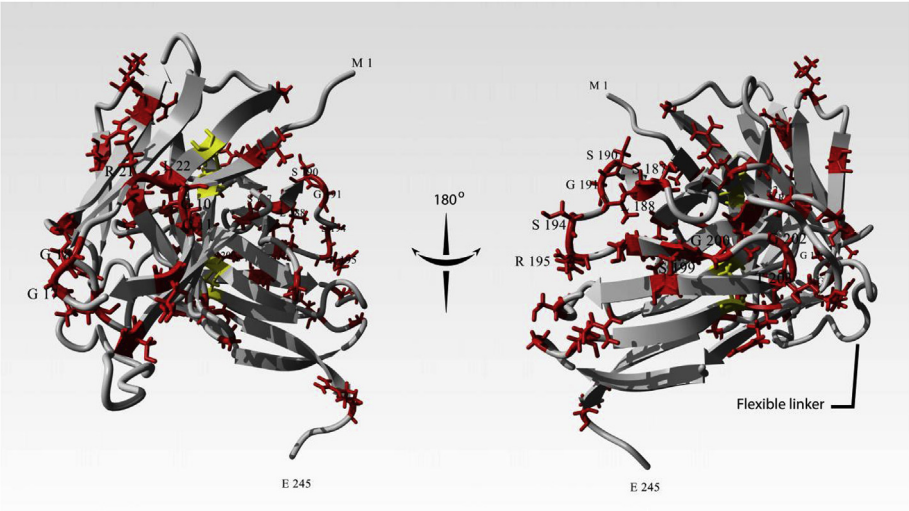

Fig. 1. Homology model of TFI-scFv and the distribution of rare codons.

fractions were pooled together and concentrated using 10,000 MWCO VIVASPIN 20 columns (Sartorius, Germany). The protein concentrations of the solubilized fractions were determined using the Q-Bit Illuminometer and Q-Bit Protein assay. All fractions were evaluated by 10% SDS-PAGE and densitometry as previously described.

2.9. Prothrombin times

Diluted prothrombin time tests (PTs) were performed using Dade C Control platelet poor plasma (Siemens Healthcare, Germany) in conjunction with the STAR-4 coagulation instrument (Diagnostica Stago). Tissue factor (Dade Tromborel S, Siemens Healthcare, Germany) was individually incubated with escalating dosages (0.0 mg ml^{-1} to 0.2 mg ml^{-1}) of TFI-scFv at 37 °C for 10 min prior to testing. PTs were performed in triplicate and the average clotting time was calculated. The inhibition effect (extended clotting time) of the TFI-scFv fractions was calculated as the difference between the average clotting time and the blank (negative control).

3. Results

3.1. Rare codon optimization

Analysis of the TFI-scFv gene was performed using *E*CUA software with a 10% codon frequency threshold based on highly and continuously expressed genes during exponential growth phase (Class II genes). The analysis of the gene identified a total of 45 (approximately 20%) rare codons distributed throughout the Heavy- and Light chains of the scFv (Fig. 1 and suppl. Fig. 3), which contribute to low expression levels. A total of 150 out of 242 codons (approximately 62%) were substituted in the optimized gene. The CAI index value for the native gene increased from 0.61 to 0.91 for expression in *E. coli* (Fig. 2).

Two ribbon diagrams of the TFI-scFv homology model are shown at 180° rotations with the flexible (GGGGS)×3 linker indicated as predicted by YASARA software. The distribution and positions rare codons in TFI-scFv are indicated in red, with the Cysteine disulfide bridges located within the heavy- and light chains in yellow. C-terminal His- and Myc-tag has been omitted from diagram.

The comparative summary of codon frequency between the native TFI-scFv and optimized TFI-scFv. A value of 0–100 was assigned to synonymous codons for each amino acid based on their frequency of utilization according to the *E. coli* codon usage database. Codons with low relative frequency values indicate less preferred or rare codons. Low frequency codons have lower expression efficiency and as with the case of rare codons may lead to the truncation of proteins. Codons with high relative frequency (highly utilized) generally improve expression efficiency.

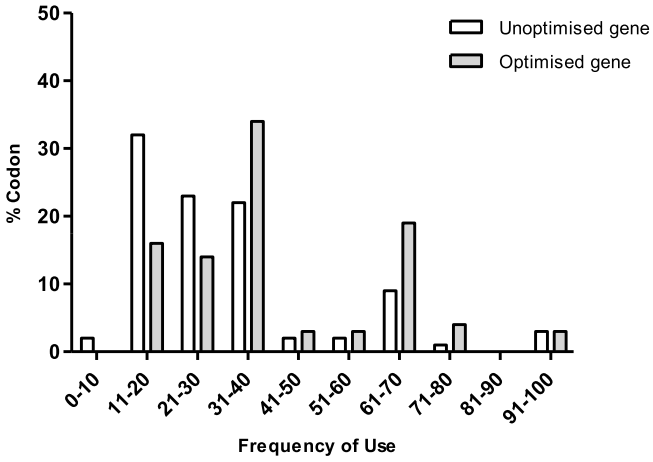

Fig. 2. Codon frequency of usage profile.

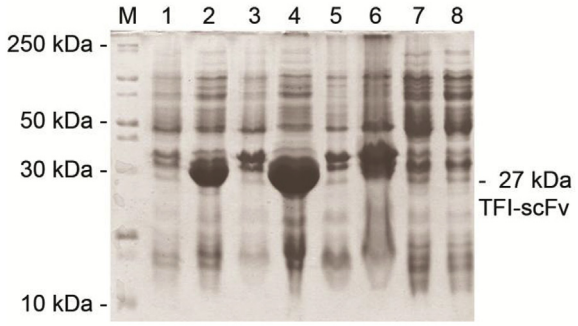

Fig. 3. Cytoplasmic expression of TFI-scFv.

Multiple alignment of the native and optimized DNA sequences reveals the extent of modification to the original sequence (Supplementary Material: Table 2) while the final amino acid sequences have remained unchanged (Supplementary Material: Table 3).

Table 2
Summary of solubilization efficacy.

Refolding Buffer	Denaturant	Aggregated TFI-scFv (μg)	Soluble TFI-scFv (μg)	% Solubility
1	Range 1	439	61.3	12
2		490	71.0	14
3		374	126	25
4	Range 2	462	37.4	7.5
5		300	200	40
6		372	128	26
7	Range 3	198	302	60
8		261	239	48
9		219	280	56

Table 3
Analysis of prothrombin times.

Refolding Buffer	Average PT	Time extended	Final Concentration $mg.mL^{-1}$	Relative Inhibition Effectiveness (%)	Specific activity Time extended/ Concentration $s/mg.mL^{-1}$
Blank	20.6	0	0	0	0
1	23.1	2.4	0.245	14	9.79
2	24.8	4.1	0.282	24	14.5
3	23.7	3.1	0.503	18	6.16
4	22.3	1.6	0.150	10	10.6
5	25.1	4.5	0.8	26	5.63
6	35.8	15.2	0.512	100	29.6
7	21.2	0.6	0.121	4	4.96
8	24.8	4.1	0.956	24	4.28
9	20.9	0.3	0.112	2	2.68

3.2. *In vitrorefolding of TFI-scFv inclusion bodies*

3.2.1. *Cytoplasmic expression of codon-optimized TFI-scFv*

SDS-PAGE analysis revealed (Fig. 3) that a protein with a molecular weight of approximately 27 kDa was highly expressed (lane 2) which correlates with the theoretical molecular weight of TFI-scFv. TFI-scFv constitutes approximately 40% of the total cellular protein. Generally, expression ranged from 20 to 30% of total cellular protein. The majority of expressed TFI-scFv accumulated in the insoluble cytoplasmic fraction (Lane 4) while the soluble fraction (Lane 8) only contained minimal amount (< 10%) of the 27 kDa protein TFI-scFv.

SDS-PAGE analysis of codon-optimized TFI-scFv expression. The expected molecular weight of TFI-scFv is 27 kDa. Lane M, protein marker. Lane 1: Whole cell fraction of *E. coli* BL21 transformed with empty pET22b (+) plasmid (negative control). Lane 2: Whole cell fraction of *E. coli* BL21 transformed with pET22 TFI-scFv. Lane 3: Insoluble cytoplasmic fraction (negative control). Lane 4: Insoluble cytoplasmic fraction (pET22 TFI-scFv). Lane 5: Membrane fraction (negative control). Lane 6: Membrane fraction (pET22 TFI-scFv). Lane 7: Ultracentrifuge soluble fraction (negative control). Lane 8: Ultra centrifuge soluble fraction (pET22 TFI-scFv).

3.3. *Isolation and purifications of TFI-scFv*

The SDS-PAGE analysis (Fig. 4A) of the insoluble cytoplasmic fraction. TFI-scFv contributed to roughly 22% of the total cellular protein of the whole cell fraction (Fig. 4A lane 1) with. The washing and denaturation steps (Fig. 4A: Lanes 2–4) removed most inclusion body associated proteins. The low-quality migration observed in Fig. 4A lanes 5–7 is due to the high concentration of guanidinium chloride (6 M) required to denature the target protein. As the dilution ratio increased (6–30-fold dilution) the migration normalized. The final purified inclusion body fraction contained TFI-scFv with greater that 80% purity (Fig. 4A: Lane 7), which correlates to production of approximately 37 $mg\,L^{-1}$ of TFI-scFv. During IMAC purification of the solubilized inclusion bodies under denaturing conditions, a protein absorbance peak, corresponding to the 70–100 mL fractions, were pooled together (Fig. 4B) and was analyzed by means of SDS-PAGE. The denatured 27 kDa TFI-scFv was thus isolated with high purity (Fig. 4C: Lane 1). Densitometry analysis (Supplementary Material: Fig. 4) indicated that the denatured 27 kDa TFI-scFv was successfully isolated and purified with a purity greater than 95%. The denatured TFI-scFv yield was approximately 4.25 $mg\,L^{-1}$ TFI-scFv. Isolation and purification of denatured TFI-scFv is summarized in Table 1.

A) SDS-PAGE analysis of the insoluble cytoplasmic fraction. Lane M: Protein marker. Lane 1: Whole cell fraction. Lane 2: Disruption buffer wash I. Lane 3: Disruption buffer wash II. Lane 4: Chelating buffer wash. Lane. 5: Solubilized inclusion bodies (12-fold dilution). Lane 6: Solubilized inclusion bodies (6-fold dilution). Lane 7: Solubilized inclusion bodies (30-fold dilution). B) AKTÄ Purification Profile of TFI-scFv under denaturing conditions. C) SDS-PAGE analysis of the purified denatured TFI-scFv (70–100 mL pooled fraction). Lane M: Protein marker. Lane 1: Purified TFI-scFv.

Refolding reactions displayed varying levels of protein aggregations in all the samples (Supplementary Material: Fig. 5). Refolding buffer 4 produced the lowest level of solubilization (7.5%) while refolding buffer 7 produced the highest level of solubility (60%). Solubilization data is summarized in Table 2.

The redox ratio of 1 to 1 oxidized/reduced glutathione (1 mM) (sample 3, 5 and 7) produced the highest level of solubility of each of the three redox ratios examined across all three denaturant ranges. The lowest levels of solubilization was achieved with oxidized/reduced glutathione ratio of 1–10 in range 1 (Buffer 1), a ratio 1 to 5 in range 2 (Buffer 4) and a ratio of 1–10 in range 3 (Buffer 8).

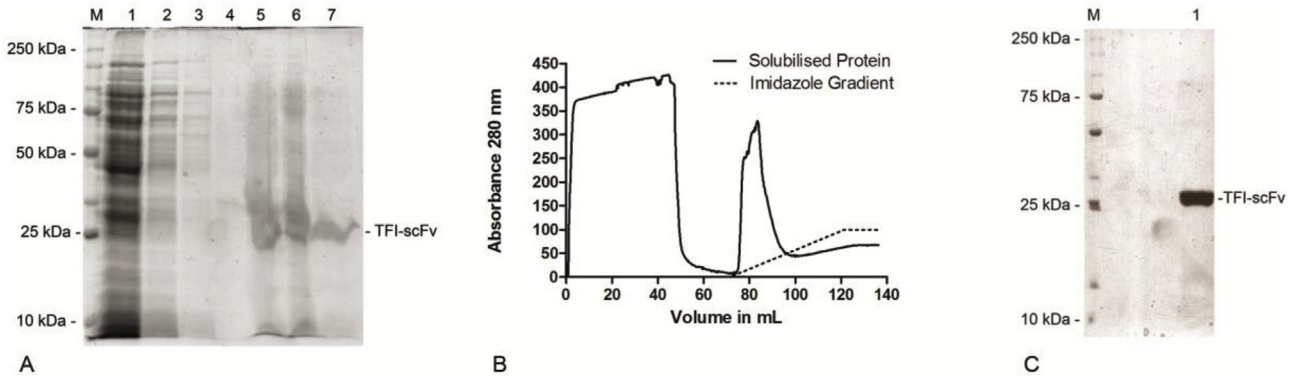

Fig. 4. Isolation and purification of scFv from insoluble cytoplasmic fraction.

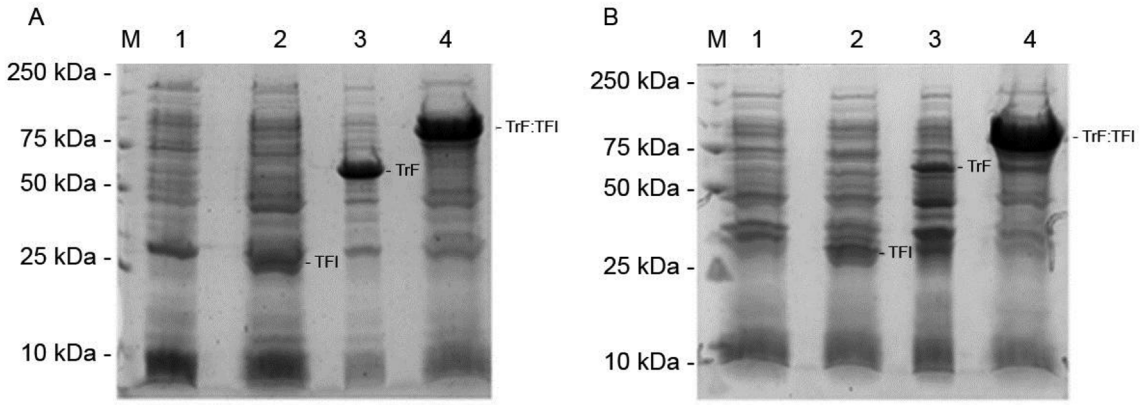

Fig. 5. Expression levels of TFI-scFv and TrF-TFI-scFv.

3.4. Prothrombin times

Diluted Prothombin Times (PTs) were performed in triplicate and the average PT was calculated (Table 3). The inhibition effect of each refolded TFI-scFv was calculated as the difference between the average PT and the blank (negative control). All refolded samples were capable of extending the baseline PT value (Negative control of 20,6 s). The highest inhibition effect was observed with sample 6 (15.2 s), which is an approximate 84% extension of the baseline PT value. As a result, inhibition effectiveness of the refolded TFI-scFv was expressed as a percentage value relative to sample 6. Refolding buffer 2, 5 and 8 were capable of relative inhibition of approximately 25% while refolding buffers 4, 7 and 9 had a relative effectiveness of less than 10% relative to refolding buffer 6. The specific activity was calculated as the time extended in seconds per $mg.mL^{-1}$ of TFI-scFv added to each reaction.

3.5. Summary of best in vitro refolding reaction

When considering the level of solubilization in conjunction specific activity of various refolding condition is clear that refolding buffer 6 (800 mM guanidium, 800 mM L-arginine, 2 mM glutathione (GSH), 0.2 mM glutathione disulfide (GSSG)) was the most effective in producing functional TFI-scFv. Although several of the refolding were more successful in producing soluble TFI-scFv, they did not display good inhibition. This suggest that despite being soluble, the TFI-scFv is misfolded and incapable of recognizing its target.

3.6. Cold shock expression of TFI-scFv

3.6.1. Expression TFI-scFv and TrF-TFI-scFv

The scFv antibody was successfully expressed by *E. coli* BL21 (DE3) using both pCOLD DNA II and pCOLD TrF vectors (Fig. 5A). Expression of the pCOLD DNA II TFI-scFv construct (lane 2) resulted in the production of TFI-scFv (27 kDa) which consisted of approximately 15% of the total protein. The over expression of modified fusion partner (Trigger factor 52 kDa) by pCOLD TrF (Empty Control) is clearly visible in lane 3. The Trigger factor constituted approximately 25% of the total protein. The 79 kDa TrF-TFI-scFv fusion construct (Trigger factor (52 kDa) + TFI-scFv (27 kDa)) is also clearly visible in lane 4. Similarly, the fusion construct constituted approximately 25% of total protein production. Both of the constructs were also successfully expressed by *E. coli SHuffle* (Fig. 5B). The 27 kDa antibody expressed from the pCOLD DNA II TFI-scFv vector was approximately 10% of total protein (lane 2). Interestingly, the production levels of the trigger factor fusion partner (lane 3) is slightly reduced to 15% of total protein in comparison to the 25% of total protein attained when expressed by *E. coli* BL21 in Fig. 5A lane 3. The TrF-TFI-scFv fusion construct (lane 4) constituted approximately 25% of total protein.

Whole cell fraction SDS-PAGE analyses of pCOLD DNA II and pCOLD TrF expression profiles in A) *E. coli* BL21 (DE3) and B) *E. coli SHuffle*. Lane M: Protein Marker, Lane 1: pCOLD DNA II (Empty Control), B) Lane 2: pCOLD DNA II TFI-scFv, Lane 3: pCOLD TrF (Empty Control) and lane 4: pCOLD TrF TF-scFv. The 27 kDa TFI-scFv is indicated as TFI (lane 2), the 52 kDa modified trigger factor is indicated as TrF (lane 3) and the 79 kDa fusion construct is indicated as TrF:TFI (Lane 4).

3.6.2. Solubilization levels of TFI-scFv and TrF:TFI-scFv

The expression of the pCOLD DNA II TFI-scFv vector by *E coli* BL21 (Fig. 6 A Lane 1 and 2) resulted in production of approximately 19% soluble TFI-scFv with the majority (> 80%) of the protein produced as insoluble inclusion bodies. The expression of the pCOLD TrF-TFI-scFv vector by *E. coli* BL21 resulted in the solubilization of approximately

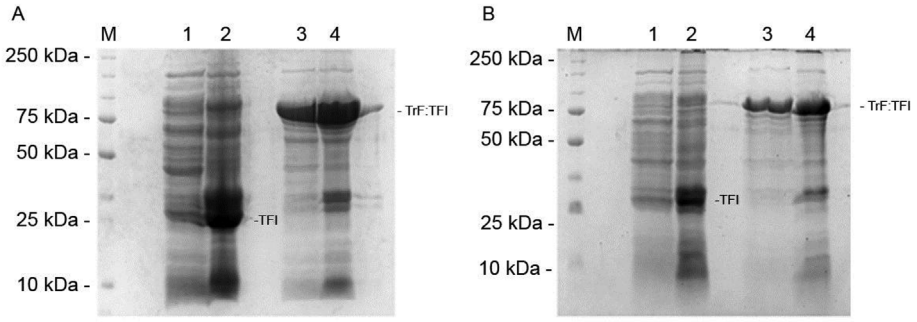

Fig. 6. Solubilization levels of TFI-scFv and TrF:TFI-scFv.

Table 4
Summary of large scale expression and protein yields.

m	Plasmids	Biomass wet weight (in g.L^{-1})	Fraction of total cellular protein (%)	Total purified TFI-scFv protein (mg.L^{-1})	Purity
E. coli BL 21 (DE3)	pCOLD DNA II	7.3	15%	2.13	> 80
	pCOLD TrF	8.2	25%	4.83	> 80
E. coli SHuffle	pCOLD DNA II	3.6	10%	1.32	> 95
	pCOLD TrF	4.2	25%	3.26	> 95

38% of the total TrF-TFI-scFv produced (Fig. 6 A Lane 3 and 4). Expression of the two vectors by *E. coli SHuffle* (Fig. 6 B) resulted in the solubilization of approximately 20% of the TFI-scFv produced by pCOLD DNA II (Lane 1 and 2) and 37% of the fusion constructs produced by pCOLD TrF TFI-scFv (lane 3 and 4).

SDS-PAGE analyses of pCOLD DNA II TFI-scFv and pCOLD TrF TFI-scFv expression profiles A) *E. coli* BL21 *(DE3)* and B) *E. coli SHuffle*. Lane M: Protein Marker, Lane 1: pCOLD DNA II TFI-scFV (Soluble Fraction), Lane 2: pCOLD DNA II TFI-scFv (Insoluble Fraction), Lane 3: pCOLD TrF TFI-scFV (Soluble Fraction) and Lane 4: pCOLD TF TF-scFv (Insoluble Fraction). The 27 kDa TFI-scFv is indicated as TFI and the 79 kDa fusion construct is indicated as TrF:TFI.

3.6.3. Cytoplasmic expression and purification

The use of the bioreactors resulted in a final yield of roughly 7 to 9 (wet weight) grams of *E. coli* BL21 per liter and 3–5 g of *E. coli SHuffle* per liter. The final protein production ranged from 2.0 mg L^{-1} to 5 mg L^{-1} for *E. coli* BL21 and 1.0 mg L^{-1} to 3.5. mg.L^{-1} for *E. coli SHuffle*. Overall the *E. coli* BL21 (DE3) strains are capable of producing much higher biomasses that the *E. coli SHuffle* strains when grown under cold shock conditions. The 27 kDa TFI-scFv was successfully isolated and purified form the cytoplasm of both *E. coli* strains (Supplementary Material: Fig. 6). The non-fusion construct was isolated with a purity greater than 95%, while the purity of the antibody purified from the fusion construct was higher than 80% for both strains utilized. Although the low purity is not ideal, it was deemed sufficiently pure for initial activity screening. However, as these fractions did not provide any notable inhibition effect on the actions of tissue factor, no additional attempts were made to improve the purity of the product. The final biomass production and protein purification details are summarized in Table 4.

3.6.4. Prothrombin times

Of the four different purified fractions that were tested only the non-fusion construct produced by *E. coli SHuffle* pCOLD DNA II was capable of notably extending the prothrombin times (Fig. 7). The prothrombin time at the highest concentration (0.2 mg ml^{-1}) of TFI-scFv was extended by 16.7 s which is an approximate 77% extension of the baseline prothrombin time with a specific activity of 83.8 s/mg.mL^{-1}. The remaining isolates of TFI-scFv fractions had very little inhibition effect, with all fractions only extending the prothrombin times less than 3 s.

The inhibition efficacy of the various isolated fraction of TFI-scFv was tested by means of prothrombin times. Only the non-fusion construct produced by *E. coli SHuffle* using pCOLD DNA II expression vector was capable of extending the prothrombin times in a dose dependent manner. The remaining isolates were incapable of notably extending the prothrombin times.

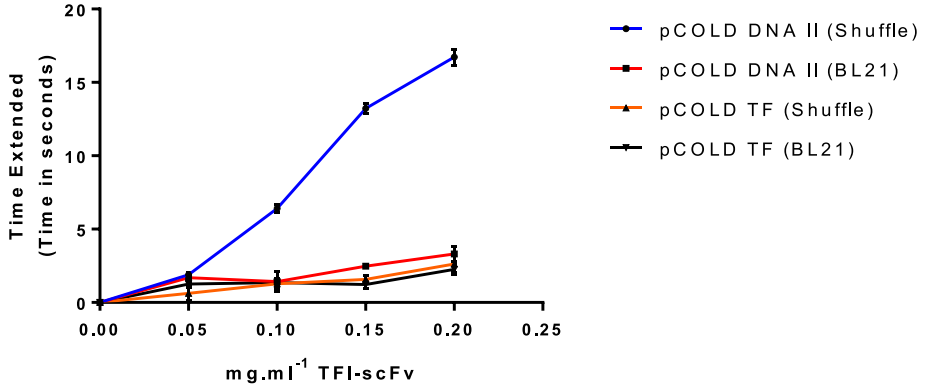

Fig. 7. Elongation of prothrombin times.

4. Discussion

It was suspected that due to the heterologous nature of the native scFv gene that the low levels of expression initially attained were due to codon bias. Codon usage has been identified as the single most important factor in prokaryotic gene expression [40]. The rare codon analysis of TFI-scFv revealed that 45 codons (approximately 20% in total) was distributed throughout the gene. Of these rare codons, 3 consecutive repeats and 2 single rare codons were identified near the N-terminal of the scFv gene a phenomenon which has been show to further exacerbated protein expression levels [40]. Expression of the codon optimized gene in *E. coli* BL21 (DE3) using pET 22 resulted in the improved expression of TFI-scFv with the antibody fragment representing approximately 22% of the total cellular protein at 40 mg L^{-1}, a 40-fold improvement on previously reported levels [3]. Although it is clear that the codon optimization improved TFI-scFv expression, the majority (> 70%) of TFI-scFv was accumulated as inclusion bodies in the insoluble cytoplasmic fraction. This result is indicative of protein aggregation due to misfolding [41], however, the formation of insoluble aggregate folding intermediate during high-level protein in the cytoplasm production is a common occurrence especially with scFv [42,43]. It is believed that the resulting high expression rates in combination with the presence of disulfide bridges, reducing cytoplasmic environment, insufficient chaperone assistance and the lack of post-translation modification contribute to protein aggregation observed during bacterial expression [8–10]. Initial attempts to alleviate the production of the inclusion bodies through expression at lower temperatures ranges, and the co-expression of chaperones in *E. coli* BL21 and Origami strains, only produced minor improvement in biomass and solubilization levels of TFI-scFv, nonetheless these techniques were unsuccessful in producing functional antibody (Unpublished data).

The generation of biologically active antibodies from insoluble aggregates has become common practice when working with scFvs [44]. The challenge is to convert the inactive and insoluble scFv aggregates into soluble biologically active products [45]. A dilution method was used for the screening of the various refolding conditions due to; low cost, availability of equipment, and relative simplicity, thus placing this method with in the capabilities of most research laboratories. The refolding matrix explores three stages of escalating concentrations of denaturant (guanidinium chloride) in combination with varying L-Arginine concentrations while maintaining the pH at 8.0. Although there is a clear increase in solubility with an increase in denaturant, it is important to note that solubilization does not necessarily translate into functionality. The addition of arginine in refolding buffers in order to suppress protein aggregation is a well-established practice in protein refolding. Is has been proposed that the guanidinium group of arginine as well as the side chains of tryptophan may be responsible for suppression of protein aggregation by arginine [46]. The addition of arginine does not, however, suppress aggregation in some disulfide-containing proteins [47]. Due to this unpredictable effect of arginine on disulfide bond formation, the effect of arginine was examined across a large concentration range (0–800 mM). In general, the refolding buffers containing the lower levels the guanidinium chloride (range 1) had the higher levels of aggregation. Interestingly, the highest level of solubilization was observed in sample 7, which contains no L-arginine, while in contrast samples 1 and 4 (also L-arginine free) produced the lowest levels of solubilization for their respective ranges. The specific activity analysis provides a good indication of which buffer produced the most functional TFI-scFv capable of recognizing the tissue factor epitope, oppose to producing soluble but non-functional TFI-scFv. Refolding Buffer 6 (29.6 s/mg.mL^{-1}) and refolding buffer 2 (14.5 s/mg mL^{-1}) had the highest specific activity. Interestingly, refolding buffer 5, 7, 8 and 9 which all produced a higher level of solubility than refolding buffer 6, had a considerable lower specific activity. This indicates that although these refolding buffers were more effective at producing soluble TFI-scFv, the antibodies were folded in incorrect intermediates that were not capable of recognizing the tissue factor epitope.

Thus, as expected, the redox potential had a significant effect on the production of functional antibody due to the presence of intra-chain disulfide bonds, although this did not correlate with solubility [11,12,48]. Disulfide bond formation plays an important role in the stabilization of folding intermediates as the protein advances to maturity [18,49,50]. At this refolding rate one could extrapolate that approximately 6 mg of active TFI-scFv can be produced during one round of expression. However, it must be mentioned that several attempts to scale up these results using an on-column refolding approach with identical experimental conditions, failed to produce functional TFI-scFv (Unpublished data). It is also clear form the purification table (Table 2) that large fraction of the inclusion bodies is lost during the isolation process. Most of the isolated TFI-scFv was lost during IMAC purification as the columns do perform optimally under denaturing and reducing conditions. This only demonstrates the complexity and sensitivity of *in vitro* refolding procedures and emphasizes need for target specific optimized procedures in order to balance target protein quality against quantity.

Due to the drawbacks of *in vitro* refolding, the feasibility of producing a functional TFI-scFv in the cytoplasm of *E. coli* was explored as an alternative approach. Solubilization of the scFv was achieved though commercial cold shock expression vectors (pCOLD DNA II and pCOLD Trigger factor) in conjunction with *E. coli* BL21 (DE3) and specialized disulfide bond promoting strain (E. *coli SHuffle)*. When comparing the expression of TFI-scFv using the pCOLD DNA II cold shock vector by *E. coli* BL21 (DE3)- and *SHuffle* strains, it is interesting to note that the expression level by *E. coli* BL21 was only marginally higher (5%), while the level of solubilization between the two strains remained similar at 20% of the target protein. This was unexpected, as the mutations to *E. coli SHuffle* to reduce aggregation did not yield a higher level of solubilization than that of *E. coli* BL21. The similarities in protein solubilization when comparing the two cell lines might be attributed to the comprehensive effect of cold shock itself. Protein expression at low temperature results in improved solubilization of the target protein likely due to the reduction in translation rate of mRNA to the polypeptides [41,51,52]. By comparison, the expression levels of the TrF-TFI-scFv fusion constructs using pCOLD TF were roughly 10% higher. This increase in protein expression is largely due to the high expression levels of the fusion partner itself. Trigger factor constitutes 15–25% of total protein yield in the respective controls. The TrF-TFI-scFv fusion constructs were expressed at similar levels (25% of the total protein). The solubilization levels were also higher than that of the pCOLD DNA II constructs. A total of 37–38% of the expressed fusion construct was produced as soluble TFI-scFv. However, of the four isolated fractions, only the TFI-scFv produced via the expression of pCOLD DNA II plasmid in *E. coli SHuffle,* was capable of tissue factor inhibition as demonstrated by the PT assay.

Functional antibody fragments were produced through a combination of cold shock, pCOLD DNA II expression vector in conjunction with *E. coli SHuffle*. The *SHuffle* strain has deletions of the glutathione reductase and thioredoxin reductase (Δ gor and Δ trxB) genes, which allows disulfide bonds to form in the cytoplasm. In addition, *SHuffle* expresses a version of the periplasmic disulfide bond isomerase DsbC which lacks a signal sequence, retaining it within the cytoplasm in order to facilitate disulfide bond formation. These specific mutations to *E. coli SHuffle* seemed to be the key to producing functional TFI-scFv in the bacterial cytoplasm. However, a major drawback to cold shock expression systems is the reduction in target protein yield as well as final biomass yields, caused by to the lower growth- and protein synthesis rates [53]. This issue becomes further compounded by the slower growth rate of *E. coli SHuffle.*

When comparing the expression strategies, the *E. coli* BL21 (DE3) strains yielded roughly double the biomass compared to *E. coli SHuffle*

when grown in the 5-L bioreactors. However, the TFI-scFv produced by cold shock had a specific activity of 83.8 s/mg.ml^{-1} opposed to 29.6 s/mg.mL^{-}1 of the refolded TFI-scFv. This difference suggests that although functional scFv was produced by *in vitro* refolding, much of the refolded fraction was soluble but inactive. We could speculate that is may be due to due incorrect refolding or may be due to partial degradation (Supplementary Material: Fig. 5). One of the major challenges of working with a novel antibody is the fact that there is no commercially available counterpart to serve as reference point. It is complications such as these that makes working with novel heterologous proteins a complex and challenging task. Overall, the cold shock expression systems provide a simpler, less time consuming, and more cost-effective approach that is simpler to scale up in when compared with *the in vitro* refolding strategy.

5. Conclusion

The challenge in recombinant protein expression is to find the balance between quantity and quality of target protein while considering practicality and the cost involved. The heterologous production of scFv in the cytoplasm of *E. coli* is notoriously difficult with various factors contributing to reduced expression and improper folding. Although various strategies exist to overcome these difficulties, they are generally protein specific and usually do not translate to other cases of scFv expression. In this study, codon optimization appears to be the most effective strategy to increase expression levels but contributed to folding complication. Subsequently, several general optimization strategies were explored to improve the production of soluble TFI-scFv but were unsuccessful. This study indicates that, at least in small scale experiments, *in vitro* refolding is feasible however not optimal. At larger scales, specialized expression hosts and vectors are required to obtain functional antibody. When comparing these two strategies the use of cold shock in conjunction with of *E. coli SHuffle* provided a simpler and more cost-effective method for the production of function TFI-scFv appose to the more complex *in vitro* refolding strategies. This strategy can be followed when expression other problematic low yielding scFv.

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Conflicts of interest

None.

Author contributions

Dr. Jan-G Vermeulen: Principle researcher and main author.

Dr. Errol Cason: Experimental design and manuscript editing.

Prof SM Meiring: Study leader, - Experimental design and manuscript editing.

Prof. FJ Burt: Co Study Leader - Manuscript editing and infrastructure support.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pep.2018.06.005.

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Research Article

The Use of Phage Display and Yeast Based Expression System for the Development of a Von Willebrand Factor Propeptide Assay: Development of a Von Willebrand Factor Propeptide Assay

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Background. The diagnosis of von Willebrand disease is complex due to the heterogeneity of the disease. About eighty percent of von Willebrand disease patients are diagnosed with a quantitative defect of von Willebrand factor (VWF) where fifty percent is due to an increased clearance of von Willebrand factor. These patients do not respond well to the treatment of choice, Desmopressin (DDAVP) due to decreased efficacy. The ratio between the VWF propeptide and the mature VWF antigen is used to diagnose these patients. Commercial VWF propeptide assays are too expensive for use in developing countries. In this study, we developed a cost-effective ELISA assay. *Methods*. We first displayed VWF propeptide on yeast. Antibody fragments were selected against the displayed VWF propeptide by using phage display technology. The antibodies were used to develop a cost-effective VWF propeptide assay and compared to a commercial VWF propeptide assay. *Results*. Two of these antibody fragments bound specific to the VWF propeptide and not to the yeast used for the expression of the propeptides. These purified antibody fragments were able to detect VWF propeptide in normal plasma. *Conclusion*. Our assay performed well when compared to a commercial kit. It also showed a higher binding affinity for VWF propeptide in plasma at especially lower plasma concentrations.

1. Background

Von Willebrand disease (VWD) is the most common bleeding disorder in the world with a prevalence of one percent in the general population [1]. It is classified into three types. Types 1 and 3 are quantitative defects of von Willebrand factor (VWF) and Type 2 is a qualitative defect VWF [1]. The diagnosis of VWD is complex due to the heterogeneity of the disease. Various mutations of the VWF gene result in a variety of phenotypes that makes the diagnoses of these patients very challenging [1]. It is therefore not surprising that patients with VWD are largely under-diagnosed or misdiagnosed. About 80% of VWD patients are diagnosed with type 1 VWD and about fifty percent of these patients present with an increased clearance rate of VWF [1]. Patients with increased clearance of VWF need to receive different treatment strategies and the correct diagnosis is crucial, since DDAVP, the treatment of choice for type 1 VWD patients, would not be effective at all in these patients. An increased ratio between von the Willebrand factor propeptide (VWFpp) and the mature VWF antigen can be used to diagnose these patients. This can be interpreted as a shortened half-life of plasma VWF and therefore increased clearance of VWF [1].

Furthermore, the level of VWFpp in the circulation can also be used as a marker of VWF synthesis. Studies showed that VWFpp levels are more reliable than mature VWF levels in the assessment of endothelial cell activation and endothelial damage, since the plasma VWFpp concentration is not influenced by blood groups or factors such as adhesive properties and catabolism [2]. Increased plasma VWFpp

concentrations have been described in vasculopathies such as hypertension and diabetes, systemic sclerosis, myocardial infarction, and cardiovascular mortality [2, 3].

The current commercially available assays that are used to test the levels of VWFpp in plasma are expensive and the price that medical aids pay does not cover the costs of the tests. With this research, we developed a cost-effective VWFpp diagnostic laboratory assay using antibodies produced by phage display technology. Phage display is an *in vitro* method that allows rapid production of antibodies during cycles of biopanning and propagation without the use of animal models [4–6].

2. Methods

2.1. Display of VWF Propeptide on Yeast. Since a commercial preparation of the VWFpp is not available, the VWFpp (P04275, amino acids 23-763 of VWF) were displayed on yeast according to the method described by Lin-Cereghino et al. (2005) [7]. In short, the VWF propeptide-encoding sequence was transferred from the pMK-RQ plasmid to pINA1317-CWP110 using the *Sfi*I and *Hind*III restriction sites. Recombinant expression vectors were used to transform the *Y. lipolytica* strain P01h. All plasmids and related reagents were purchased from TaKaRa, Japan. A negative control strain was constructed by transformation of *Y. lipolytica* strain P01h with the original pINA1317-CWP110 vector. Transformants were randomly selected from yeast nitrogen base selective plates [yeast nitrogen base without ammonium sulphate and amino acids: 0.17%, ammonium chloride: 0.4%, glucose: 1%, casamino acids: 0.2%, agar: 2%, and pH: 6.5]. Chromosomal integration in transformants was confirmed by PCR using genomic DNA as template. *Y. lipolytica* transformants were cultivated in yeast extract peptone dextrose broth [yeast extract: 1%, peptone: 2%, and glucose: 2%] on a rotary shaker at 30°C. After 48h cultivation, cells were harvested by centrifugation at 5000 *g* for 5 min. Cell pellets were washed with phosphate buffered saline (PBS) and resuspended to a final concentration of 50 g/L wet cell weight prior to assay. A control yeast with no VWFpp displayed on it was also prepared.

To detect the VWFpp on the yeast cells, VWFpp displaying yeast cells and control yeast cells were coated onto a 96-well plate (Nunc, Thermo Scientific, USA) at decreasing concentrations ranging from 27.5 g/L to 0 g/L (only PBS) wet cell weight and fixed onto the plate by adding ice cold methanol (100%) to each well and dried again overnight at 37°C. After blocking with PBS/0.1% Tween-20 with 1% Bovine Serum Albumin for 2hrs at 37°C, the plate was washed three times with PBS/0.1% Tween-20. The bound VWFpp was detected with a commercial anti-VWFpp horseradish peroxidase-conjugated antibody (CLB-Pro 14.3, 1:1000 dilution in PBS/Tween/BSA, Biocom Africa, SA) at 37°C for 2hrs. The reaction was developed with *o*-phenylenediamine dihydrochloride (OPD) substrate (10ml 0.1M Citric acid, 10 ml 0.1M Na_2HPO_4, 200 μl OPD (1mg/ml), and 8μl H_2O_2) and stopped with 30μl of 4M sulphuric acid after 15min. Absorbance was measured at 450 – 650nm using the SynergyHT ELISA reader (Biotek, USA).

2.2. Selection of Antibody Fragments. Two phage display libraries (Tomlinson I & J) from the Medical Research Council Centre for Protein Engineering, Cambridge in London, UK, were amplified. The I library consists of diversified side chains on 18 residues while the J library has NNK side chains on these residues (N=GACT and K=GT). Antibody selection was done as previously described [8]. In short, Nunc-Immuno™ tubes (Thermo Scientific, USA) were coated with VWFpp displaying yeast cells (110 g/l in PBS) and rotated overnight at 4°C. After washing (3 times) and blocking with 2% skimmed milk powder in PBS at room temperature for 2 hours, 10^{12} phages of each library (I and J) were added to the tubes and rotated at room temperature 2 hours. After 10 times washing, the unbound phages were discarded and the bound phages were eluted with 500 μl trypsin-PBS (10mg/ml trypsin, 50mM Tris-HCl, 1mM $CaCl_2$ in PBS, and pH7.4) for 10min at room temperature. TG1 *E-coli* cells (1.75ml) were infected with the eluted phages for 30 minutes at 37°C in a water bath until OD_{600} reach 0.4-0.6. The infected TG1 *E-coli* cells were then centrifuged at 11600g in a microcentrifuge for 5min; the pellet suspended in 50μl 2TY medium (16g bacto-tryptone, 10g yeast extract, and 5g NaCl in 1 liter distilled water) and grown on trypsin-yeast plates (15g agar, 10g bacto-tryptone, 8g NaCl, and 5g yeast extract in 1 liter distilled water) containing 100 μg/ml ampicillin and 1% glucose overnight at 37°C. Cells were loosened from the plates with 2ml 2TY medium containing 15% glycerol and a small volume (50μl) was grown in 50ml 2TY containing 100 μg/ml ampicillin and 1% glucose at 37°C until OD_{600} of 0.4-0.6 was reached. The culture was then infected with 5×10^{10} helper phages, incubated at 37°C in a water bath for 30 min, centrifuged at 3000g for 10min and resuspended in 50ml 2TY medium containing 100 μg/ml ampicillin, 50μg/ml kanamycin, and 0.1% glucose, and amplified overnight shaking at 30°C. After centrifugation at 3300g for 15min, poly-ethylene glycol was added to 40ml supernatant and mixed well and the phages were precipitated on ice for 2hrs. The phage mixture was centrifuged again at 3300g for 30min at room temperature, resuspended in PBS, and centrifuged shortly to remove access bacterial cells. The precipitated phages were used for the next round of selection. Three selection rounds were performed.

Ninety-six single colonies from the third selection round of the I and J libraries, respectively, were amplified in 96-well microculture plates. The individual colonies were grown overnight shaking (250 rpm) at 37°C in 100μl 2TY media containing 100 μg/ml ampicillin and 1% glucose. Two μl of the above cultures was inoculated into each well of the plates containing 200 μl 2TY with 100 μg/ml ampicillin and 1% glucose and grown shaking at 37°C until an OD_{600} of 0.4-0.6 was reached. After infection with 1×10^9 helper phages for 30 min, the cultures were pelleted and resuspended in 2TY medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin and grown overnight at 30°C. The cultures were then centrifuged at 1,800g for 10 min and the supernatant from each of the single colonies was tested for binding to the VWFpp.

Two ELISA plates (one of each library) were coated with the VWFpp displaying yeast and another two plates with

the control yeast. Fifty μl of the supernatant of each of the different monoclonal phage colonies was added to both plates and incubated for 2 hours at 37°C. The plates were then washed and 100μl of a 1:5000 dilution of HRP-anti-M13 antibody (Amersham, South Africa) in PBS/2% skimmed milk was added and incubated for 1 hour at 37°C. After washing again, the reaction was developed as mentioned in previous section. The six colonies with the highest binding affinity ($OD_{490-630nm}$ > 0.5) were upscaled and tested for concentration depended binding to VWFpp. The following phage concentrations were used: 5×10^{10} phages/ml, 2.5×10^{10}, 1.25×10^{10}, 6.25×10^{9}, 3.125×10^{9} and a blank, etc. The two colonies with the strongest and most specific binding to the VWFpp on the displaying yeast were used to produced soluble antibody fragments.

2.3. Production of Soluble Antibody Fragments. Phages from each of the two strongest binders were used to infect exponentially growing HB2151 bacteria (OD_{600} of 0.4-0.6). In order to obtain soluble antibody fragments, isopropyl-β-D-thiogalactoside (IPTG) (Thermo Scientific, USA) was added to a final concentration of 1mM to the cultures and grown overnight at 30°C. The overnight cultures with IPTG were centrifuged at 1,800 g for 10min and the scFv in the supernatant was concentrated with the minimate™ TFF filtration system (Thermo Scientific, USA).

2.4. Protein Purification. A Protein A IgG purification kit (Thermo Scientific, USA) was used according to the instructions of the manufacturers. Elution fractions with the highest absorbance values were pooled together and dialysed in PBS overnight using a Slide-A-Lyzer Dialysis cassette (Thermo Scientific, USA) with 10 000kD cut-off and stored at 4°C for further use. The two purified antibody fragments were then characterised in a VWFpp assay and compared to commercial anti-VWFpp antibodies.

2.5. Assay Development. A sandwich ELISA was performed to show that the antibody fragments are able to identify the VWFpp in normal human plasma (WHO 6th International standard for FVIII and VWF in plasma). One antibody fragment (A9) was used to coat the plate and the other one (G7) was conjugated with HRP (EZ-Link activated peroxidase antibody labelling kit, Thermo Fisher Scientific, USA) and used as the detection antibody. We also used our scFv as coating antibodies with commercially available antibody as the detection antibody and *vice versa*. Furthermore, the commercially available antibodies were also used alone for comparison. The outline of these assays was as follows:

(1) Coating antibody: CLB-Pro 35, Detection antibody: CLB-Pro 14.3

(2) Coating antibody: CLB-Pro 35, Detection antibody: A9

(3) Coating antibody: A9, Detection antibody: CLB-Pro 14.3

(4) Coating antibody: G7, Detection antibody: CLB-Pro 14.3

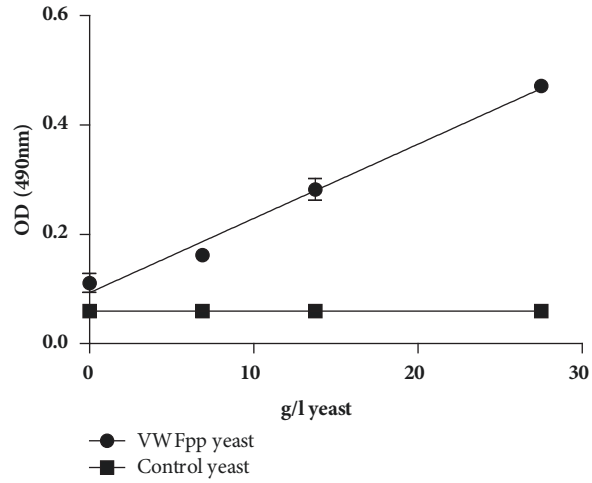

Figure 1: Existence of the displayed VWFpp. The VWFpp displayed yeast is indicated by dots and the control yeast indicated by squares. The binding is expressed as average ± 1 standard deviation (SD) where $n = 2$.

(5) Coating antibody: A9, Detection antibody: G7.

Protein A-HRP was used as previously described for detection in the second scenario. The assay conditions were again similar to that of the polyclonal ELISA. This assay was repeated twice. As validation of our assay, we compared the standard curves of using both commercial antibodies and of using our two scFv. The sensitivity and interrun accuracy of the assay were also determined from the standard curve.

3. Results

The displayed propeptide onto the yeast is confirmed in Figure 1. The displayed propeptide binds to the commercial antibody (CBL-Pro 35) that is directed against the VWFpp. The control yeast however did not bind to the anti-VWFpp antibody.

From the third selection round, three colonies from the I library and 6 from the J library were identified as strong binders to the VWF displayed yeast and not the control yeast, but only two of them bound specific to the VWFpp yeast and not the control yeast. The results of the concentration dependent ELISA are shown in Figure 2. The two monoclonal phages, JA9 and JG7, showed the most specific affinity for VWFpp.

Soluble antibody fragments grown from these two colonies bound concentration dependently to the VWFpp displayed on the yeast. Neither of them bound to the yeast itself.

These two colonies were then purified on a protein A columns. Both proteins appeared in the first 2 fractions during the elution process. The protein concentrations of these antibody fragments were calculated as 185 μg/ml for JA9 and 191 μg/ml for JG7 after dialysis with PBS.

Our VWFpp assay where standard plasma (6th WHO VWF: FVIII standard, NIBSC, UK) was used and compared

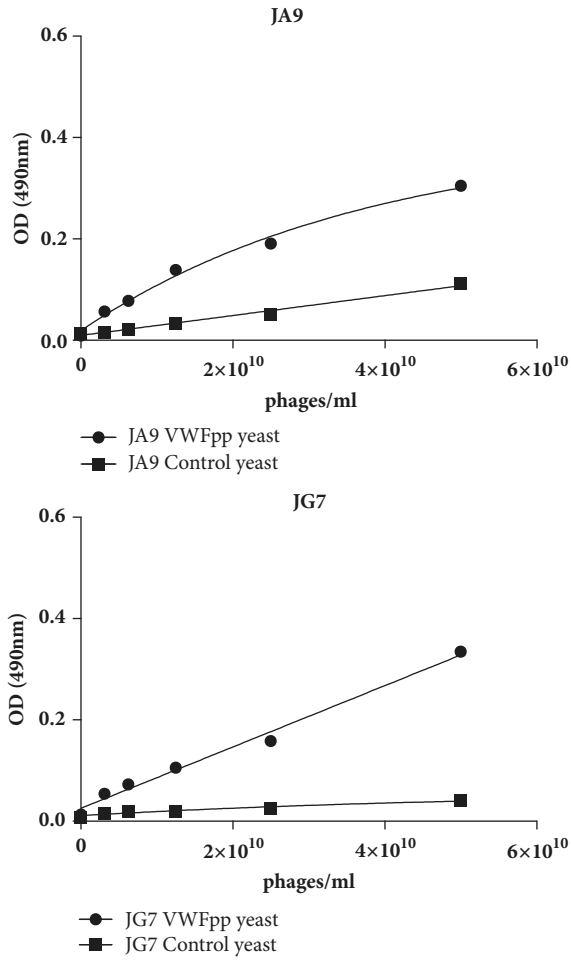

Figure 2: Concentration dependent binding of single colonies that showed specific binding affinity for VWFpp displayed on yeast. Phage binding to the VWFpp yeast is indicated by dots and to the control yeast by squares. The binding is expressed as average ± 1 standard deviation (SD) where $n = 2$.

to a commercial kit with antibodies (CLB-Pro 35 & CLB-Pro 14.3, Biocom Africa, SA). Figure 4 showed that our assay using both our antibody fragments compared well to that of the assay using commercial antibody fragments. Our assay detects VWFpp concentrations form 1.5625%, while the commercial assay only detects VWFpp from 6.25%. Furthermore, a total %CV of 16% between our duplicate runs were found. Thus this standard curve serves as proof that our two antibody fragments are suitable to be used in a VWFpp assay.

The differences in the protein sequences of our JG7 and JA9 antibody fragments are shown in Box 1. The light and heavy chains of the two antibody fragments do differ and thus are suitable to be used in a sandwich ELISA assay, since it is unlikely that they might bind to the same binding site on the VWFpp.

The cost of producing 1 mg of a monoclonal antibody amounts to approximately 45 US Dollar (4500 USD for 100 mg). However, it will be much more cost-effective to produce single chain variable fragments in large scale using metal-affinity columns (about 3000 USD for 100 mg) [9]. The cost of our assay amounts to approximately 60% of the cost of a commercial assay kit. This will allow our medical aids to support funding for patients with increased VWF clearance who needs the outcome assay for diagnostic purposes.

4. Discussion

The VWFpp assay has recently been included in the diagnostic setup of von Willebrand disease (VWD) [10]. The ratio of the propeptide to antigen (VWFpp/VWF:Ag) is used to determine the clearance rate of VWF from the circulation [11]. The VWFpp assay also measures endothelial cell activation during endothelial damage [12, 13].

Since the current commercially available assays that measure the levels of VWFpp in plasma are so expensive, we developed a cost-effective VWFpp diagnostic laboratory assay using antibodies produced by phage display technology. Phage display technology was used because it is a molecular diversity technology that allows the presentation of large amounts of various proteins on the surfaces of filamentous phages. It also allows rapid production of antibodies during cycles of biopanning and propagation without the use of animal models. Phage display libraries thus permit the selection of peptides and proteins, including antibodies, with high affinity and specificity for almost any target [14]. The use of phage display also allows the user to manipulate the protocol according to the requirements of the target antigen. As long as the target is immobilised to a support and the exposed solutions containing phage are immobilised to the target, the changes of the experiment succeeding are almost a hundred percent [6]. We used two single chain variable antibody fragment libraries (Tomlinson I and J libraries) from which we selected the VWFpp binding antibody fragments [15].

The advantage of using small antibody fragments is that they easily penetrate the cellular or tissue membranes without compromising their affinity and specificity. Antibodies fragments are easier and faster to produce. They can be easily purified with commonly used purification systems such as protein A [16, 17].

The VWFpp is not commercially available and therefore we needed to express the recombinant protein. *E.coli* protein expression systems are widely used but lack proper protein folding and posttranslational modifications [17]. Mammalian cells produce very low yields and are also very costly [18]. We made use of a yeast display system to display the VWFpp antigen (amino acids 23-763 of VWF). Yeast display is more likely to produce soluble functional proteins with the appropriate posttranslational modifications [19]. Furthermore, proteins displayed on the surface of the yeast can be purified easily as an active yeast particle by centrifugation. A yeast cell is also capable of displaying up to 50–2000 copies of the antigen on its surface [20].

The combination of phage display and yeast expression systems in protein expression provides the opportunity to produce antibodies without the need of soluble target protein [21]. The reason why we did not use yeast display for the selection process is because the library sizes are smaller than those of other display systems. The existence of the displayed VWFpp on the yeast was confirmed (Figure 1).

JG7
PIT2 vector: DSGNSYDHDYAKLACKFYFKETVIMKYLLPTAAAGLLLLAAQPAMAEV
Lightchain:QLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSSIVETGPETSYADSVKGRFTIS
RDNSKNTLYLQMNSLRAEDTAVYYCAKSPQL
Linker sequence:FDYWGQGTLVTVSSGGGGSGGGGSGGGGSTDIQMTQ
Heavychain:SPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYRASSLQSGVPSRFSGSGSGTDFTLTIS
SLQPEDFATYYCQQGHPTPGTFGQGTKVEIKR
PIT2 vector:AAAHHHHHHGAAEQKLISEEDLNGAAEITS
JA9
PIT2 vector:DSGNSYDHDYAKLACKFYFKETVIMKYLLPTAAAGLLLLAAQPAMAEV
Lightchain:QLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSSIYEEGVLTMYADSVKGRFTIS
RDNSKNTLYLQMNSLRAEDTAVYYCAKRSVIR
Linker: FDYWGQGTLVTVSSGGGGSGGGGSGGGGSTDIQMTQ
Heavychain:SPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASCKVGSPSRFSGSGSGTDFTLTISS
LQPEDFATYYCQQENCEYTFGQGTKVEIKR
PIT2 sequence: AAAHHHHHHGAAEQKLISEEDLNGAXEITS

Box 1: **Protein sequence of antibody fragments JA9 and JG7**. The differences between the two antibody fragments are underlined.

We used the Tomlinson I and J Phage display single chain variable fragment libraries since they have a diversity of over 100 million different antibody fragments (Tomlinson I & J protocol). Three selection rounds are mostly used in literature [22] so we also performed 3 rounds of selection. Ninety-six colonies of each library's selection were chosen from the third selection round. Only two colonies (JA9 and JG7) showed specific binding to the VWFpp and not to the yeast (see Figure 2).

Some studies showed that phage colonies might lose their specificity after conversion into soluble antibody fragments [16, 22]. This however did not happen in our study. The antibody fragments of both colonies (JA9 and JG7) still bound concentration dependently to the displayed VWFpp and not to the control yeast (see Figure 3).

The availability of purification tags that can be used to purify antibodies generated with phage display makes the purification process easy and reliable [21]. The soluble scFv antibodies JA9 and JG7 were purified on protein A column and successfully eluted with 3-5ml elution buffer and aliquots pooled into single tube.

The amount of purified antibodies was just enough to perform a VWFpp assay where standard human plasma was used. The WHO 6th International standard for FVIII and VWF in plasma was used, since it is the only standard with a given true value for the VWFpp. Although there is not a gold standard assay available to compare our assay with, we compared the standard curve of the VWFpp assay using our 2 antibody fragments to the standard curve of the VWFpp assay using two commercial antibodies to the VWFpp. Our assay shows higher binding affinity at especially lower plasma levels and might thus be more specific than the commercial one. Due to funding constraints and a lack of upscaling facilities, the amount of purified and concentrated antibody fragments was not enough to test patient samples and to do a full validation. However, the plasma that we used to set up a standard curve is of human origin and was pooled from between 20 and 40 human plasmas with an assigned value specific for the VWFpp.

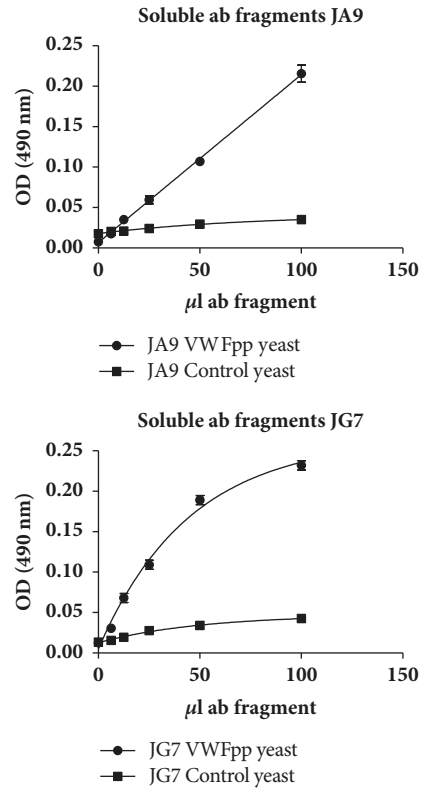

Figure 3: Concentration dependent binding curves of soluble single chain variable antibody fragments that bind specific to the VWFpp on the yeast. Binding to the VWFpp yeast is indicated by dots and to the control yeast by squares. The binding is expressed as average ± 1 standard deviation (SD) where $n = 2$.

The sensitivity of our assay was determined at 1.5625% VWF:pp in plasma, which is better than those of the commercial assay of 6.25%. The robustness of the assay was not determined in this study; however, it is known that the full-length VWF levels are influenced by cold storage at 4°C, but

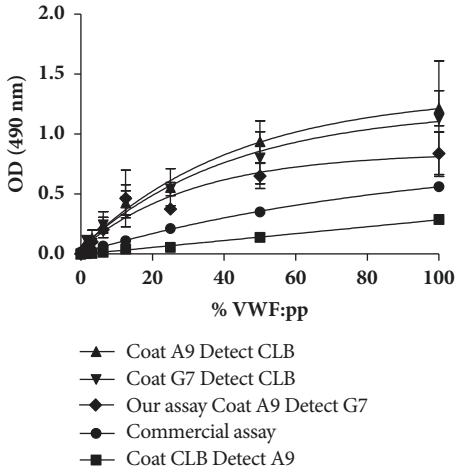

Figure 4: VWFpp ELISA assays with our 2 purified antibody fragments, commercially available VWFpp antibodies and combinations of both. Our assay (diamonds), commercial assay (dots), commercial coating antibody (ab) and JA9 detection ab (squares), JG7 coating ab and commercial detection ab (triangle upside-down) and JA9 coating ab, and commercial detection ab (triangle upright). The data are expressed as average ± 1 standard deviation (SD) where $n = 2$.

not by freezing at -80°C [23]. In this study, all plasma samples were stored at -80°C.

Specificity was however not determined in the final assay. We however showed that the soluble antibody fragments bind to the propeptide yeast and not to the control yeast; that indicated the specificity of the antibody fragments (Figure 3).

Commercial assays, however, show a high total %CV of 25% for the between-run accuracy [24]. We found a total %CV of 16% between our duplicate runs.

Since the amino acid sequence of the two antibody fragments differs significantly (Box 1), it might be used in a sandwich ELISA. The sequences were also compared to other scFv sequences in a database and more than 90% homology was found. It is known that all scFv from the I and J libraries are more than 90% homologous. No exact sequences to ours were found.

In conclusion, our antibody fragments can be used in a sandwich ELISA to determine the VWFpp levels in plasma. A follow-up study is however needed to fully validate the assay.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Performance and utility of a cost-effective collagen-binding assay for the laboratory diagnosis of Von Willebrand disease

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Abstract

Background: The collagen-binding assay, a functional assay of Von Willebrand factor (VWF), discriminates the subtypes of Von Willebrand disease (VWD). Commercial collagen binding assays have the advantage of immediate use for fast results, but are expensive.
Methods: In this study we evaluated an in-house collagen-binding assay using type III collagen. We included it in the diagnostic work-up of 44 patients with VWD and 40 normal subjects. Other assays included VWF antigen, ristocetin cofactor activity, ristocetin-induced platelet agglutination and VWF multimeric analysis.
Results: The cost of this collagen-binding assay is 10-fold lower than that of commercial kits. The intra- and inter-assay coefficients of variation were <8% and <9% for normal values, respectively, and the normal reference range varies between 51% and 143%. This assay is sensitive to large VWF multimer representation, with a mean collagen-binding activity/antigen level (CB/Ag) ratio of 0.18 and 0.45 for type 2A and type 2B VWD, respectively, indicating functional discordance. It correlates with the antigen levels in type 2M and type 1 VWD, with mean CB/Ag ratios of 1.1 and 1, respectively.
Conclusions: Our cost-effective in-house collagen-binding assay produced reliable results. We recommend the use of this test together with the ristocetin cofactor test in the diagnostic work-up of VWD.
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Keywords: collagen-binding assay; Von Willebrand disease; Von Willebrand disorder; Von Willebrand factor.

Introduction

Von Willebrand disease (VWD) is a commonly inherited bleeding disorder caused by either a qualitative or quantitative defect of Von Willebrand factor (VWF) (1). VWF, an adhesive plasma protein required for effective primary haemostasis, has a multimeric structure with binding sites for platelets, collagen, factor VIII and heparin (2). In the current classification scheme, VWD is subdivided into three major types based on a quantitative (types 1 and 3) and a qualitative (type 2) defect in VWF (3). Type 1 is associated with low VWF levels. Type 2 VWD is subdivided into four subtypes (2A, 2B, 2M and 2N). According to the subdivision scheme, type 2A is characterised by reduced platelet-dependent function resulting from the absence of high-molecular-weight multimers of VWF, while type 2B is characterised by the increased ability of VWF to bind glycoprotein 1b receptor on platelets. A decreased platelet-dependent function that is not caused by the absence of high-molecular-weight multimers characterises type 2M VWD, while type 2N is defined by decreased binding of VWF to factor VIII. Type 3 manifests the most severe symptoms of VWD because of a lack of VWF in such patients (3).

Laboratory diagnosis of VWD is based on measurement of a battery of tests including the plasma VWF (VWF:Ag) assay; the ability of VWF to interact with platelet receptors, namely, the ristocetin cofactor assay (VWF:RCo); the collagen-binding assay (VWF:CB); the ristocetin-induced platelet agglutination (RIPA) test; analysis of the multimeric structure of VWF; and the factor VIII binding assay of plasma VWF (3).

The standard assessment of VWF functional activity is the VWF:RCo assay in which dilutions of the patient's plasma are tested for the ability to promote platelet agglutination in the presence of the antibiotic ristocetin. Recently, measurement of VWF:CB was introduced as another functional test (4), which is based on the ability of the large multimers of VWF to bind to collagen. This is an ELISA in which the patient's plasma is added to a collagen-coated ELISA plate and the amount of bound VWF is evaluated using a horseradish peroxidase-conjugated anti-human VWF antibody. This assay has been shown to be sensitive in the discrimination of types 1, 2A and 2B VWD (5). Casonato et al. demonstrated that the VWF:CB was consistently more sensitive to large and intermediate VWF multimer representation than was the case for the VWF:RCo (6). This became clear because the decreased values for VWF:CB in type 2A patients were also more consistent than those of the VWF:RCo assay. VWF:CB is recommended for use not as an alternative, but rather as a complement to the VWF:RCo assay (7). Although the collagen-binding assay is commercially available, it is still an expensive test for small laboratories and for patients. We devel-

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oped a cost-effective ELISA using human collagen type III for measuring VWF:CB in plasma from 44 patients with VWD and 40 normal subjects. We also evaluated the technical performance of this assay and its usefulness in conjunction with other assays for the laboratory diagnosis of VWD.

Materials and methods

Subjects

Patients with a history of a tendency to bleeding were screened for VWD. VWF:CB and other screening tests were performed on plasma collected from 40 normal subjects and 44 patients to be screened for VWD. This study was approved by the Ethics Committee of the University of the Free State and consent was given by all participants.

Sample collection

Blood samples were collected into Vacutainer™ tubes (BD Vacutainer Systems, Paymouth, UK) containing 0.105 M sodium citrate at a ratio of 1:9 to blood. Platelet-poor plasma was prepared by centrifugation of whole blood at 2000×*g* for 20 min at room temperature. Samples were stored in polypropylene tubes at −70°C until analysed. All tests were performed on original aliquots that were not previously thawed.

Laboratory tests

Screening tests included plasma factor VIII levels, VWF:Ag, VWF:RCo and VWF:CB. Plasma factor VIII levels were determined using a one-stage assay on an ACL coagulation analyzer (Instrumentation Laboratories, Lexington, MA, USA).

VWF:Ag and VWF:CB were measured using ELISAs run in parallel. For VWF:Ag, an ELISA plate (Nunc Maxisorp, Rochester, NY, USA) was coated overnight at 4°C with a specific rabbit anti-human VWF antibody (DAKO, Glostrup, Denmark) [1:6000 dilution in phosphate-buffered saline (PBS)] that captures the VWF to be measured (100 µL per well). For VWF:CB, 5 mg of human collagen type III (collagen type X, Sigma, St. Louis, MO, USA) was dissolved in 5 mL of 3% acetic acid and diluted in PBS (0.02 M phosphate buffer with 0.15 M NaCl) to obtain a concentration of 1 mg/mL. The samples were then dialysed in PBS for 2 days with four refreshments and diluted in PBS to a concentration of 100 µg/mL. A 96-well ELISA plate (Nunc) was coated overnight at 4°C with 10 µg/mL collagen (100 µL per well). The two assays were completed in almost exactly the same way. After three washing steps with PBS containing 0.1% Tween-20 (Sigma), the plates were blocked with 4% skimmed milk (SM; Difco Laboratories, Detroit, MI, USA) in PBS for 2 h at room temperature. The washing steps were done manually. After washing three times, plasma samples were added. Plasma samples from each patient were diluted in PBS/2% SM by serial dilution to yield VWF concentrations of 20% and 10% (1:50 and 1:100 dilutions), of which 100 µL was added to two wells each. In other words, the samples were tested in duplicate. The Fifth International Standard for VWF (NIBSC, Potters Bar, UK) was used as a calibrator and was added in serial dilutions, i.e., 100%, 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56% VWF. A blank was added to each plate. A plasma control with a known VWF level (DAKO) was added to the VWF:Ag plate and a plasma control with a known collagen-binding value (Life Therapeutics, Frenchs Forest, NSW, Australia) was added to the VWF:CB plate. Plates with plasma were incubated for 2 h at 37°C. After seven washing steps, a peroxidase-conjugated rabbit polyclonal anti-human VWF (DAKO) was added to the VWF:Ag plate at a concentration of 1:8000 in PBS/2% SM and to the VWF:CB plate at a concentration of 1:3000 and incubated for 1 h at room temperature. After eight washing steps, the colour was developed by adding a substrate for peroxidase, ortho-phenylenediamine (0.05% OPD in 0.1 M citrate-phosphate buffer, pH 5.0 containing 0.03% H_2O_2, 90 µL per well) from Sigma, where the colour intensity indicates the amount of VWF bound to collagen. The colour reaction was stopped by addition of 4 M H_2SO_4 (30 µL/well) after 3 min for VWF:Ag and after 5 min for VWF:CB. The optical density was read at 490 nm with an EL312e microplate bio-kinetics reader (Bio-Tek Instruments, Winooske, VT, USA). An eight-point standard curve was drawn with the known VWF concentrations or collagen-binding values of the International Standard on the x-axis and the optical density values on the y-axis. The VWF:Ag level or VWF:CB activity for each patient was read off the corresponding standard curves.

The VWF:RCo assay was performed using a ristocetin cofactor assay kit from Chronolog (Havertown, PA, USA). Formalin-fixed washed platelets do not agglutinate in the presence of the antibiotic ristocetin unless normal plasma is added as a source of VWF. The agglutination follows a dose-response curve that is dependent on the amount of plasma VWF added. The test was performed on a Chronolog 560 CA whole blood aggregometer using the Aggrolink software provided. Plasma concentrations of 1:2 and 1:4 in Tris-buffered saline (TBS) were used. A standard curve of calibrated human plasma is used as the standard against which the patient's plasma is measured.

The RIPA test and VWF multimer patterns were assessed to diagnose the type 2 subtypes. These tests were not conducted together with the other tests, but only when type 2 VWD was suspected. RIPA is measured by mixing different concentrations of ristocetin ranging from 0.2 to 2 mg/mL in increments of 0.1 mg/mL with the patient's platelet-rich plasma (PRP) on a Chronolog 560 CA aggregometer. The results are expressed as the concentration of ristocetin (mg/mL) able to induce 30% agglutination.

The multimeric structure of VWF in plasma was determined by a highly sensitive and rapid method described by Krizek and Rick (8). This method utilises submerged horizontal agarose gel electrophoresis, followed by VWF transfer to a polyvinylidine fluoride membrane, and immunolocalisation and luminographic visualisation of the VWF multimer pattern. This method distinguishes type 1 from types 2A and 2B VWD. The density of the high-, intermediate- and low-molecular-weight multimers of each pattern were determined using a Geldoc XR gel documentation system (Bio-Rad, Hercules, CA, USA).

Statistical analysis

We evaluated the technical performance of the VWF:CB assay on the results for 40 healthy volunteers (20 men, 20 women, age range 21–55 years). Reference intervals (mean±2 SD), the intra and inter-assay coefficients of variation (CVs), and the limits of detection and of quantification were calculated for the 40 normal samples (Table 1). To determine the usefulness of our assay in conjunction with other assays, we determined the RCo/Ag and CB/Ag ratios for all samples (Table 2 and Figure 1).

Results

The standard curve for collagen-binding activity (Figure 1) shows a good dose-response, with fast sub-

Table 1 VWF:CB assay performance.

Variable tested	Result
Intra-assay precision, CV, %	<8
Inter-assay precision, CV, %	<9
Reference range (n=40)	
VWF:CB activity, %	51–143
VWF:CB/VWF:Ag ratio	0.8–1.24
Limit of detection, %	0.6
Limit of quantification, %	4

strate colour development, high ELISA optical density readings and steep upward linearity with respect to VWF concentration. The goodness of fit shows an r^2 value of >0.99.

Table 1 shows the technical performance data for the method. The reference interval (mean±2 SD) was established internally on the basis of results for 40 healthy volunteers. The intra- and inter-assay CVs were <8% and <9%, respectively. The reference range for the 40 normal samples was 51%–143% and that of the CB/Ag ratio was 0.8–1.24. VWF:CB showed good analytical sensitivity and precision, since the limit of detection was 0.6% and the limit of quantification was 4%.

Table 2 summarises the results for all normal subjects, as well as for the patients. It shows the mean values and SD for normal values and for each type and subtype of VWD. Figure 2 shows the RCo/Ag and CB/Ag ratios for the different patients, who were classified as type 2A, type 2B, type 2M and type 1 VWD. We used 0.7 as the cut-off for the RCo/Ag and CB/Ag ratio points for the diagnosis of types 2A and 2B VWD. For type 2M, we used 0.7 as the cut-off point for only the RCo/Ag ratio. In normal subjects, the VWF:CB and VWF:RCo assays correlated well with the antigen levels, with a mean RCo/Ag ratio of 0.85 and a mean CB/Ag ratio of 1. In type 2A patients, the VWF:CB assay and the ristocetin cofactor activity were low because of the functional defect in VWF. The VWF:CB assay was even more sensitive to the absence of large multimers than the ristocetin cofactor activity, since the mean CB/Ag ratio was 0.18, which is lower than the mean RCo/Ag ratio of 0.4 (Table 2). In type 2B, both the functional assays had low values, with a mean RCo/Ag ratio of 0.36 and a mean CB/Ag ratio of 0.45. The VWF:CB assay correlated well with the antigen levels in type 2M patients because of the presence of large multimers in plasma. VWF:CB did not show any discrepancy from the VWF:Ag level in type 2M patients. The ristocetin cofactor level in these patients was low because of a functional defect (low platelet binding capacity) in this subtype and is therefore an essential test in the diagnosis of these patients. The mean RCo/Ag ratio was 0.47, while the mean CB/Ag ratio was 1.10 in type 2M VWD patients. In type 1 VWD, both functional assays correlated with the antigen level, and the mean values for all tests were low because of the low antigen levels. The ratios were normal and no discrepancy was found. The range of data for VWF:RCo tends to show lower scatter than VWF:CB. This is in contrast to the literature (12–15).

The RIPA test was performed separately from the other laboratory tests for VWD and at a later stage. It was unfortunately not possible to locate all the patients for whom RIPA testing would be useful to diagnose the appropriate subtype of VWD. The RIPA test was carried out to distinguish type 2B from types 2A and 2M VWD. We found lower RIPA results for all six of the ten type 2A patients tested. At ristocetin concentrations of >1.5 mg/mL, 30% agglutination occurred. In eight of the 14 type 2M patients tested for RIPA, we found normal to decreased levels, with 30% agglutination at ristocetin concentrations ranging between 1.0 and 2.0 mg/mL. The RIPA test was primarily used to distinguish type 2B patients and all of these patients showed higher RIPA results, with 30% agglutination at 0.5–0.7 mg/mL ristocetin.

Figure 3 shows the relationship found between the collagen-binding activity and multimer distribution of VWF. The collagen-binding activity is closely correlated with the VWF multimeric distribution in subjects' plasma, and was low in type 2A VWD, which is characterised by an absence of large and intermediate multimers. In type 2B VWD, for which only the large multimers are absent, collagen binding was also reduced, but not as much as for type 2A VWD. Collagen-binding activity was correlated with the antigen level and showed normal levels in type 2M VWD.

Discussion

The VWF:RCo assay is still the most popular method for determination of the functional activity of VWF. However, the literature shows that the VWF:CB assay is more sensitive and reproducible than VWF:RCo (9, 10). We determined the relationship between VWF antigen levels, ristocetin cofactor activity and the collagen-binding activity for 44 VWD patients from our clinic and 40 normal subjects.

We developed an in-house ELISA method for determination of the collagen binding activity in plasma. We coated the ELISA plates overnight at 4°C with

Table 2 Correlation between VWF:Ag, VWF:RCo and VWF:CB for 44 VWD patients and 40 normal subjects (mean±SD).

Type	Number of patients	Correlation, %			RCo/Ag mean ratio	CB/Ag mean ratio
		VWF:Ag	VWF:RCo	VWF:CB		
Normal	40	97±28	82±23	97±23	0.85	1.00
2A	10	60±18	24±9	11±8	0.40	0.18
2B	10	77±27	28±5	35±21	0.36	0.45
2M	14	64±16	30±12	72±24	0.47	1.10
1	10	37±11	36±18	37±14	0.97	1.00

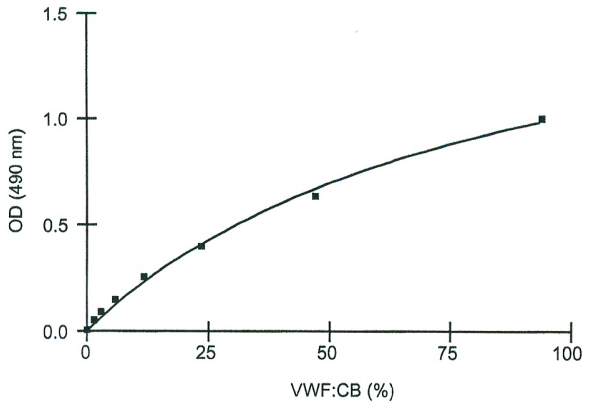

Figure 1 Standard curve for the collagen-binding activity of Von Willebrand factor.
The Fifth International Standard for VWF from the NIBSC was used as a standard and VWF concentrations ranging from 0% to 100% on the x-axis were tested for collagen-binding activity. Each point represents the mean of duplicate readings for a single representative experimental data set. The final collagen concentration was 10 μg/mL.

human type III collagen (type X collagen, Sigma) since this type of collagen shows, according to the literature, the highest affinity for VWF and commercial collagen-binding assays to also use type III collagen (11–13). However, the literature often indicates that type I/III collagen mixtures sometimes show better performance in such assays than pure type III collagens (12).

Coating conditions of overnight incubation at 4°C gave the best VWF binding results (11). Furthermore, type III collagen showed a good dose-response (calibration) curve and achieved discrimination between type 1 and types 2A and 2B VWD, with CB/Ag ratios of <0.6, similar to that reported in the literature (12).

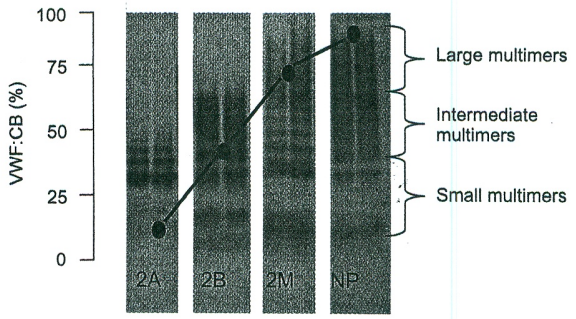

Figure 3 Relationship between the percentage collagen-binding activity (VWF:CB, %) and the multimer distribution of VWF in types 2A, 2B and 2M VWD and normal plasma (NP).

Our results showed that VWF:CB was more sensitive to large VWF multimer representation than VWF:RCo in type 2A patients, as indicated by lower values for VWF:CB (Figure 3). A similar relationship between collagen-binding activity and multimer degradation in plasma was reported in the literature (14). However, in type 2B patients, we found that VWF:RCo activity was less than VWF:CB, thus indicating greater functional discordance. This is in contrast to some recent literature (15), where VWF:CB was found to have greater functional discordance than VWF:RCo. VWF:CB correlated well with the antigen levels in type 2M and type 1 VWD. Consequently, this contributes to the diagnosis of these subtypes. The reason for this concordance is the presence of large functional multimers in these subtypes (Figure 3). This concordance also helps in the diagnosis of type 2M VWD.

We did not compare the results of our assay with those for commercial kits or in-house VWF:CB methods performed with a type I/type III collagen mixture,

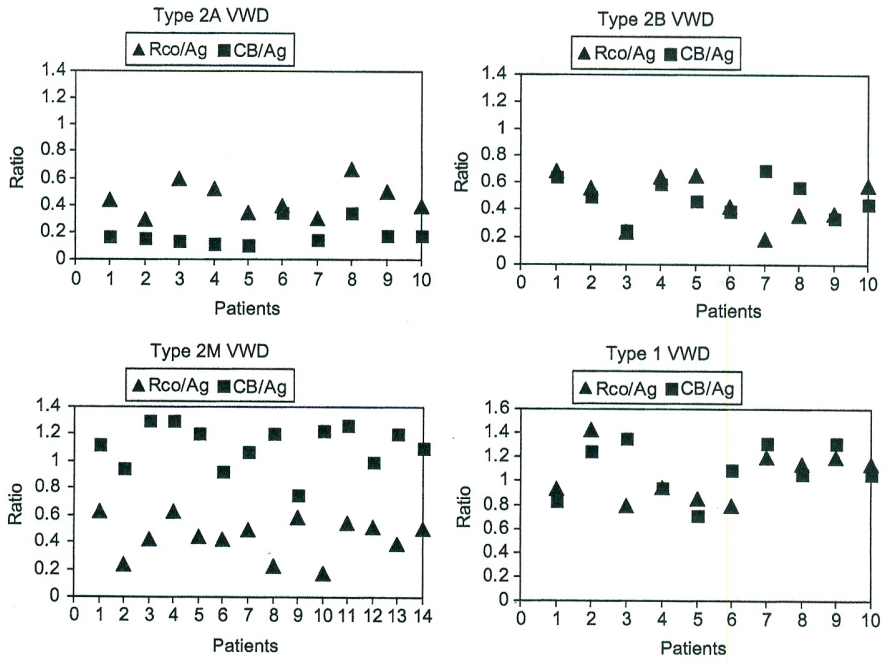

Figure 2 Ratio of VWF:RCo to VWF:Ag (RCo/Ag, triangle) and VWF:CB to VWF:Ag (CB/Ag, square) for the different patients classified as type 2A, type 2B, type 2M and type 1 VWD.

since such a study has already been carried out (12). Our results, however, compare well with literature findings on VWF:CB using type III collagen from Sigma, for which the CB/Ag ratio varied between 0.2 and 0.5 for type 2 patients, correlating well with our findings of 0.18 and 0.45 for types 2A and 2B, respectively.

We used 0.7 as the cutoff value for the RCo/Ag and the CB/Ag ratios and found that both VWF:RCo and VWF:CB tests are needed in the diagnosis of VWD. In type 2M patients the VWF:RCo test plays a vital role in diagnosis, since the VWF:CB assay did not show any discrepancy. The VWF:CB assay, on the other hand, is more sensitive to large multimer presentation than the VWF:RCo, as observed for type 2M VWD. In type 2B VWD patients, both tests confirm the diagnosis, since both CB/Ag and RCo/Ag ratios are less than 0.7. Furthermore, the RIPA test confirms the diagnosis of type 2B VWD.

Since the RIPA tests were carried out at a later stage than the other tests, we experienced difficulties in locating some of the patients again. Thus, the RIPA test could not be performed for all patients classified as type 2 subtypes. Multimeric analysis also plays a vital role in the diagnosis of these subtypes. It was also difficult to determine to which test the reduced RIPA (e.g., in type 2A patients) relates to, since the number of patients was so low. A larger study will be required in the future.

Validation data for the VWF:CB assay show low inter- and intra-assay variation. The upper limit of the reference range for normal samples is, however, lower than values reported in the literature for commercial kits. Since our VWF:CB assay was carried out in parallel to the VWF:Ag assay, we found very little variation between the two assays for normal samples and our normal VWF:Ag levels did not exceed 150%. Since the age of the normal subjects ranged from 21 to 55 years, with a mean age of 35 years, it is possible that age distribution had an influence on the lower VWF:Ag levels.

Compared to the cost of the commercial kits from Life Therapeutics, our method shows a cost reduction of 90% when all the reagent costs are taken into account. Labour costs are calculated to be the same for all test methods.

To conclude, most routine laboratories in developing countries do not use the VWF:CB assay as a screening test for VWD mainly because of the cost of the reagents involved. We developed a simple and cost-effective ELISA with good functional discordance of VWF in types 2A and 2B VWD. The ELISA technique described here should be accessible to all routine haemostasis laboratories in developing countries and should be used as a complement to the VWF:RCo test in the diagnostic work-up of VWD.

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A rapid and cost-effective method to visualize von Willebrand factor multimers in plasma

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Abstract

The multimeric analysis of von Willebrand factor (VWF) is utilised for von Willebrand factor disease (VWD) subclass identification and is important for treating purposes. A highly sensitive and rapid method for the visualisation of the multimeric structure of VWF in plasma and platelets was described by Krizek et al in 2000 [1]. This method uses a western blot technique where horizontal agarose electrophoresis is followed by the transfer of the VWF onto a polyvinylidine fluoride (PVDF) membrane. The multimeric pattern of VWF is visualized by immunolocalisation and luminographic detection and no radioactivity is used. We modified this method com prehensively to increase its sensitivity and to reduce the cost and duration of the test. We used one in stead of two localisation anti bodies and thereby reduced the immunolocalisation time by more than two hours. This also reduced the cost of the procedure. We further reduced the cost by using two carbon plates for blotting in stead of a blotter instrument. A total cost reduction of 40% could be achieved. A higher sensitivity was obtained by degassing the agarose before the casting process. The higher sensitivity is reflect ed by the fact that differences between the multimer patterns of type 2M and normal patients could be detected.

Introduction

Von Willebrand factor (VWF) is a multimeric glycoprotein that plays a dual role in haemostasis. Firstly, it forms a complex with coagulation factor VIII and it protects it from degradation. Secondly, VWF contributes to platelet ad hesion and aggregation by acting as a molecular bridge between sub endo thelial collagen and platelets [2]. The ability of VWF to support platelet ad hesion and aggregation increases with multimer size. In the circulation mul timer size is controlled by several mechanisms including proteolytic cleav age by the VWF cleaving protease, ADAMTS 13 [3, 4]. The steady state concentration of plasma VWF reflects the equilibrium between synthesis and clearance. In addition, the multimer distribution of plasma VWF depends on competition between the mechanisms of clearance and of proteolysis by ADAMTS 13 [4, 5].

Inherited VWD has been subdivided into three types that reflect its patho physiology. Types 1 and 3 VWD reflect respectively, the partial or virtually complete deficiency of VWF. Type 2 VWD is a qualitative defect that is sub divided into 4 subtypes (2A, 2B, 2M and 2N). Type 2A refers to variants with decreased platelet dependent function and is associated with the absence of high molecular weight multimers. Type 2B refers to variants with increased affinity for platelet glycoprotein 1b. Type 2M refers to variants with decreas ed platelet dependent function not caused by the absence of high molecular weight multimers and Type 2N to variants with markedly decreased affinity for factor VIII.

No single test is available that provides appropriate information about the various functions of VWF and the laboratory diagnosis of VWD is based on a panel of tests that includes the bleeding time, the measurement of factor VIII coagulant activity (VIIIC), VWF antigen (VWF:Ag), VWF activity as measured by the ristocetin cofactor activity (VWF:RCo) and the collagen binding assay (VWF:CBA), VWF multimer analysis, ristocetin induced pla telet agglutination (RIPA) and the factor VIII binding assay of plasma VWF.

The evaluation of the multimeric analysis of VWF is utilised for subclass identification of VWD. It is important to identify the specific subtype, be cause treatment of VWD is based on the classification of the disease. A high ly sensitive and rapid method for the visualisation of the VWF multimers is described [1], which we modified significantly to make it even more rapid, sensitive and cost effective.

Materials and Methods

Preparation of SDS Agarose Gel

A 0.65% agarose gel is prepared in 100ml Tris acetate electrophoresis buffer (40 mM Tris, 0.1% SDS, 1 mM EDTA, pH 7.8) by melting the agarose until clear. After degassing by vacuum suction, the agarose is poured into a hori zontal gel apparatus with a 20 tooth comb in place and after solidification, the gel is placed at 4°C for 30 min.

Sample preparation

Whole blood of patients with a history of a bleeding tendency who was re ferred to our Haematology Clinic is collected into two vacutainer tubes con taining 0.105 M sodium citrate in a ratio of 1:9 with blood. Platelet poor plasma is prepared by centrifugation of whole blood at 2000 g for 20 min utes at room temperature. Samples are stored in 200 μl aliquots in poly propylene tubes at 70°C until analysed. All tests are performed on aliquots that were not previously thawed. Each plasma sample is thawed at 37°C and diluted 1:30 in sample buffer (0.01M Na2HPO4, 37 mM iodoacetamide and 1% SDS, pH 7.0). After incubation at 37°C for 60 minutes, 10 μl bromphe nol blue is added 1:10 to the diluted plasma and centrifuged at 14000 rpm for 1 minute in an Eppendorf centrifuge

Electrophoresis

The gel is set in place and the comb removed. Pre cooled electrophoresis buffer is poured onto the gel to overlay it with no more than 2 3 mm and 10 μl of diluted sample is loaded into each well. The power is set on 30 mamps (constant amp) for 30 minutes where after it is set at 50 mamps and run for a further 4 6 hours until the dye front had migrated (±8cm).

Western Blot

Two litres of transfer buffer (2.5 mM Tris, 19.2 mM glycine, 20% methanol, 0.01% SDS, pH 8.8) are prepared and stored at 4°C. After electrophoresis is complete, the gel is placed in transfer buffer and equilibrated for 30 minutes. Polyvinylidene fluoride (PVDF) 0.45 μm membrane are cut only a bit small er than the size of the gel, pre soaked in methanol for 1 2 minutes and stored in transfer buffer until use. A western blot "sandwich" is assembled by plac ing the cathode carbon block with black electrode at the bottom, 2 Scotch Brite pads on top of it, 2 thick filter papers (Whatman CHR3, Whatman International, England), 2 thin filter papers (Whatman no 1), the gel, the PVDF membrane, 2 thin filter papers, 2 thick filter papers, 2 Scotch Brite pads and the anode block with red electrode on top. The pads and filter papers are soaked in transfer buffer before the assembly. The whole sand wich is placed into a plastic container at 4°C and covered with plastic wrap to prevent evaporation. The transfer conditions are 70 mille ampere (con stant ampere) overnight (15 17 hours).

Blocking and immunolocalization of VWF multimers

After blotting, the PVDF membrane is placed in the blocking agent that con tains 5% skimmed milk powder in TBS for 1.5 hours at room temperature. Three washing steps are done with 50 ml TBS Tween for 1 minute and 3 times 100 ml TBS Tween for 7.5 minutes each. The membrane is then placed into a 1:4000 dilution of an anti human VWF HRP conjugated antibody in TBS Tween for 1 hour 15 minutes. Washing steps are done as before where after the membrane was placed onto a mat surface with the protein side upwards. Equal volumes of ACL western blot detection reagent 1 and 2 (AEC Amersham, UK) are mixed and poured onto the membrane. The rea gent must cover the entire surface of the membrane and must be held to the surface by surface tension. After 1 minute incubation, the excess detection reagent is drained off, the membrane wrapped into plastic wrap and placed in the dark on an x ray film and exposed for 1 2 minutes. The film is then removed and developed in an automated film developer (Kodak, USA). The density of the high, intermediate and low molecular weight multimers are determined using a Geldoc XR geldocumentation system (Bio Rad, CA, USA). The total duration of this multimer analysis procedure is less than 28 hours.

Results

Typical patterns of VWF multimers of normal pooled plasma and vWD types 1, 2A, 2B, and 2M are shown in figure 1. The density of the different bands was plotted in figure 2 against the relative front (Rf value) of the different lanes. The relative front is the distance of a band from the top of its lane, divided by the total length of the lane. The larger multimers are represented by the smallest values on the x axis in figure 2. The first set of lanes in fig ure 1 contains plasma from a pool of 20 normal human volunteers. The high est density is in the region of the larger multimers (broken line in figure 2).

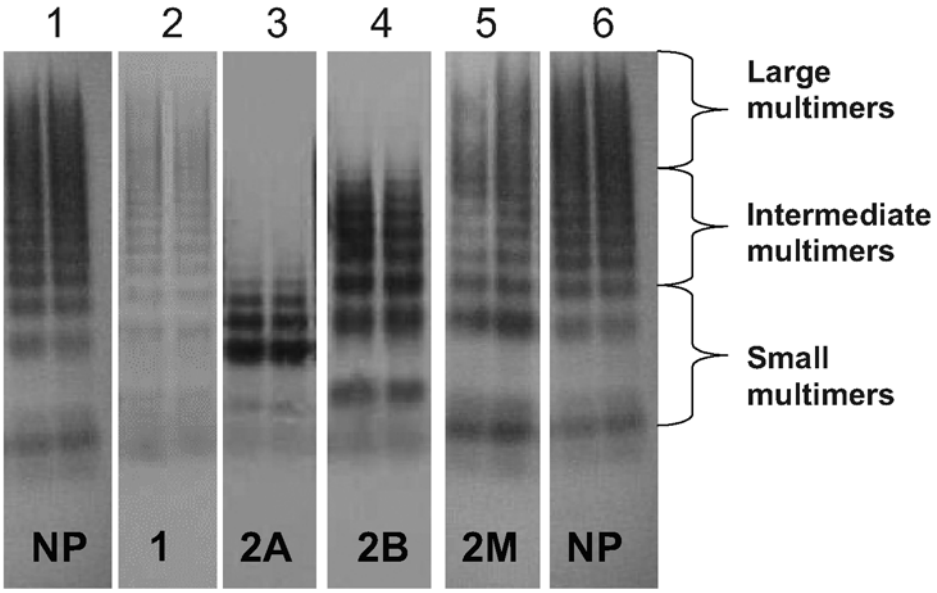

Figure 1

The multimeric structure of VWF in normal plasma (NP) (lane sets 1 and 6), type 1VWD (lane set 2). Type 2A VWD (lane set 3), Type 2B VWD (lane set 4) and type 2M VWD (lane set 5).

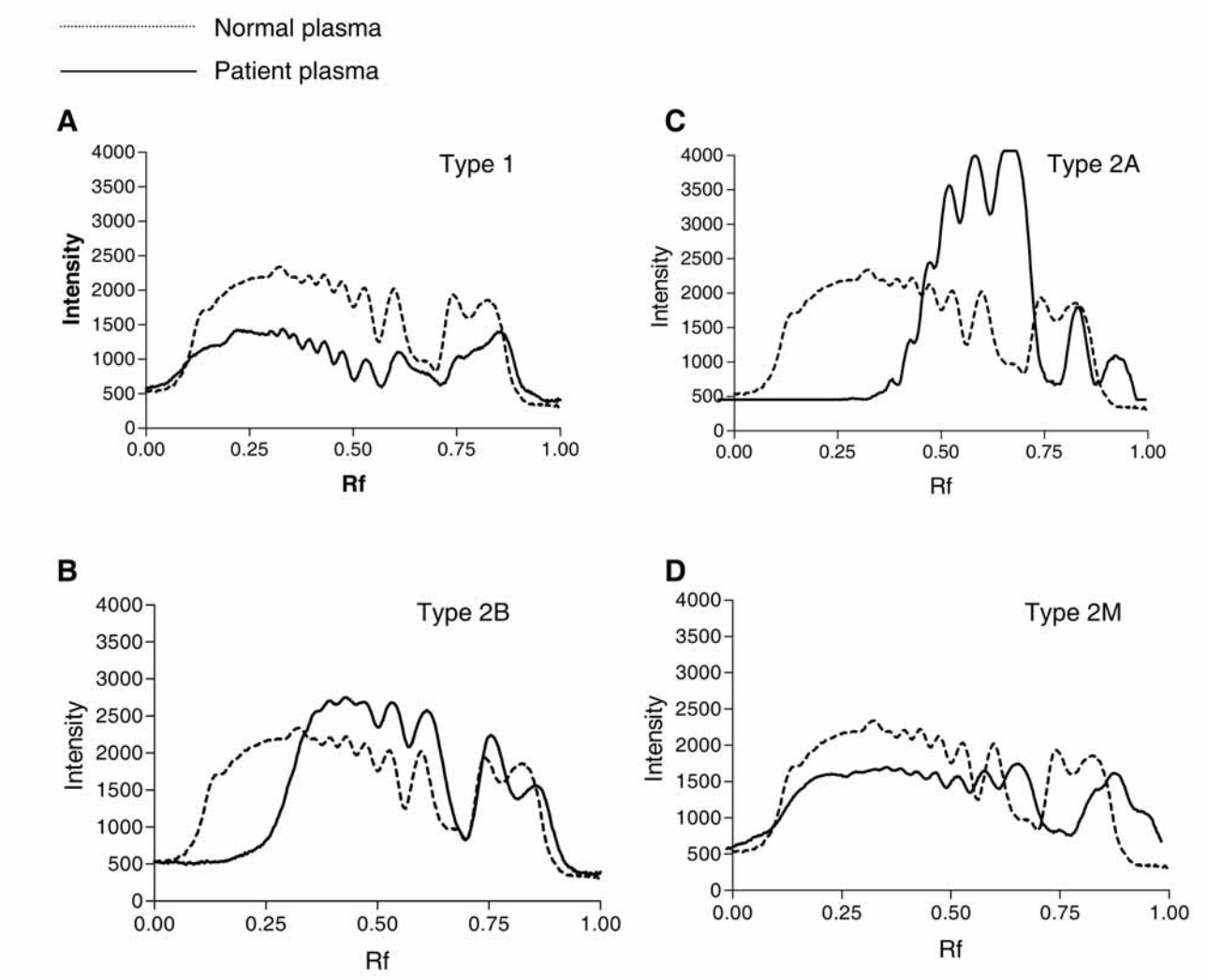

Figure 2

Densitometric tracing of lane sets 2 to 5. The density of the different bands is plotted against the relative front (Rf value) of the different lanes.

Plasma from a patient with type 1 VWD was run in the second set of lanes in figure 1. The highest density is also in the range of the larger multimers and due to the low VWF:Ag levels, the bands are much lighter (lower density) than those of normal persons (figure 2A). Plasma from a patient with type 2A vWD was run in the lane set 3 (figure 1). No high molecular weight multimers can be detected and an increase in the small multimers is visible. The low molecular weight multimers also show a higher density than those in normal plasma (figure 2C). Plasma from a patient with type 2B VWD was run in lane set 4 in figure 1. Only the intermediate and small multimers are visible. The density of these multimers is also higher than those in normal plasma (figure 2B). Lane set 5 in figure 1 contains plasma from a type 2M patient. It is interesting to note the difference in distribution of the different size multimers compared to that of normal plasma. The density profile shows an almost even distribution of all multimers (figure 2D). The density of the small multimers is higher than those of normal plasma and those of the large multimers less.

Discussion

This method distinguishes type 1 from type 2A and 2B VWD. Type 2A shows a total absence of the high and intermediate molecular weight multimers and type 2B shows an absence of only the highest molecular weight multimers. Type 1 VWD shows a multimer pattern similar to that of normal plasma. Plasma from type 2M VWD patients contains a higher concentration of small multimers than normal plasma, although large, intermediate and small multimers are present. Perhaps the greatest advantages of our method are the rapid processing (less than 28 hours), high sensitivity to low concentrations of VWF, no radioactivity and the low cost of this procedure. A cost comparison between this method and those described in the literature shows a reduction of 40% in favour of our method when all the apparatus and reagent costs are taken into account. We could also demonstrate the difference in multimer pattern between normal plasma and that of type 2M patients, which is not the case with the method of Krizek et al in 2000 [1]. By using only one antibody to VWF instead of the two that is described in the literature, we shortened the duration of the test with more than 2 hours and further reduced the cost of the method.

In conclusion, our modified luminographic method is a rapid, highly sensitive and cost effective visualisation method for VWF multimers in plasma and it will be wise to include the density profiles in the diagnostic work up of von Willebrand disease.

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ORIGINAL ARTICLE

Differential sensitivity of von Willebrand factor (VWF) 'activity' assays to large and small VWF molecular weight forms: a cross-laboratory study comparing ristocetin cofactor, collagen-binding and mAb-based assays

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Summary. *Background:* von Willebrand disease (VWD), the most common inherited bleeding disorder, is caused by deficiencies and/or defects in von Willebrand factor (VWF). An effective diagnostic and VWD typing strategy requires plasma testing for factor VIII, and VWF antigen plus one or more VWF 'activity' assays. VWF activity is classically assessed by using VWF ristocetin cofactor activity (VWF:RCo), although VWF collagen-binding (VWF:CB) and VWF mAb-based (VWF activity [VWF:Act]) assays are used by some laboratories. *Objective:* To perform a cross-laboratory study to specifically evaluate these three VWF activity assays for comparative sensitivity to loss of high molecular weight (HMW) VWF, representing the form of VWF that is most functionally active and that is absent in some types of VWD, namely 2A and 2B. *Methods:* A set of eight samples, including six selectively representing stepwise reduction in HMW VWF, were tested by 51 different laboratories using a variety of assays. *Results:* The combined data showed that the VWF:CB and VWF:RCo assays had higher sensitivity to the loss of HMW VWF than did the VWF:Act assay. Moreover, within-method analysis identified better HMW VWF sensitivity of some VWF:CB assays than of others, with all VWF:CB assays still showing better sensitivity than the VWF:Act assay. Differences were also identified between VWF:RCo methodologies on the basis of either platelet aggregometry or as performed on automated analyzers. *Conclusions:* We believe that these results have significant clinical implications for the diagnosis of VWD and monitoring of its therapy, as well as for the future diagnosis and therapy monitoring of thrombotic thrombocytopenic purpura.

Keywords: diagnosis, high molecular weight sensitivity, laboratory testing, von Willebrand disease, von Willebrand factor.

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Introduction

von Willebrand disease (VWD) is considered to be the most common inherited bleeding disorder. VWD is suspected following clinical and physical examination in individuals with personal and familial evidence of mucocutaneous bleeding, and the diagnosis is confirmed by laboratory testing [1,2]. VWD reflects quantitative or qualitative defects or deficiency in plasma von Willebrand factor (VWF), and is classified into six types. Types 1 and 3 define quantitative disorders with partial or complete loss of VWF, respectively, and types 2A, 2B, 2M and 2N define qualitative disorders [1,2]. Notably, type 2A VWD is characterized by decreased VWF-dependent platelet adhesion, owing to selective deficiency of high molecular weight (HMW) VWF multimers, type 2B VWD by increased affinity of VWF for platelet glycoprotein (GP)Ib, often also associated with loss of HMW VWF, and type 2M VWD by impaired VWF-dependent platelet adhesion without selective deficiency of HMW VWF [2].

Focused laboratory testing generally includes assessment of plasma factor VIII coagulant activity (FVIII:C), and VWF antigen (VWF:Ag) and VWF 'activity', supplemented with additional tests on a case-by-case basis [1–3]. VWF 'activity' is classically assessed with the VWF ristocetin cofactor (VWF:RCo) assay, originally described in the early 1970s [3–5]. Problems with this assay, including complexity, performance time, poor reproducibility, and poor sensitivity to low levels of

VWF (reviewed elsewhere [3]), has seen attention focused on newer assays, primarily the VWF collagen-binding (VWF:CB) assay and various so-called VWF 'activity' (VWF:Act) assays based on mAb technology. Used in conjunction with VWF:Ag and FVIII activity, VWF activity assays have the capacity to identify and discriminate the various types of VWD. For example, VWF:RCo/VWF:Ag (RCo/Ag) and VWF:CB/VWF:Ag (CB/Ag) ratios can be used to differentiate type 1 from types 2A, 2B and 2M VWD [1–3,6], given that these assays are sensitive to loss of HMW VWF and may also reflect VWF function (i.e. binding of VWF to platelet GPIb or collagen, respectively). Activity assays are also used as surrogate laboratory markers of HMW VWF, either for VWD diagnosis [1–3], VWF concentrate production, therapy, and pharmacokinetic studies [7], or assessment of VWF protease (e.g. ADAMTS-13 activity or inhibition for investigation of thrombotic thrombocytopenic purpura [TTP]) [8].

The VWF:CB assay is an ELISA-based assay first described by Brown and Bosak in 1986 [9]. mAb-based VWF:Act immunoassays, originally described in 1995, utilize a mAb directed against a functional epitope on VWF as the capture and/or detection antibody [3,10–12]. Later independent validation studies suggested inferiority of the commercial mAb ELISA-based assays to VWF:RCo and VWF:CB or even in-house mAb-based ELISA assays for discrimination of HMW VWF-deficient VWD types such as 2A and 2B [13–15], and as most recently highlighted for a case study of type 2A VWD [16]. The latex agglutination assay developed by Instrumentation Laboratory is currently the most popular mAb-based VWF:Act assay [17,18]. The true utility of this particular assay in VWD is only now emerging. Despite early reported high correlation with the VWF:RCo assay [17,18], these two assays are increasingly recognized as providing different values with many cases of VWD, notably qualitative variants [19,20].

Assessments of the respective performance of VWF activity assays within cross-laboratory testing exercises are limited. The Royal College of Pathologists of Australasia (RCPA) Haematology Quality Assurance Program (QAP) last reported findings in this area in 2007 [21]. At that time, the mAb latex-based VWF:Act assay had recently emerged, and was performed by approximately 20% of participant laboratories, often as a replacement for the VWF:RCo assay. Nevertheless, discrepant behavior of the three different activity assays (i.e. VWF:RCo, VWF:CB, and VWF:Act) for discrimination of several type 2B VWD cases was reported. More recently, the North American Specialized Coagulation Laboratory Association (NASCOLA) reported their experience [22], highlighting the emerging trend of the mAb VWF:Act assay replacing the VWF:RCo assay in many North American laboratories, and despite any evidence of equivalence. In that study, overall diagnostic interpretation error rates ranged from 3% for normal samples, to 28% for type 1 VWD, and to a staggering 60% for type 2 VWD. Notably, the type 2 VWD samples were identified correctly by all laboratories using CB/Ag ratios, but by only one-third using RCo/Ag or VWF:Act/VWF:Ag (Act/Ag) ratios.

A systematic, specific and comparative evaluation of VWF activity assays in terms of sensitivity to specific loss of HMW VWF has not, to our knowledge, ever been performed. Accordingly, we describe a study that aimed to compare results obtained with the three most commonly used activity assays (VWF:RCo, VWF:CB, and VWF:Act) by testing of a normal sample as well as samples selectively and sequentially depleted of HMW and intermediate molecular weight (IMW) VWF forms, using a cross-laboratory exercise involving 51 laboratories across a broad geographic region.

Materials and methods

Preparation and initial testing of main study samples

The main study sample set is summarized in Table 1, and extended information is provided in Table S1 and Data S1. The samples were derived primarily from a plasma pool of individual normal plasmas. A series of eight plasma samples was then initially produced from this pool by one of us (E.J.F.). These comprised the otherwise unmodified pool (identified as sample V1 in Table 1), and seven similar-volume aliquots of this normal pool, each of which was differentially treated to produce increasing stepwise loss of HMW and then IMW VWF forms (Data S1, Table S1), This was achieved through a propriety process of disulfide bond reduction with *N*-acetylcysteine (NAC), similar to that recently described by Chen *et al.* [23]. Although such treatment results in the loss of HMW VWF, and also IMW VWF with continued application, the native VWF is otherwise essentially normally active [23]. The generated study samples were later anonymized to permit blinded testing for the main study (Data S1, Table S1). The first few samples in the series (e.g. V2 and V3 in Table 1) represent a loss of HMW with a minor loss of IMW VWF, which might occur with a normal sample subsequent to a preanalytic event (e.g. filtration of a sample also intended for lupus anticoagulant testing, or refrigeration of a whole blood sample) [24–26]. Subsequent samples (e.g. V4 and V5 in Table 1) would then show increasing loss of HMW and IMW VWF, potentially reflective of type 2A or 2B VWD-like plasma, but with a relatively high level of VWF, which is a pattern that might occur in type 2A or 2B VWD during pregnancy. The final sample in the series (V6 in Table 1) was meant to be depleted of HMW and IMW VWF, and could reflect a type 2A VWD pattern.

The initial set comprised eight plasma samples (Table S1), which were tested for FVIII activity and various VWF test parameters at the host laboratory (Institute of Clinical Pathology and Medical Research [ICPMR], Westmead), prior to being frozen in both aliquot form and as large volume sets with stabilizers in preparation for lyophilization and later stability and homogeneity testing. Samples were subsequently lyophilized in vials in 0.5-mL volumes by a commercial lyophilization process, and thereafter stored refrigerated at 4 °C until required. All samples were retested by the host laboratory in a validation study, including homogeneity and

Table 1 Summary of test sample set and characteristics

Sample ID (current report)	Sample comprised:	Sample intended to, or that could feasibly, represent:
V1	Pool of normal plasma samples	Normal plasma
V2	Sample V1 treated to yield minor loss of HMW VWF	Normal plasma with minor loss of HMW VWF, as might be caused by a preanalytic event (see main text)
V3	Sample V1 treated to yield loss of HMW and minor loss of IMW VWF	Normal plasma with mild loss of HMW VWF, as might be caused by a preanalytic event (see main text)
V4	Sample V1 treated to yield loss of HMW VWF and moderate loss of IMW VWF	Type 2A or 2B VWD-like plasma as might be obtained in pregnancy
V5	Sample V1 treated to yield loss of HMW VWF and high loss of IMW VWF	Type 2A or 2B VWD-like plasma as might be obtained in pregnancy
V6	Sample V1 treated to yield complete loss of HMW and IMW VWF	Type 2A VWD-like plasma.
V7	Mixture of V1 and VWF-deficient plasma	Moderate type 1 VWD-like plasma with target VWF:Ag and FVIII:C = 20–30 U dL^{-1}
V8	Mixture of HMW VWF-deficient sample and VWF-deficient plasma	Type 2A or 2B VWD-like plasma with target VWF:Ag and FVIII:C = 20–30 U dL^{-1}

FVIII:C, factor VIII coagulant; HMW, high molecular weight; IMW, intermediate molecular weight; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:Ag, von Willebrand factor antigen. Sample V1 represents a normal pool plasma and the main plasma used to generate subsequent test samples. Samples V2–V6 were derived from sample V1, and reflect sequential increasing depletion of HMW VWF, followed by sequential loss of IMW VWF. Samples V7 and V8 were, respectively, derived from mixtures of VWF-deficient plasma and sample V1 or an HMW VWF-deficient sample to produce samples reflective of moderate type 1 VWD and 2A/2B VWD, but with a similar level of VWF:Ag.

stability, as extensively detailed in the Data S1. There were no obvious deleterious effects observed. Sample vials were also subsequently sent to four collaborative laboratories for local blind testing of FVIII:C and VWF test parameters including multimer analysis to further validate study samples (details in Data S1). This testing also confirmed the selective and sequential loss of HMW and, in some cases, IMW VWF (Fig. S1A,B), as well as the comparative reduction in functional VWF test parameters (Fig. S1C,D). A subset of six samples (Table 1, V1–V6) was finally selected as representing the best differential stepwise pattern for distribution to participants of the RCPA Haematology QAP for the main cross-laboratory study.

On the basis of the preliminary testing, another two samples were subsequently prepared to mimic a moderate type 1 VWD-like and a more classical type 2A VWD-like plasma (Table 1; V7 and V8). These were differentially and respectively prepared by dilution of either the normal pool sample or an HMW and IMW VWF-depleted sample into commercial VWF-deficient plasma (see Data S1). Notably, the respective plasmas were specifically designed to produce two test plasmas with similar levels of VWF:Ag (target of $\sim$ 25 U dL^{-1}) and FVIII:C (target of 20–25 U dL^{-1}), but with comparably differing VWF activities, reflective, respectively, of a type 1 or 2A VWD (see Table 1 and Results).

Further confirmation of test sample integrity

mAb-based assay ELISA testing The sample production process was intended to produce an incremental loss of HMW VWF, followed by a loss of IMW VWF, but was not intended or expected to substantially affect the core structure or activity of VWF [23]. In order to help confirm this, the samples were tested with several various well-characterized mAbs against VWF [14,27,28] (Data S1). These mAbs bind to various (different) sites on the VWF molecule, and include several that are reactive with the platelet GPIb-binding site on VWF, and which otherwise differentially block various VWF activities [27,28]. Notably, there was no evidence that production of the samples adversely affected the core VWF structure, as represented by a structurally intact GPIb-binding site, despite samples reflecting a sequential loss of HMW and IMW VWF (Data S1). However, it was also noted that: (i) mAb data were not identical to each other or to polyclonal antibody (RαVWF) data; (ii) the sample reflecting the greatest loss of HMW and IMW VWF (i.e. V6) yielded a lower VWF 'protein' level than the other samples, with all VWF detection test systems (i.e. RαVWF and all mAbs; Fig. S2) – thus, some alteration of core structure or internal disulfide reduction cannot be excluded for this sample; and (iii) the levels of VWF protein appeared to initially rise slightly in the first few samples, with a loss of HMW and IMW VWF (Fig. S1C). This would be consistent with the test sample generation process providing improved access for VWF antibody binding in these samples, rather than elevation of VWF as such (see Data S1 and [29]).

Another series of experiments were later performed with the same mAbs as VWF capture antibodies (i.e. rather than as detection antibodies as above) (see Data S1). We have shown in the past that, used in this way, some mAbs show some HMW selectivity [14], as is also shown by the commercial mAb-based 'activity' assays.

Main cross-laboratory study

All laboratories (n = 55) enrolled in the standard VWF/VWD test module (C) of the RCPA Haematology QAP (http://www.rcpaqap.com.au/haematology/) were invited to participate

in the main cross-laboratory study. This included three laboratories that also participated in the validation study described above (see also Data S1). Two additional international collaborating laboratories that were also involved in the validation study were similarly invited to participate in the main study. All invited laboratories (total $n = 57$) were sent three vials of each of eight anonymized samples (see Data S1; reidentified as V1 to V8 in Table 1), with sample reconstitution and test instructions, requesting laboratories to perform their normal test panels for testing of VWD. Laboratories were also asked for test methodology details and to interpret their test results. The choices offered included normal, equivocal, type 1 VWD (mild, moderate, or severe), type 2A or 2B VWD, type 2M VWD, type 2N VWD, type 3 VWD, or other (with a request to specify). Some of these choices (e.g. type 2N VWD and type 3 VWD) did not reflect any of the samples, but a complete range of possible interpretations was provided to avoid any potential biasing of returned data. Laboratory numerical data were used as reported by participants, except that values reported as '<' a given value were corrected downwards to permit numerical analysis (for example, an assay value of < 10 U dL^{-1} was corrected to 9 U dL^{-1}, and an assay ratio of < 0.2 was corrected to 0.1). This is consistent with our normal External Quality Assurance (EQA) practice. These events occurred only for samples with high loss of HMW VWF (i.e. V6 and V8), and only for VWF:RCo and VWF:CB testing (respectively: five events for V6 VWF:RCo, four events for V6 VWF:CB, four events for V8 VWF:RCo, and three events for V8 VWF:CB).

Statistical analysis

Numerical data were analyzed by means of comparative medians, means, ranges and interassay (interlaboratory) coefficients of variation, with GraphPad Prism (GraphPad Software, La Jolla, CA, USA; http://www.graphpad.com).

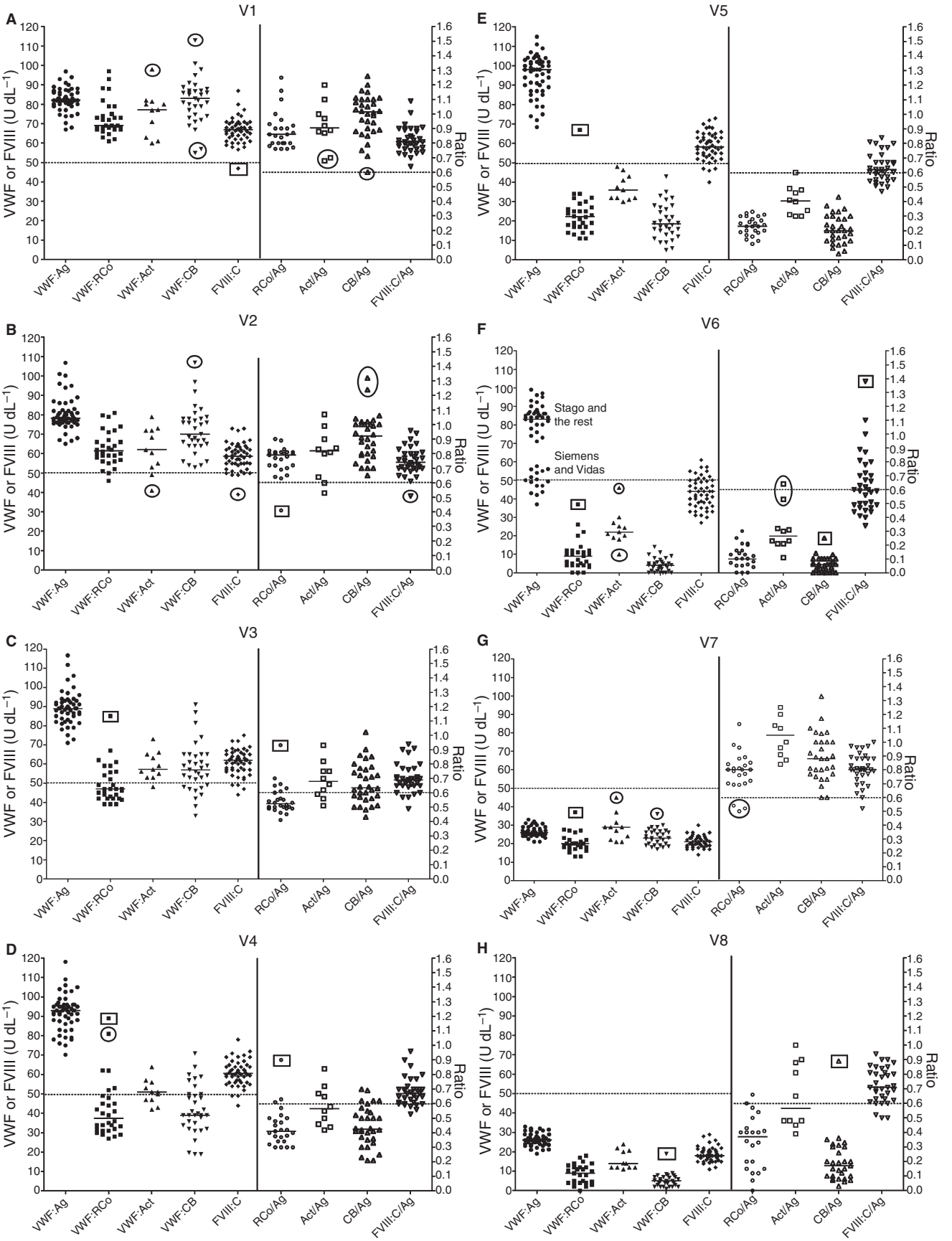
Results

Validation data for the samples prepared and dispatched in the main study are detailed in Data S1 and Figs 1–3. The main study data are derived from 51 participant laboratories (as not all invited laboratories [$n = 57$ in total] returned data), and are presented in Figs 1–3, with multimer patterns from one of the collaborating laboratories used as a reference site shown in Fig. 4. A breakdown of laboratory tests as used by laboratories, as well as summary statistical data, is given in Table 2.

All data for all samples and all laboratories for the main study test indices, namely VWF:Ag, VWF:RCo, VWF:CB, VWF:Act, and FVIII:C, as well as assay ratios, are shown in Fig. 1 as a scatter plot. This shows individual data and general trends, but also permits identification of outlier data, which may reflect aberrant testing from a few laboratories or potential transcription errors. In brief, outlier data were only identified in the case of activity assays, and values > 3 standard deviations from the mean were thereafter excluded from analysis. Outlier data were also checked for data source. It was noted that five laboratories were responsible for nearly half of all the data outliers identified in Fig. 1. These outlier data were also thereafter excluded from analysis. Summarized data (excluding outliers noted above), including corresponding assay ratios, are shown in Fig. 2, which attempts to focus on assay performance rather than participant performance. Note that VWF:Ag and FVIII:C testing identified similar levels across all samples tested except for sample V6, similar to the trend identified prior to the main study by collaborative laboratories (Fig. S1). In contrast, and as expected, VWF:RCo, VWF:CB and VWF:Act levels fell sequentially according to increasing loss (or decreasing levels) of HMW and IMW VWF. Notably, VWF:RCo and VWF:CB showed the greatest (similar) falls, particularly for samples showing the highest loss of HMW VWF, whereas VWF:Act showed intermediate results. Consistent with the trends observed for VWF:Ag and FVIII, the FVIII/VWF:Ag (FVIII:C/Ag) ratio showed only a moderate trend to reduction. Act/Ag ratios also showed a gradual trend to reduction, but this was overshadowed by the greater reductions observed in RCo/Ag and CB/Ag ratios. Comparative data for samples V7 and V8, intended to represent a moderate type 1 VWD and a type 2A VWD sample, respectively, are shown in Fig. 2C,D. The type 1 VWD mimic sample (V7) yielded similar numerical data for all test parameters (between 20 and 30 U mL^{-1}), with normal assay ratios (all > 0.7). In contrast, although the type 2A VWD mimic (V8) yielded similar numerical data for VWF:Ag and FVIII:C (around 20–30 U mL^{-1}) and similar data to sample V7, the VWF activity data (VWF:RCo, VWF:CB, and VWF:Act) showed decreased values, as did respective ratio data, and as expected for this sample. Consistent with the findings in Fig. 2B, VWF:RCo and VWF:CB (and thus RCo/Ag and CB/Ag ratios) provided the lowest comparative values.

A subanalysis of data according to type of VWF:CB and VWF:RCo assay is shown in Fig. 3. Although data should be interpreted cautiously, given low subsample test numbers, some VWF:CB assays appeared to be more sensitive to the loss of

Fig. 1. Main study data as derived from all samples tested where reported by 51 participating laboratories and shown as a scatter plot. The data for the main study test indices, namely von Willebrand factor (VWF) antigen (VWF:Ag), VWF ristocetin cofactor (VWF:RCo), VWF collagen binding (VWF:CB), VWF activity (VWF:Act), and factor VIII coagulant activity (FVIII:C), are shown on the left side of each figure. The horizontal dashed line represents a nominal 'normal' cut-off value of 50 U dL^{-1}. Data for assay ratios are shown on the right side of each figure. The horizontal dashed line represents a nominal 'normal' cut-off value of 0.6. (A)–(H), respectively, show data for samples V1–V8 (see Table 1). Some outlier data are evident; data in squares indicate values that are > 3 standard deviations from the mean. Data in circles show other visually appearing outlier data, in general between 2 and 3 standard deviations from the mean. All identified outlier data were checked for transcription error and for source of data.



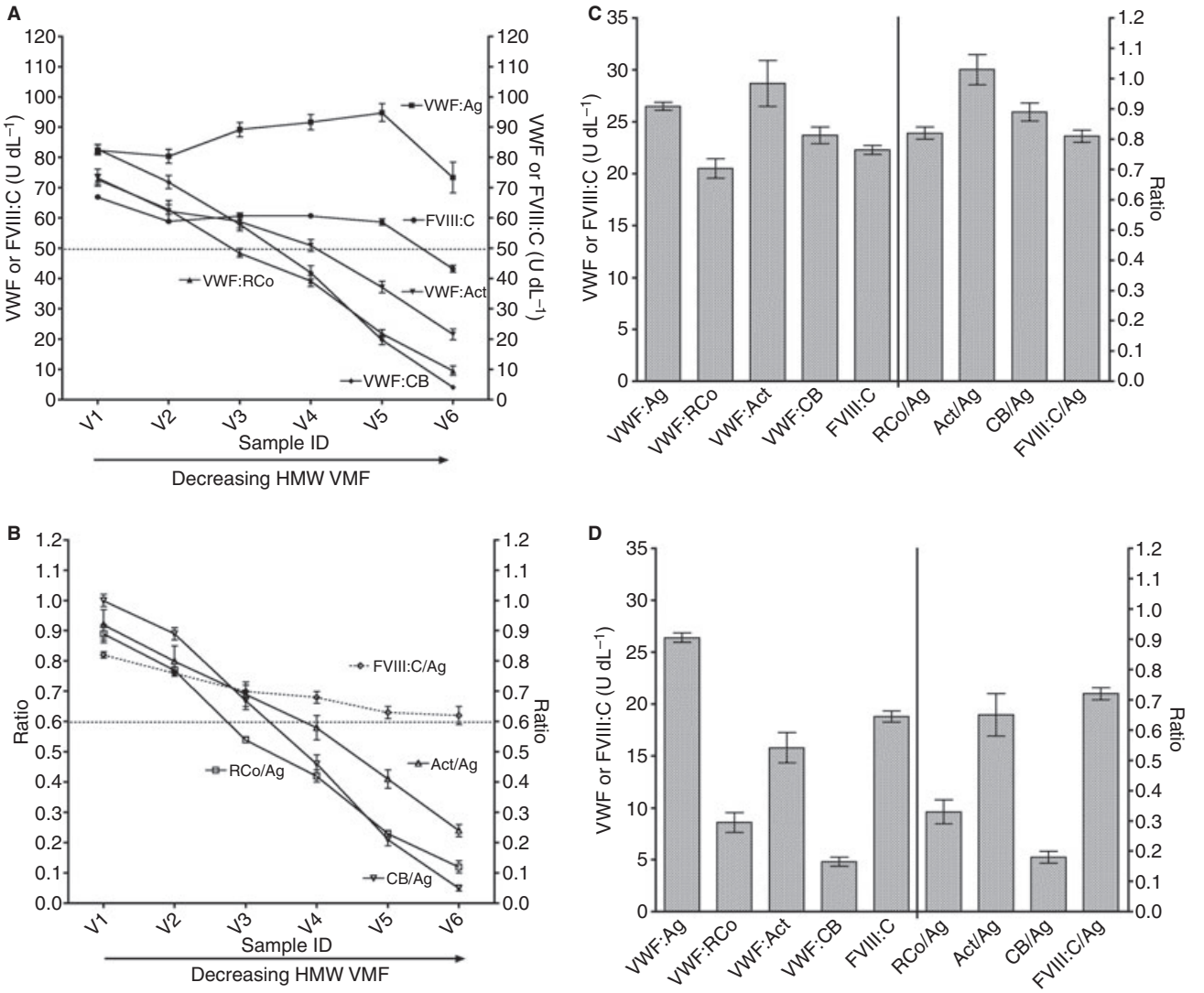


Fig. 2. Summarized main study data as derived from all samples tested where reported by 51 participating laboratories and shown as mean ± standard error of the mean. Some outlier data identified in Fig. 1 have been removed (refer to text) to permit a focus on method-based differences (i.e. to exclude potential participant-based problems). (A) Main study test indices, namely von Willebrand factor (VWF) antigen (VWF:Ag), VWF ristocetin cofactor activity (VWF:RCo), VWF collagen binding (VWF:CB), VWF activity (VWF:Act), and factor VIII coagulant activity (FVIII:C), as reported by all laboratories. The horizontal dashed line represents a nominal 'normal' cut-off value of 50 U dL^{-1}. (B) Data for corresponding assay ratios. The horizontal dashed line represents a nominal 'normal' cut-off value of 0.6. (C, D) Comparative data for samples V7 (C) and V8 (D), intended to, respectively, represent a moderate type 1 von Willebrand disease (VWD) and a type 2A VWD sample.

HMW VWF than others. Similarly, there also appeared to be a small difference in sensitivity between aggregometer-based and automated test processes for VWF:RCo. Figure 3C shows comparative data between the 'best' and 'least well' performing commercial VWF:CB assays, with VWF:RCo (aggregometry) and VWF:Act.

The reference laboratory multimer analysis shown in Fig. 4 is consistent with previous observations, and confirms the sequential loss of HMW and IMW VWF in the generated samples from V1 to V6, as well as the loss in sample V8 (type 2A VWD mimic), but not in sample V7 (moderate type 1 VWD mimic). The multimer analysis results submitted by two study participant laboratories yielded similar findings (data not shown).

In order to help explain some of the above observations, further studies (as outlined in Materials and methods) were performed by the host laboratory using several mAbs, including those known to react with VWF at the functional GPIb-binding site, which are presumed to be analogous to the mAb used in the commercial VWF:Act assay. The data are shown in Data S1 (Fig. S3). In brief, when used as the VWF capture system, some mAbs could be shown to yield HMW sensitivity data that were very similar to those of the VWF:Act assay. This sensitivity was increased by manipulation of the assay, most notably by reducing the concentration of coating mAb used (see Discussion).

Interpretative data from participants are shown in Fig. 5. Notably, all participants identified sample V1 as being normal,

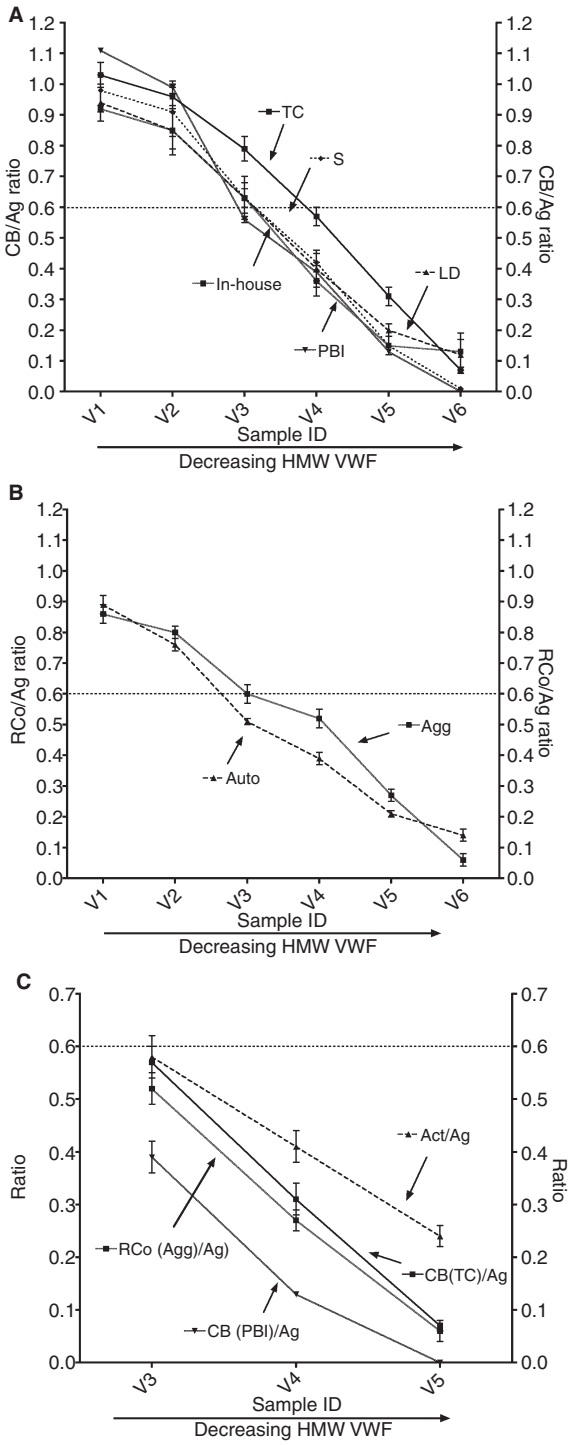


Fig. 3. Main study data from Fig. 2, but now showing submethod analysis for ratios of von Willebrand factor (VWF) collagen binding (VWF:CB)/VWF antigen (VWF:Ag) (CB/Ag) (A) and VWF ristocetin cofactor activity (VWF:RCo)/VWF antigen (VWF:Ag) (RCo/Ag) (B). (C) Comparative data for 'best' (Precision BioLogic Inc. [PBC]) and 'least well' (TechnoClone [TC]) performing method in the VWF:CB group vs. the RCo (Agg)/Ag and VWF activity (VWF:Act)/VWF antigen (VWF:Ag) (Act/Ag) groups for main three samples (V4, V5, and V6) showing the greatest loss of high molecular weight (HMW) VWF (see Fig. 4). Dashed horizontal line at 0.6 represents a typical nominal normal cut-off value. Agg, agglutination; Auto, automated; LD, Life Diagnostics; S, Stago.

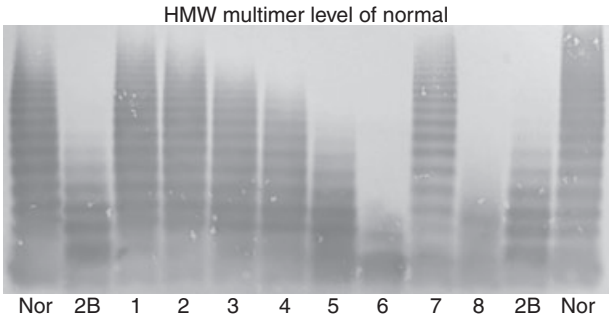


Fig. 4. Multimer patterns obtained on the main study sample set from one collaborating laboratory, and used as the reference multimers, and confirming sequential loss in high molecular weight (HMW) and intermediate molecular weight VWF in samples V1, V2, V3, V4, V5, and V6, as well as the loss in sample V8 (type 2A von Willebrand disease [VWD] mimic), but not sample V7 (moderate type 1 VWD mimic). Nor, normal plasma control; 2B, type 2B VWD plasma control.

which was reassuring. The vast majority of participants identified sample V6 (devoid of HMW VWF) as being type 2A, 2B or 2M VWD-like. There was also a trend for decreasing identification of the samples as normal, to that of them being increasingly identified as type 2A, 2B or 2M VWD-like, as the samples progressively lost HMW and then IMW VWF. Most participants identified sample V7 as type 1 VWD-like, and most also identified sample V8 as type 2A, 2B or 2M VWD-like. Somewhat concerning, perhaps, was that eight participants identified HMW VWF-deficient sample V4 as normal, with three of these same participants also identifying sample V5 as normal. Similarly concerning was that 10 participants identified the HMW VWF-deficient sample V8 as a type 1 VWD.

Discussion

To our knowledge, this study represents the first comparative assessment of the most widely used VWF 'activity'-based assays (i.e., VWF:Co, VWF:CB, and VWF:Act) for their specific sensitivity to loss of HMW VWF. VWF:RCo, the original functional VWF assay described in the early 1970s [4,5], reflects the ability of VWF to bind to its major platelet receptor (GPIb). The VWF:CB assay, first described in 1986 [9], represents another activity of VWF, namely its ability to bind to collagen, a subendothelial matrix component. The VWF:Act assay, first reported as an immunoradiometric assay in 1985, and later as an ELISA assay, is now most commonly performed with an immunolatex procedure [10–13,16,17].

The mAb-based VWF:Act assay is marketed as an 'activity' assay on the basis that the mAb used to capture VWF in the test sample recognizes the GPIb-binding site of VWF. Whether this then bestows functionality to the assay is debated. Nevertheless, the assay has been embraced by a high proportion of laboratories, and in many cases inappropriately used as a surrogate for the VWF:RCo assay. Although the RCPA Haematology QAP has noted such a trend in Australia [21], this was also recently noted in North America by the

Table 2 Summary test statistics and breakdown of main study methods used by study participants

Test/ methodology	No. of participants reporting results*	% of total†	% of method‡	Median (range); CV (of all data)§							
				V1	V2	V3	V4	V5	V6	V7	V8
FVIII:C	51	100	100	66.9 (47.0–87.0); 9.5	58.7 (39.0–73.0); 11.2	62.0 (44.0–75.0); 10.7	60.6 (44.0–78.0); 10.4	58.2 (40.0–73.0); 11.9	44.0 (27.0–61.0); 18.7	21.0 (14.0–30.0); 14.5	18.0 (11.0–29.0); 20.1
VWF:Ag	52	100	100	82.1 (67.0–96.9); 7.2	78.3 (65.0–107); 10.5	89.0 (71.0–117); 9.6	93.0 (70.2–118); 10.0	98.0 (68.4–115); 11.1	74.0 (37.0–99.0); 24.3	26.0 (21.0–33.0); 10.1	26.1 (19.0–33.0); 12.0
LIA	41	80.4	80.4	–	–	–	–	–	–	–	–
ELISA	6	11.8	11.8	–	–	–	–	–	–	–	–
ELFA	4	7.8	7.8	–	–	–	–	–	–	–	–
VWF:RCo	30	60.8	100	69.0 (61.0–97.0); 12.4	61.5 (46.0–81.0); 14.1	47.0 (39.0–85.0); 20.3	37.5 (27.0–89.0); 35.6	22.2 (11.0–67.0); 27.3	9.0 (0–37.0); 85.3	20.0 (13.0–37.0); 24.1	9.0 (0–18.0); 58.1
Aggregometer	9	17.6	29.0	–	–	–	–	–	–	–	–
Automated	22	43.1	71.0	–	–	–	–	–	–	–	–
VWF:CB	33	60.8	100	83.0 (55.0–113); 14.2	70.0 (53.0–107); 17.2	57.0 (33.0–91.0); 22.2	39.0 (19.0–71.0); 30.7	18.5 (5.0–43.0); 45.5	4.0 (0–14.0); 84.7	23.0 (17.0–36.0); 18.6	5.0 (1.0–19.0); 65.2
In-house	6	11.8	19.4	–	–	–	–	–	–	–	–
Stago	7	13.7	22.6	–	–	–	–	–	–	–	–
Technoclone	11	21.6	35.5	–	–	–	–	–	–	–	–
Life Therapeutics Group¶	5	9.8	16.1	–	–	–	–	–	–	–	–
Precision Biologic Inc.	2	3.9	6.5	–	–	–	–	–	–	–	–
VWF:Act**	11	21.6	100	77.2 (60.0–98); 14.9	62.0 (41.0–79.0); 19.0	57.2 (48.0–73.0); 12.2	51.0 (47.0–64.0); 12.6	36.0 (30.0–48.0); 17.4	22.0 (10.0–46.0); 37.9	28.9 (21.0–45.0); 25.4	14.0 (11.0–24.0); 30.6
Multimers	4	7.8	NA	NA	NA	NA	NA	NA	NA	NA	NA

CV, coefficient of variation; ELFA, enzyme-linked fluorescence assay; FVIII:C, factor VIII coagulant activity; LIA, latex immunoassay; NA, not applicable; VWF:Act, von Willebrand factor activity; VWF:Ag, von Willebrand factor antigen; VWF:CB, von Willebrand factor collagen binding; VWF:RCo, von Willebrand factor ristocetin cofactor. *A total of 57 laboratories were invited to participate, but only 51 laboratories provided test data for main study samples. Some laboratories reported results for multiple test systems and methodologies, whereas other laboratories reported data only for selective tests and methodologies; thus, test numbers will differ in each case, and do not always add up to 51. †Percentage of total participants reporting results for each test group. ‡Percentage of participants reporting results for specific test or methodology groups (VWF:Ag, VWF:RCo, or VWF:CB, etc.). §Includes outlier data; data not reported for submethodology. ¶Comprises group of Life Therapeutics Ltd, Reaads Corgenix (Corgenix Medical Corporation) and Nusep Inc. **All participants reported using the Instrumentation Laboratory assay.

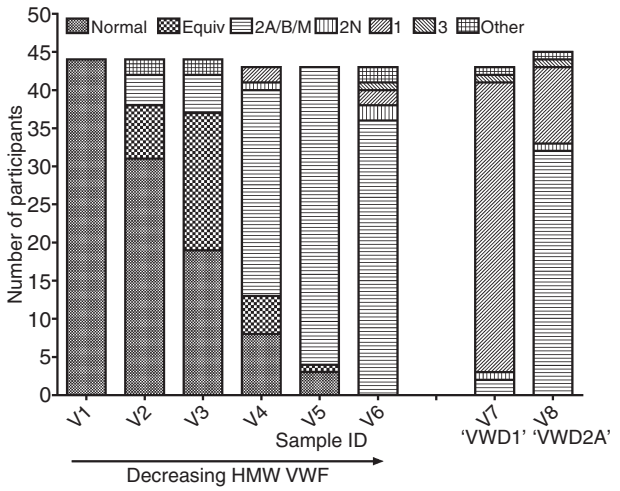


Fig. 5. Participant interpretations for tested samples. Note that: (A) not all participant laboratories provided an interpretation for test samples, so numbers are generally < 51; (B) all participants providing an interpretation identified sample V1 as being normal; (C) the vast majority identified the high molecular weight (HMW) von Willebrand factor (VWF)-devoid sample (V6) as being type 2A, 2B or 2M von Willebrand disease (VWD)-like; (D) there was a trend for decreasing identification of the samples as normal, to them increasingly being identified as type 2A, 2B or 2M VWD-like, as the samples progressively lost HMW and then intermediate molecular weight VWF (i.e. from sample V2 to sample V6); (E) most participants identified sample V7 as type 1 VWD-like; (F) most participants identified sample V8 as type 2A, 2B or 2M VWD-like. However, some participants identified HMW VWF-deficient samples V4 and V5 as normal, and some participants identified HMW-deficient sample V8 as a type 1 VWD.

NASCOLA [22]. This trend is presumably related to the assay's ease of use, and early (but incomplete) studies showing behavior similar to that of the VWF:RCo assay by regression analysis of selected samples. In some cases, preferential usage may also be driven by regulatory requirements. For example, although the VWF:Act assay has been cleared by the FDA for in vitro diagnostic use in North America, a VWF:CB assay has not. As clearly shown in the current study, the VWF:Act assay does show some selective discrimination of HMW VWF, but the VWF:RCo and VWF:CB assays are much more effective in this regard. This sensitivity of the VWF:Act assay for HMW VWF may be related to the manner in which the manufacturer has controlled the assay conditions, something that the host laboratory for the current study has shown is possible for ELISA mAb-based capture systems, simply by reducing the mAb concentration used (Fig. S3, Data S1, and [14]). Thus, the use of a lower concentration of mAb as a capture system makes the ELISA-based assay more selective for HMW VWF, presumably because low molecular weight VWF does not contain sufficient binding sites to permit stable binding of the VWF to the microplate. In a latex agglutination assay, it can be hypothesized that a similar process may occur; that is, cross-latex agglutination may also require VWF of a certain mass, according to the amount of latex-bound mAb. We have previously shown that, in discrimination of type 2 vs. type 1 VWD cases, the VWF:RCo and VWF:CB assays (and thus the RCo/Ag and CB/Ag ratios) generally perform better than the VWF:Act assay (and Act/Ag ratios) [21]. Interestingly, the NASCOLA study also identified better performance of the VWF:CB assay than either the VWF:Act or VWF:RCo assay in the context of such discrimination [22,30,31]. The current study expands on these findings, and in part may also explain possible reasons behind them, given that the VWF:Act assay appears to be less sensitive to the loss of HMW VWF than both the VWF:RCo and VWF:CB assays.

Although the VWF:CB and VWF:RCo assays (and thus the CB/Ag and RCo/Ag ratios) showed similar trends, the data were not identical. Methodology subanalysis was also performed, and although small subgroup numbers prevent any definitive conclusions, there did appear to be some differences in relation to methodology (Fig. 3). For example, the least sensitive VWF:CB assay for HMW VWF appeared to be that produced by Technoclone GmbH, an observation that is quite consistent with many previous evaluations of VWD cases by the host laboratory [15,32]. Even so, this 'least sensitive' VWF:CB assay for HMW VWF still appeared to be more sensitive than the Act/Ag ratio (Fig. 3C). For RCo/Ag ratios, the automated VWF:RCo method was as sensitive, if not more so, to the increasing loss of HMW and IMW VWF, except for sample V6, where it is suspected that low limit of VWF sensitivity issues compromise the assay's utility (that is, many laboratories cannot report assay values below 10 U dL^{-1} with this assay [33]). This lower limit of sensitivity can be improved by use of a low assay curve, as recently reported [34,35].

Participants in the main study provided interpretations of their data that were generally consistent with the sample type tested (Fig. 5). On occasion, however, interpretations appeared to be at odds with the sample type. Notably, several participants identified HMW VWF-decreased samples V4 (eight laboratories) and V5 (three laboratories) as 'normal', and sample V8 (10 laboratories) as 'type 1 VWD'. Interestingly, an analysis of these findings appears to identify the major problem as being that of limited test panels, rather than problems with 'activity' assays as such. Thus, these 21 occasions reflected testing by 15 laboratories, all of which performed the VWF:Ag assay. Three of the 15 laboratories performed no activity assay of any kind, eight laboratories performed the VWF:RCo assay, five performed the VWF:CB assay, and three performed the VWF:Act assay, with eight of 15 (53.3%) therefore performing only a single activity assay, and only four performing two activity assays. Thus, 11 of 15 (73.3%) laboratories performed only one or no VWF activity assays, compromising their ability to identify a loss of HMW VWF in this study (and a potential qualitative type 2 VWD otherwise). This finding is also consistent with our previous experience [36].

The current study utilized a propriety process that employed NAC to achieve a stepwise reduction in HMW and then IMW VWF. This in vitro process is thought to mimic a natural in vivo process, described but incompletely characterized, that permits reduction of VWF in the absence of ADAMTS-13 [37], and also possibly that assists in the formation of VWF complexes at a thrombus by a process of self-association [38].

The in vitro processing of VWF with NAC has most recently been described by Chen *et al*. [23] as a potential therapeutic aid in TTP, and, indeed, we believe that clinical trials of this agent have recently begun or being planned [37]. Should this agent become a treatment of choice for TTP, it can be envisaged that laboratories may be called upon to monitor treatment in TTP by using VWF assays, including the VWF:Ag assay and an HMW VWF 'activity surrogate'. The current study would then suggest caution in regard to the use of the VWF:Act assay for this purpose.

Finally, VWD therapy primarily involves the use of either desmopressin (DDAVP) or VWF factor concentrate [1,2]. The same assays that are used to diagnose VWD are also used to monitor therapy for VWD, and to assess the potential clinical utility of factor concentrates. Of additional interest, DDAVP therapy can also be used to assist in VWD diagnosis and typing [6], with various test patterns being observed in different VWD cases. Recently, the VWF:Act assay was proposed as a possible suitable 'alternative' to the VWF:RCo assay in terms of assessing VWF factor concentrates [39]. The current study, however, would caution against the expectation that the VWF:Act and VWF:RCo assays will provide the same information in such assessments. Indeed, although a given factor concentrate may provide the same value for VWF:RCo and for VWF:Act, this is not the same as identifying these assays as being equivalent for this purpose. Thus, on the basis of the current study, it can be predicted that the VWF:Act assay will provide higher VWF values than the VWF:RCo assay for VWF concentrates that are somewhat devoid of HMW VWF. Although potentially favorable to manufacturers of VWF concentrates, as this gives the impression of more favorable characteristics, this is not recommended practice.

Although the sample production used in this study reflected an in vitro process to reduce the levels of HMW and IMW VWF, we do not believe that this caused any substantial reduction in core VWF function as such. This was shown by testing with various mAbs against VWF, including functional sites on VWF, which showed comparable data to those obtained with polyclonal RαVWF material (Data S1). This would, in essence, suggest an intact VWF functional GPIb-binding site. This is also consistent with current knowledge that identifies very few cysteine molecules within the A domains of VWF [38]. Moreover, the FVIII/VWF:Ag ratio also remained fairly stable across the range of samples, again suggesting an intact VWF functional FVIII-binding site. Although there was a slight drop in the FVIII/VWF:Ag ratio across the samples, this was more likely related to the slight increase in VWF:Ag observed than to any fall in FVIII:C (Fig. 2A), with this presumably reflecting greater accessibility of the antibodies to the VWF in the samples [29] rather than an increase in VWF:Ag as such.

In conclusion, we report on a cross-laboratory evaluation of VWF testing with a range of assays and samples selectively depleted in HMW and, in some cases, IMW VWF. Differences in the ability of 'activity' assays to detect this loss were observed, with the VWF:RCo and VWF:CB assays being similar and showing the highest sensitivity, and the VWF:Act assay showing lower sensitivity. We believe that these findings have significant implications for clinical practice in a variety of settings, namely diagnostic and therapy management practice for both VWD and TTP. In particular, diagnostic and therapy management of both VWD and TTP rely, in part, on assays that are defined as being sensitive to the loss of HMW VWF, but not all 'activity' assays show similar sensitivities to the loss of HMW VWF. There now remains a stock of reserved samples prepared for this study that can be used for ongoing EQA, or for a more extensive, perhaps expert laboratory-based study, similar to that previously reported for patient samples [40]. A clinical validation study, showing comparative findings of plasma samples from NAC-treated TTP patients in a clinical trial setting and cotested by different VWF activity assays, would also seem to be warranted.

Addendum

E. J. Favaloro: conceived, designed and coordinated the study, prepared the study samples, undertook preliminary testing of study samples, participated in method validation, undertook data analysis, and wrote the manuscript; R. Bonar: arranged and oversaw the logistics of the main study, arranged for sample lyophilization, and contributed to data analysis; K. Chapman: assisted in study design, participated in preliminary testing of study samples and method validation, and participated in the main study; M. Meiring: participated in preliminary testing of study samples and method validation, and participated in the main study. D. Funk (Adcock): participated in preliminary testing of study samples and method validation, and participated in the main study. D. Funk (Adcock)'s laboratory acted as the study reference center for multimer analysis. All coauthors contributed to manuscript revision, and have approved the final manuscript for publication.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Extended description of materials and methods related to the validation study.
Figure S1. Main initial validation dataset.
Figure S2. VWF:Ag testing of test samples by the host laboratory with either rabbit antibody (RaVWF) or various mAbs (CR1, CR2, 5D2, 6G1) used as VWF detection antibodies.
Figure S3. VWF testing by ELISA with mAbs as VWF capture antibodies.
Table S1. Detailed summary of test sample set and characteristics.

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Full Length Article

Differential sensitivity of von Willebrand factor activity assays to reduced VWF molecular weight forms: A large international cross-laboratory study

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ABSTRACT

Introduction: von Willebrand disease (VWD), the most common inherited bleeding disorder, is due to deficiencies/defects in von Willebrand factor (VWF). Effective diagnosis requires testing for FVIII, VWF antigen and one or more VWF 'activity' assays. Classically, 'activity' is assessed using ristocetin cofactor (VWF:RCo), but collagen binding (VWF:CB) and/or other assays are used by many laboratories. This extensive international cross-laboratory study has specifically evaluated contemporary VWF activity assays for comparative sensitivity to reduction in high molecular weight (HMW) VWF, and their ability to differentiate type 1 vs 2A VWD-like samples.

Materials and methods: A set of four samples representing step wise reduction in HMW VWF were tested by over 400 laboratories worldwide using various assays. A second set of two samples representing type 1 or type 2A VWD-like plasma was tested by a subset of 251 laboratories.

Results: Combined data identified some differences between VWF activity assays, with sensitivity for reduction of HMW being highest for VWF:CB and VWF:GPIbM, intermediate for VWF:RCo and VWF:GPIbR, and lowest for VWF:Ab. 'Within' method analysis identified the Stago method as the most sensitive VWF:CB assay. A large variation in inter-laboratory CV (e.g., 7–24% for the normal sample) was also demonstrated for various methods. Although performance of various methods differed significantly, most laboratories correctly differentiated between type 1 and 2 samples, irrespective of VWF activity assay employed.

Conclusions: These results hold significant clinical implications for diagnosis and therapy monitoring of VWD, as well as potential future diagnosis and therapy monitoring of thrombotic thrombocytopenic purpura (TTP).

1. Introduction

von Willebrand disease (VWD) is reportedly the most common inherited bleeding disorder, and suspected following clinical and physical examination in individuals with personal and familial evidence of mucocutaneous bleeding, as later confirmed by laboratory testing [1,2]. VWD is classified into six types, representing quantitative or qualitative defects/deficiency in plasma von Willebrand factor (VWF). Types 1 and 3 VWD respectively define partial or complete loss of VWF, and types 2A, 2B, 2M and 2N define qualitative disorders [1,2]. Particularly, 2A VWD is characterized by decreased VWF-dependent platelet adhesion due to selective deficiency of high-molecular-weight (HMW) VWF multimers, 2B VWD by an increased affinity of VWF for platelet glycoprotein Ib (GPIb), often also associated with loss of HMW VWF, 2M VWD by impaired VWF-dependent platelet adhesion without a selective deficiency of HMW VWF, and 2N VWD by impaired VWF-Factor VIII binding [2]. For type 1 VWD, most guidelines now advise that diagnosis of VWD should be restricted to those with VWF levels below 30 U/dL,

Abbreviations: ELISA, enzyme linked immunosorbent assay; GPIb, glycoprotein Ib; HMW, high molecular weight (VWF); NAC, *N*-acetycysteine; TTP, thrombotic thrombocytopenic purpura; VWF, von Willebrand factor; VWF:Ab, VWF activity assays based on the binding of a monoclonal antibody (MAB) to a VWF A1 domain epitope; VWF:Ag, VWF antigen (protein level); VWF:CB, VWF collagen binding; VWF:GPIbM, VWF activity assays based on spontaneous binding of VWF to a gain-of-function mutant GPIb fragment; VWF:GPIbR, VWF activity assays based on ristocetin-induced binding of VWF to a recombinant wild type GPIb fragment; VWF:RCo, VWF ristocetin cofactor; VWD, von Willebrand disease

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with those having levels above 30, but lower than the normal cut-off value, be considered as representing 'low VWF' as a risk factor for bleeding [1,3]. VWD can also be identified as an acquired disorder, where it is usually termed acquired von Willebrand syndrome (AVWS).

Laboratory testing is focused on assessment of plasma factor VIII coagulant (FVIII:C) activity, VWF antigen (VWF:Ag), and various VWF 'activities', supplemented as required with other tests on a case by case basis [1–4]. Classically, VWF 'activity' was assessed using ristocetin cofactor (VWF:RCo), originally described in the early 1970s [4–6]. However, inherent problems, including complexity, long performance time, poor reproducibility, and poor sensitivity to low levels of VWF (recently reviewed in [4]), has focused attention on newer assays, including collagen binding (VWF:CB), and several others based either on GPIb binding or monoclonal antibody (MAB) technology [4,6]. This has also seen recent emergence of new proposed assay classifications for the 'platelet dependent VWF activity' group of assays [4,7], with the additional recommended abbreviations VWF:GPIbR, VWF:GPIbM and VWF:Ab. These assays may or may not utilize ristocetin, or even platelets, to identify VWF 'activity' [4,6,7].

Used in conjunction with VWF:Ag and FVIII:C, VWF activity assays have the capacity to identify and discriminate various types of VWD. In particular, low levels of VWF with concordance of antigen and 'activity' suggests a type 1 VWD (or 'low VWF'), but discordance of VWF 'activity' to antigen (usually expressed as a ratio below 0.6) suggests a type 2 VWD. Also relevant here is the increasing recognition of AVWS [1], especially those associated with cardiac defects or cardiac assist devices, where loss of HMW VWF is associated with increased risk of bleeding [8].

Nevertheless, the comparative behavior of different VWF 'activity' assays is not well recognized, either in diagnosis of VWD or for relative sensitivity to HMW VWF multimers. In the current study, we have evaluated the comparative sensitivity of different VWF 'activity' assays to reduction in HMW VWF multimers, as well as for relative utility to discriminate type 1 vs type 2 VWD-like samples. This study represents a large international cross-laboratory investigation comprising over 400 laboratories distributed all over the world.

2. Material & methods

2.1. Study aims and initial preparation and testing of study samples

The study sample set as prepared and later dispatched to laboratories is summarized in Table 1. The samples were produced by the lead author (EJF) in the laboratory of Haematology (Institute of Clinical Pathology and Medical Research, ICPMR, Westmead), primarily following treatment of a plasma pool of normal individual plasmas. There were two parts to this study. In study 'Part 1', four samples ('V1' to 'V4'; Table 1) were prepared to reflect sequential reduction of HMW VWF multimers, whilst aiming to retain levels of VWF relatively constant. These samples comprised the normal plasma pool, and three similar volume aliquots of this pool after treatment to produce step-wise reduction of HMW VWF multimers, using a propriety process of disulphide bond reduction with *N*-acetycysteine (NAC), as per a previous smaller study [9]. Although such treatment results in reduction of HMW VWF multimers, the native VWF remains normally active [9,10]. Given our desire to purpose generate material with predefined characteristics, namely a starting sample with ~100 U/dL VWF:Ag, and because the pool initially provided VWF levels closer to 115 U/dL, all samples were diluted slightly (~15%) with saline. Minor dilution of samples with saline or else dilution with plasma deficient in VWF is now a standard tool for preparation of 'artificial VWD' test samples for use in the Royal College of Pathologists (RCPAQAP) Haematology external quality assessment (EQA) practice, and in such experience, does not adversely affect lyophilization or selectively affect functional VWF properties. The first sample in the series of four (i.e., 'V1'; see Table 1) thus represented an essentially normal pool sample for the purpose of this exercise, with starting VWF:Ag level of ~100 U/dL, and similar levels of VWF 'activity' and FVIII. The second sample ('V2') would represent a minor loss of HMW VWF multimers, which might occur with a normal sample subsequent to a pre-analytical event (e.g., refrigeration of whole blood sample; provision of serum instead of citrate plasma; provision of clotted plasma sample) [11]. The last two samples (i.e., 'V3', 'V4') would show increasing reduction of HMW VWF multimers and then intermediate molecular weight (IMW) VWF multimers, potentially reflective of type 2A or 2B VWD-like plasma, but with a relative high VWF level, which is a pattern that might occur in type 2A or 2B VWD during pregnancy [12], or in AVWS associated with cardiac defects/assist devices [8]. The main aim of the 'Part 1' study was to identify the comparative sensitivity of different VWF activity assays to identify the (sequential) reduction in HMW VWF multimers in these samples, with associated inference on VWD diagnostics.

Secondly, 'Part 2' comprised cross-laboratory testing of two additional plasma samples, also derived subsequent to the above described process. Both samples were purpose constructed to generate samples with similar levels (~25–30 U/dL) of VWF:Ag, but one sample was engineered to behave like a type 1 VWD sample (VWD-1 'mimic'), with VWF activity remaining concordant to VWF:Ag, and with normal VWF activity/antigen ratios (i.e., > 0.7; identified as 'V5' in Table 1), whilst the second sample was engineered to behave like a type 2A VWD sample (VWD-2A 'mimic'), with VWF activity discordant to VWF:Ag, and low VWF activity/antigen ratios (i.e., < 0.5; identified as 'V6' in Table 1). These samples were also produced in a similar manner to those of the earlier smaller study [9]. There were several aims to the 'Part 2' study, including: (i) identifying the differential utility of diverse VWF activity assays to identify type 1 vs 2 VWD test patterns; (ii) whether artificially generated samples could be utilized in cross-laboratory studies (e.g., for EQA purposes) instead of native patient samples, especially given ethical and practical difficulties in obtaining

Table 1
Summary of test sample set and characteristics.

Sample ID	Sample comprised	Sample intended to, or could feasibly, represent
V1	Pool of normal plasmas	Normal plasma. VWF:Ag level of ~100 U/dL, and similar levels of VWF 'activity' and FVIII.
V2	Sample V1 treated to yield minor loss of HMW VWF	Normal plasma with minor loss of HMW VWF as might be caused by a pre-analytical event (see main text)
V3	Sample V1 treated to yield greater reduction of HMW VWF	Type 2A (or 2B) VWD like plasma as might be obtained in pregnancy.
V4	Sample V1 treated to yield high reduction of HMW VWF	Type 2A (or 2B) VWD like plasma as might be obtained in pregnancy.
V5	Mixture of V1 and VWF deficient plasma	Moderate/mild type 1 VWD like plasma with target VWF:Ag 25–30 U/dL and normal VWF:Activity/Ag ratio (> 0.7)
V6	Mixture of HMW VWF deficient sample and VWF deficient plasma	Type 2A/2B VWD like plasma with target VWF:Ag 25–30 U/dL and low VWF:Activity/Ag ratio (< 0.5)

Sample V1 represents a normal pool plasma and the main plasma used to then generate subsequent test samples. Samples V2, V3, V4 were derived from sample V1 and reflect sequentially increasing reduction of HMW/IMW VWF. Samples V5 and V6 were respectively derived from mixtures of VWF deficient plasma and V1 or a HMW VWF deficient sample to produce samples reflective of moderate type 1 VWD and 2A/2B VWD, but with a similar level of VWF:Ag. Abbreviations: HMW, high molecular weight; IMW, intermediate molecular weight; VWD, von Willebrand disease; VWF, von Willebrand factor.

Table 2
Summary test statistics and breakdown of main study methods used by study participants.[a]

Test/methodology	No. of study results[b]	% of total[c]	% of method[d]	Mean values in U/dL (CV; %)				Mean low reference value in U/dL (CV; %)[e]
				V1	V2	V3	V4	
VWF:Ag	406	100.0	100.0	97.1 (8.0)	92.7 (9.8)	74.4 (13.8)	92.9 (19.6)	51.5 (13.0)
LIA	328	80.8	80.8	97.3 (6.9)	92.0 (7.4)	74.2 (11.9)	92.7 (18.5)	51.8 (12.3)
ELISA (E)	30	7.4	7.4	96.6 (14.4)	96.2 (17.5)	73.6 (16.0)	90.5 (25.4)	48.3 (14.4)
VWF:RCo	222	54.7	100.0	74.3 (16.7)	63.4 (18.1)	27.3 (30.1)	15.8 (> 40)	51.2 (11.6)
Aggregometer (Agg)	63	15.5	28.4	71.4 (20.7)	63.2 (23.8)	30.2 (30.9)	13.7 (> 40)	51.1 (13.0)
Automated (Auto)	88	21.7	39.6	71.1 (14.4)	61.5 (14.5)	24.1 (32.3)	15.1 (> 40)	51.4 (10.9)
VWF:GPIbR	53	13.1	23.9	83.4 (9.9)	67.3 (10.5)	28.6 (17.0)	19.3 (26.9)	50.6 (11.9)
VWF:CB	128	31.5	100.0	89.2 (18.2)	76.5 (21.2)	29.0 (> 40)	11.0 (> 40)	51.2 (14.7)
In-House	14	3.4	10.9	85.4 (19.2)	72.1 (21.7)	24.1 (> 40)	8.8 (> 40)	48.5 (19.9)
Stago	21	5.2	16.4	90.4 (24.4)	66.6 (15.1)	17.2 (27.3)	6.9 (> 40)	53.7 (12.6)
Technoclone	41	10.1	32.0	94.1 (13.8)	86.7 (15.4)	37.4 (22.7)	16.5 (37.0)	49.7 (13.6)
VWF activity other								
VWF:GPIbM	87	21.4	100.0	74.1 (8.1)	62.3 (8.3)	24.9 (27.0)	11.5 (33.5)	49.3 (15.8)
VWF:Ab	85	20.9	100.0	80.2 (13.6)	66.7 (13.9)	35.2 (28.0)	25.0 (> 40)	50.3 (15.6)
Multimers	36	8.9	100.0	NA	NA	NA	NA	

Abbreviations: CV, coefficient of variation; VWF:Ag, von Willebrand factor antigen; LIA, latex immuno assay; ELISA, enzyme linked immunosorbant assay; N/A, not applicable; VWF:CB, von Willebrand factor collagen binding; VWF:RCo, von Willebrand factor ristocetin cofactor. VWF:GPIbR, VWF:GPIbM and VWF:Ab refer to methodologies as recently proposed abbreviations from the VWF ISTH SSC [7] – refer to Material & methods text for additional details.

[a] Summary data for the 'part 1' study, comprising data from all participants.

[b] From all participants; all provided test data for VWF:Ag, mostly as performed using LIA, with smaller group using ELISA (E) and other/non specified methods (data not shown). Thereafter, varied tests for VWF 'activity' performed by various laboratories as shown.

[c] Percent of total participants reporting results for respective test group.

[d] Percent of participants reporting results within a specific methodology group (e.g., for VWF:Ag, or VWF:RCo, or VWF:CB). VWF:GPIbM, VWF:Ab and Multimers are considered as individual overall methods (i.e. = 100% of that method group).

[e] Mean of study participant lower reference range value (=cut-off for normal/abnormal), and inter-laboratory CVs for these values.

large quantities of diverse patient plasma for such purpose.

The entire set of six samples (i.e., V1–V6; Table 1) were lyophilized in vials in 1.0 mL volumes, and thereafter stored refrigerated at 4 °C, prior to sending to three collaborative EQA organizations (as anonymized, sequence relabeled sets), for further distribution to participants of the cross-laboratory study. Lyophilized samples passed internal homogeneity and stability testing prior to use in the study (data not shown). However, it was noted that sample 'V3' yielded an unexpectedly lower level of VWF:Ag than samples V1, V2 and V4. We cannot explain this finding, although it is feasible that during production, sample 'V3' was inadvertently double diluted with saline to ~30% (i.e., 2× ~ 15%). If this is the explanation, then this would not expectedly affect the main study aim, being the comparative sensitivity of VWF activity assays to reduction in HMW VWF or even activity/Ag ratios, since all test analytes would be similarly 'diluted'. Moreover: (a) all samples were tested blind by participants, using a relabeled sequence of vials, and who did not know that samples V1 to V4 were 'related'; (b) all samples were also interpreted as discrete samples, and so the interpretations for sample V3 would be for the sample as presented, and thus still valid; (c) there would be no expected adverse outcome in terms of coefficients of variation (CVs).

2.2. Cross-laboratory study

All laboratories performing VWF testing and currently enrolled in any of the noted EQA programs (i.e., RCPAQAP Haematology; UK NEQAS (National External Quality Assessment Schemes) coagulation; and ECAT (External quality Control for Assays and Tests)) were invited to participate in this cross-laboratory study. Participants of RCPA (n = 68) and ECAT (n = 197) tested all six samples (V1–V6); participants of NEQAS (n = 163) tested only the four samples (V1–V4) comprising study Part 1. All invited laboratories received vials of the anonymized samples, together with sample reconstitution and test instructions, essentially requesting laboratories to perform their normal test panels for investigation of VWD. Laboratories were also asked for details of test methodology and to attempt to interpret their test results. Choices offered included normal/not VWD, VWD type 1 (mild, moderate, severe or unspecified), VWD type 2 (2A, 2B, 2M, 2N or undefined), VWD type 3, or 'other interpretation' (with a request to accordingly specify). Some of these choices (e.g., type 2N VWD, type 3 VWD) did not reflect any of the samples, but a complete range of possible interpretations was provided to avoid any biasing of returned data. The various type 2 choices were largely aimed to identify participant responses for samples V3, V4 and V6, with 'type 2 undefined' permitted because respective identification of type 2 'subtypes' would require additional investigations to those reported in this study (e.g., ristocetin induced platelet aggregation to differentiate 2A and 2B VWD). The various type 1 choices were largely aimed to identify participant responses for sample V5, and to identify laboratory perceptions around VWD 'severity'.

2.3. Data/statistical analysis

Numerical data was largely analyzed by way of comparative medians, means, ranges and inter-assay (inter-laboratory) CVs, using GraphPad Prism (GraphPad Software Inc., La Jolla, CA 92037 USA; [www.graphpad.com]). When reported, statistical comparisons reflect non-parametric assessment using the Mann-Whitney test and a two-tailed analysis, with a p-value < 0.05 being considered statistically significant. Laboratory numerical data was used as reported by participants, except that values reported as ' < ' a given value were adjusted downwards to permit numerical analysis (e.g., an assay value of < 10 was adjusted to 9, and an assay ratio < 0.2 was adjusted to 0.1). This is consistent with EQA practice of some programs. These events generally occurred only for samples with high loss of HMW VWF multimers. Data for FVIII has been excluded from this report, which has focused on VWF activity assays.

Although different laboratories apply different normal references ranges to VWF tests, values above 50 U/dL are generally considered as 'normal', and so this 'cut-off' is used in this report as a general guide. Similarly, laboratories apply different ranges to VWF activity to antigen ratios; however, values above 0.7 are generally considered as 'normal',

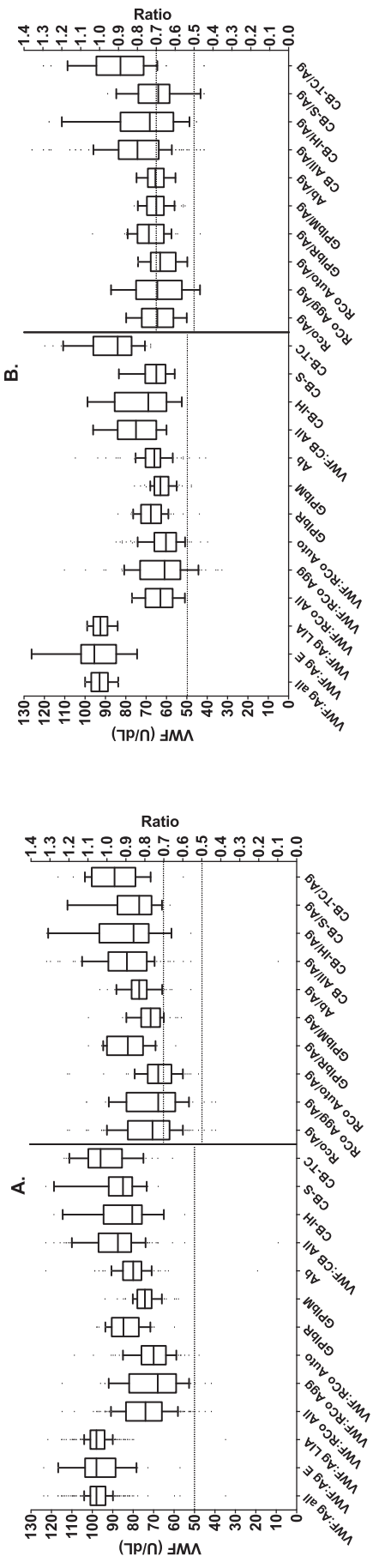


Fig. 1. Main study ('Part 1') data as derived from samples V1–V4, as tested by all participating laboratories and shown as a box plot (median, with 25th and 75th percentile identified by the box, and 10th and 90th percentile identified by the error bar), for assay groups as per Table 2. VWF test indices on left portion of each figure with values on left y-axis and the horizontal dashed line representing a nominal 'normal/abnormal' cut-off value of 50 U/dL. Assay ratios shown on the right portion of each figure, with horizontal dashed lines at 0.5 and 0.7 representing nominal 'abnormal' and 'normal' cut-off values. Panels A–D respectively show data for samples V1–V4 (see Table 1).

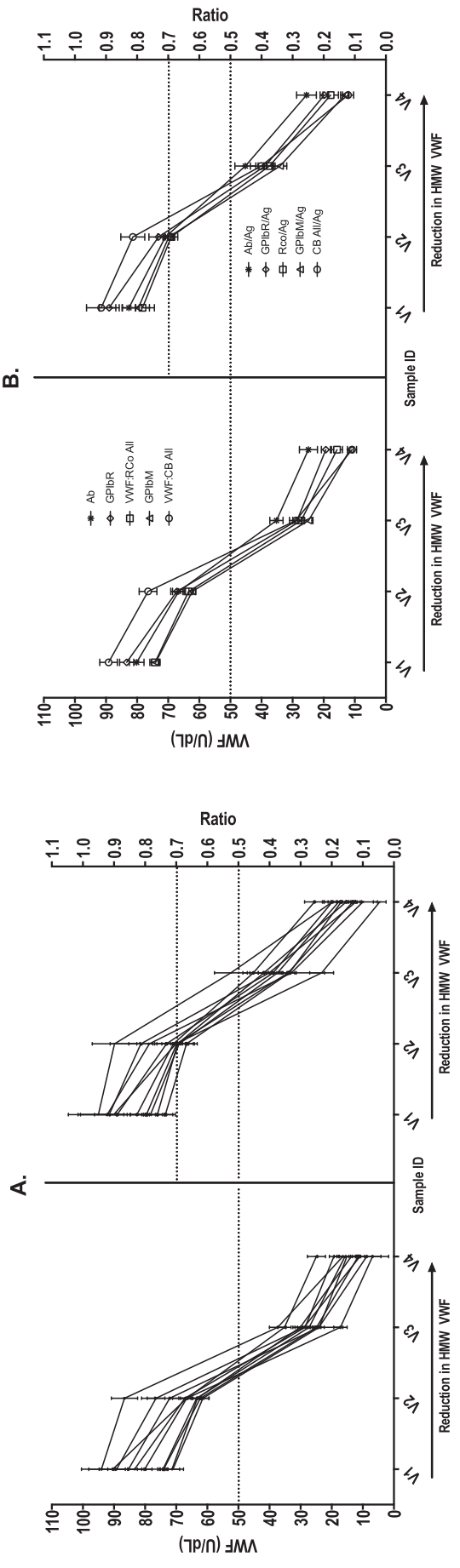


Fig. 2. Main study ('Part 1') data as derived from samples V1-V4, as tested by all participating laboratories and shown as plots of mean data (with 95% confidence intervals), for assay groups as per Table 2. VWF test indices on left portion of each figure with values on left y-axis and the horizontal dashed line representing a nominal 'normal/abnormal' cut-off value of 50 U/dL. Assay ratios shown on the right portion of each figure, with horizontal dashed lines at 0.5 and 0.7 representing nominal 'abnormal' and 'normal' cut-off values. Panels A–D respectively show data for all assay ratios to reflect spread of all data (A), main VWF activity groups (B), GPIb binding assays (C), VWF:CB (D).

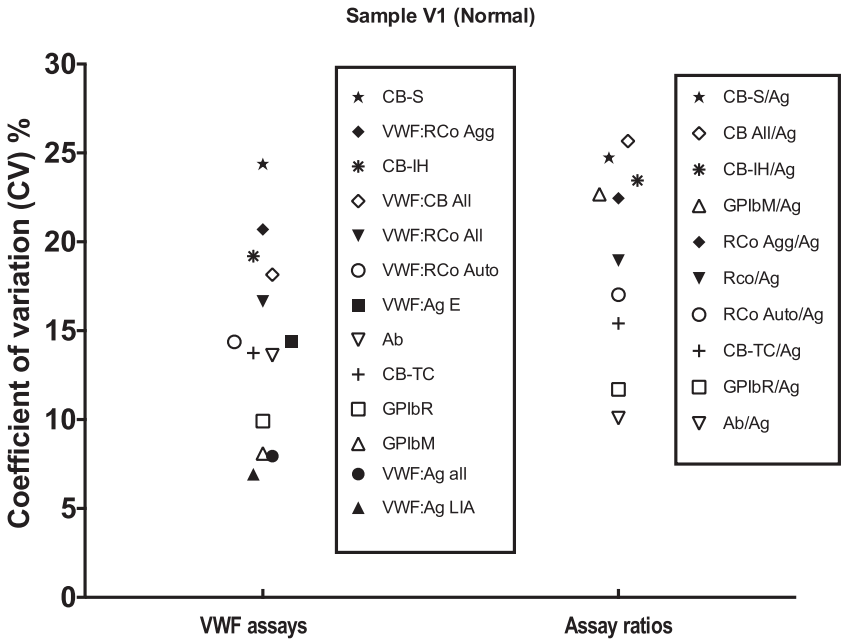


Fig. 3. Inter-assay variation as co-efficient of variation (CV; %) for main (Part 1) study data for sample V1 for different assays and assay ratios.

values below 0.5 are generally considered as 'abnormal', and values between 0.5 and 0.7 are variably assigned to either category depending on the laboratory and the VWF activity assay [4].

2.4. Additional notes on 'VWF activity assays' and nomenclature

For the purposes of this study, we have essentially followed the recommended nomenclature and abbreviations of the VWF ISTH SSC, as previously published [7,13] and recently reviewed [4], in order to compare study outcomes. We have also assessed for differential sensitivity for major 'sub-methodologies', essentially as summarized in Table 2. We have included in the overall 'VWF:RCo' group, all methodologies that employ ristocetin, whether or not employing platelets, but also show data separately for methods employing platelets and tested by aggregometer ('Agg') or automated instrument ('Auto'), as well as ristocetin based methods not employing platelets ('VWF:GPIbR'). For VWF:CB, we have assessed 'all VWF:CB data', as well as the three main 'sub-methodologies' in use (namely, 'in-house', Diagnostica Stago, and Technoclone), although also recognizing that 'in-house' VWF:CB assays are quite heterogeneous in methodology. Finally, we have also separately analyzed the other major VWF activity assays (VWF:GPIbM and VWF:Ab [4,6,7]).

3. Results

The main study data is summarized in Table 2 and main findings for both study Parts 1 and 2 shown in Figs. 1–4. Sample VWF multimer patterns are shown in Fig. 5, together with participant interpretations for VWF multimer patterns for all test samples, and final participant interpretations for all test samples. Densitometry patterns for VWF multimers were as expected (data not shown). As expected, normal test results were generally reported for sample V1 for all VWF tests, and for VWF:Ag for all samples in Study Part 1 (i.e., V1, V2, V3, V4; Table 2 and Fig. 1). However, VWF activity assay values for the 'Study Part 1' samples showed sequential reductions, in line with sequential reduction in HMW VWF multimers (Table 2; Figs. 1 and 2). Relative sensitivity of the various VWF activity assays for the reduction in HMW VWF multimers can be compared by determining which assay measured the lowest VWF activity level in the samples with the highest reduction in HMW VWF (i.e., V4). Of relevance, a true patient sample will be defined as having VWD earlier, and overall more consistently, should the utilized assay measure lower VWF activity levels compared to an assay that measures much higher levels in the same sample, and having a similar reference interval. Of interest, all assay reference range lower cut-off values had similar mean values (all close to 50 U/dL; Table 2). Similarly, VWF activity to antigen ratios also fell sequentially in samples V1 to V4, being essentially within normal limits for V1, but abnormal for sample V4 (Table 2; Figs. 1 and 2). Better detail around VWF activity to antigen ratios is provided in Fig. 2, where data has been separated according to VWF activity assay. Although all VWF activity/Ag ratios fell sequentially according to reduction in HMW VWF, there was notable variability in sensitivity according to VWF activity assay. More specifically, sensitivity for reduction of HMW was overall highest for VWF:CB, as compared with VWF:Ab ($p < 0.0001$), VWF:GPIbR ($p < 0.0001$), VWF:RCo ($p < 0.0001$), but was similar to VWF:GPIbM ($p = 0.053$). Moreover, 'within' method analysis identified better HMW VWF sensitivity of the Stago VWF:CB assay, which yielded for sample V4 the lowest values compared to other separate methods (e.g., $p < 0.0001$ for comparison with Technoclone).

Assay variation is also identified (as inter-laboratory/inter-method CVs) in Table 2 (summarized for all samples V1–V4) and Fig. 3 (for sample V1). In brief, CVs ranged from 7 to 24% for the normal sample (V1), but not unexpectedly increased as the VWF activity level fell (Table 2). The lowest CVs (below 12.5%) were observed for VWF:Ag (LIA), VWF:GPIbM and VWF:GPIbR methods.

Summary test results for the type 1 and 2A VWD-mimic samples (respectively V5 and V6; Table 1) are shown in Fig. 4. As expected, both samples yielded similar VWF:Ag test results (~25–30 U/dL). However, whilst V5 expressed similar VWF activity levels, and thus normal VWF activity/antigen ratios, consistent with a type 1 sample, sample V6 yielded discordant VWF activity levels, and thus abnormal VWF activity/Ag ratios, consistent with a type 2 sample.

Interpretations for VWF multimer patterns is shown in Fig. 5. As expected, most participants identified sample V1 as expressing normal multimers, and samples V3, V4 and V6 as expressing 'abnormal multimer patterns' or 'type 2A/2M/2B/2U' VWD (NB: U for 'undefined'). Furthermore, most participants identified sample V5 as expressing 'normal multimer patterns' or type 1 VWD.

Lastly, final interpretations for all test samples is also shown in Fig. 5. As expected, most participants identified sample V1 as normal,

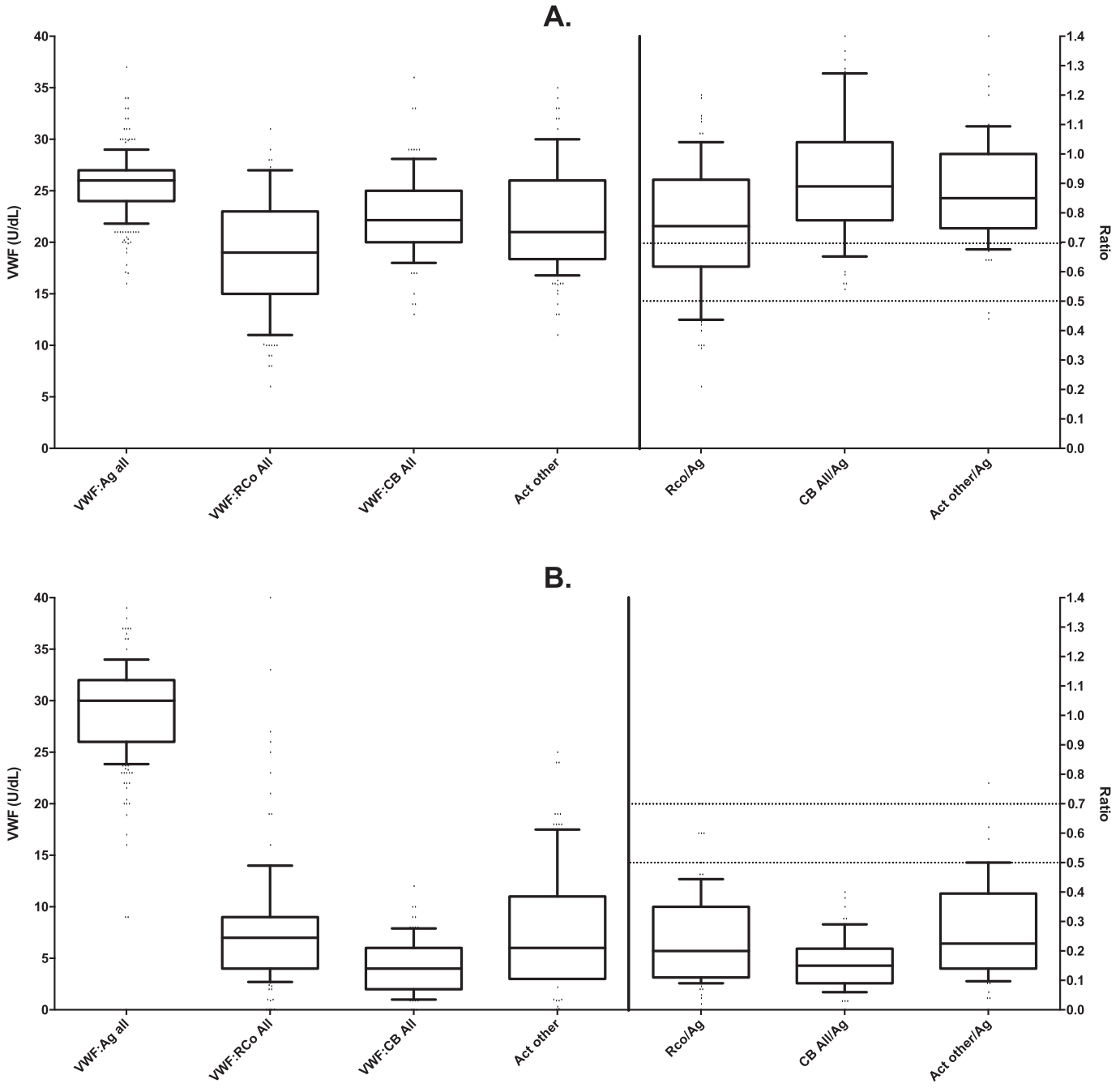


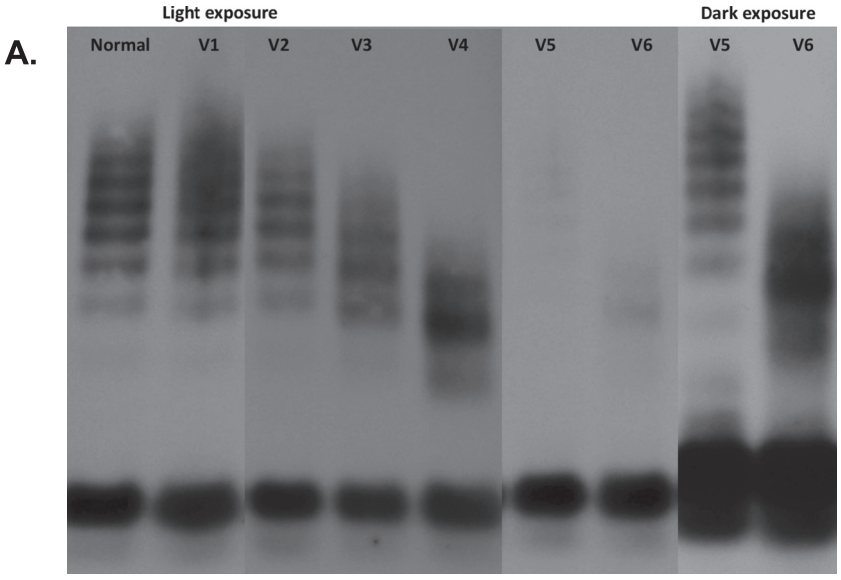
Fig. 4. Summary of 'Part 2' study data as derived from samples V5 (A) and V6 (B), as tested by participating laboratories and shown as a box plot (median, with 25th and 75th percentile identified by the box, and 10th and 90th percentile identified by the error bar), for main VWF assay groups. VWF test indices on left portion of each figure with values on left y-axis and assay ratios shown on the right portion of each figure, where horizontal dashed lines at 0.5 and 0.7 represent nominal 'abnormal' and 'normal' cut-off values.

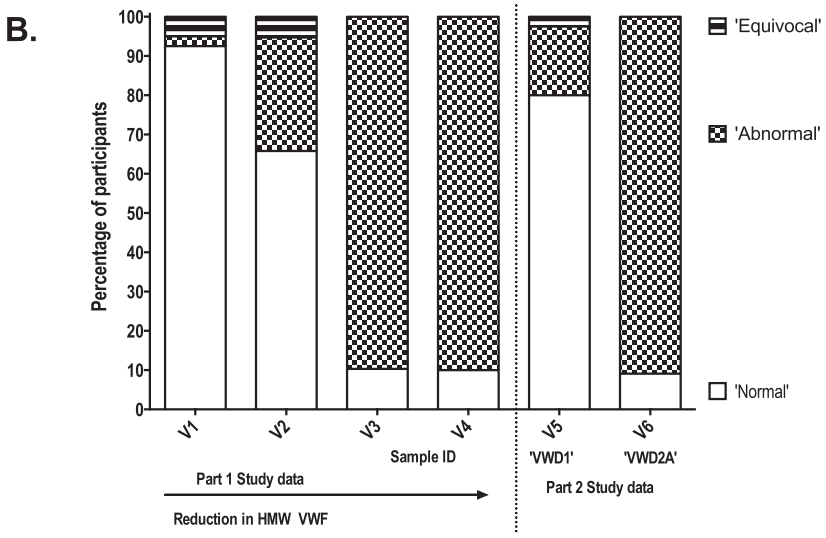
and samples V3, V4 and V6 as 'type 2A/2M/2B/2U' VWD. Most participants identified sample V5 as type 1 VWD, with perceived 'severity' sub-analysis also reported by some participants.

4. Discussion

To our knowledge, this represents the largest comparative multicenter assessment of the most widely used VWF 'activity' assays for their relative sensitivity to reduction of HMW VWF multimers. VWF:RCo, the first functional VWF assay described in the early 1970s [5,6], reflects a VWF - GPIb binding assay, albeit facilitated by ristocetin. VWF:CB, first described in 1986 [14], reflects a different VWF activity, namely collagen (sub-endothelial matrix) binding. A plethora of new VWF 'activity' assays have emerged [4,6,7], many of which also reflect GPIb binding, but may or may not involve ristocetin, or even platelets. Irrespective, laboratories are now utilizing a wide variety of VWF assays, and appreciation of relative behaviors is lacking.

Some of us previously reported on a similar comparative assessment with fewer study participants, a single EQA provider, and the VWF activity assays as then available [9]. The current study in part has similar design, but a much larger global distribution (N = 409 participants: 67.7% Europe, 10.5% North America and 17.4% Asia/Australasia) and an extensive panel of evaluated VWF activity assays, as contemporarily available. Similar to before [9], these assays show





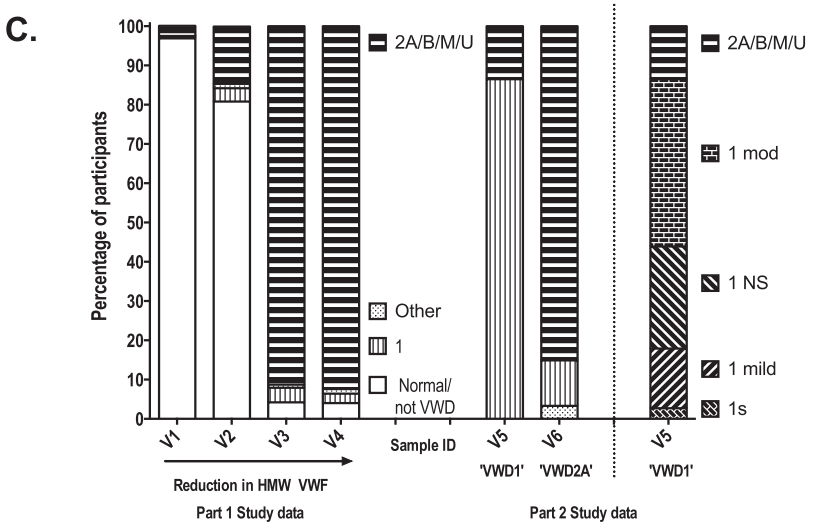


Fig. 5. A. Representative VWF multimer patterns obtained with study samples, and confirming sequential reduction in HMW and IMW VWF multimers in generated samples from V1 to V4, as well as type 1 VWD pattern for V5, and type 2 VWD pattern for V6. VWF multimers shown with light (V1–V6) and dark (V5, V6) gel exposure. Arising densitometry patterns were consistent with shown multimers (data not shown).
B. Participant interpretations for VWF multimers, expressed as a percentage of returned responses. Importantly, most participants identified samples V1 and V5 as expressing normal multimer distribution, and samples V3, V4, and V6 as expressing abnormal multimer distribution (or being type 2 (2A, 2B, 2M or 2U) VWD). However, occasional participants identified the multimer pattern of the normal sample V1 and the type 1 VWD sample as being 'abnormal' or 'equivocal', and some participants identified the multimer pattern of the HMW VWF deficient samples V3, V4 and V6 as being 'normal'. Sample V2 yielded a mixture of interpretations, with some laboratories identifying abnormal multimer patterns, but most reporting normal multimer patterns.
C. Participant 'final interpretations' expressed as a percentage of returned responses. Importantly, most participants identified sample V1 as being normal/not VWD; V3, V4 and V6 as being type 2 (2A, 2B, 2M or 2U) VWD; V5 as being type 1 VWD. However, occasional participants identified HMW VWF deficient samples V3 and V4 as normal, the type 1 VWD mimic sample (V5) as type 2 (2A, 2B, 2M or 2U) VWD, and the HMW deficient sample/type 2A VWD mimic (V6) as a type 1 VWD. More detailed information for V5 is shown in the far-right portion of the figure (S - severe, Mod – moderate, NS – not specified).

different sensitivity to reduction in HMW VWF multimers, with VWF:CB still 'maintaining' greatest sensitivity, and VWF:Ab showing least sensitivity. The current study further identifies the lower sensitivity of VWF:GPIbR and intermediate sensitivity with VWF:RCo and VWF:GPIbM, as well as method 'subgroup' differences. For VWF:CB, the most popular method (Technoclone), incongruously showed highest assay variation (Table 2) and least HMW VWF sensitivity (Fig. 2D), which replicates our previous conclusion [9], and is consistent with prior evaluation of commercial VWF:CB assays [15]. Nevertheless, this relatively 'insensitive' VWF:CB assay still appeared more sensitive than VWF:Ab (Table 2; Figs. 1 and 2). Notably, overall patterns of HMW VWF sensitivity data in this study are similar to that of genuine VWD samples [16].

Our data also demonstrates large variations in CVs among activity methods, with VWF:GPIbM and VWF:GPIbR assays showing lowest CVs, and CVs being highest for VWF:RCo and VWF:CB methods. For VWF:RCo, high CVs are well recognized, in part reflect the variability of 'submethods', and potential non-automation, also helping to drive the development of other assays such as VWF:GPIbM and VWF:GPIbR. For VWF:CB, relative high CVs may be due to use of ELISA technology, and manual and semi-automated processes. In contrast, VWF:GPIbM and VWF:GPIbR both reflect highly automated assays, with homogeneous reagents. In total, such data is also comparable to previous reported values derived from various EQA reports, including genuine VWD samples [16–19]. As heterogeneity of VWD requires performance of multiple diagnostic tests [1–4,6,7,20], since no single test completely defines VWD, the current study will help laboratories and clinicians better understand respective performance specifications of the various assays used in daily practice.

Participants usually interpreted test results appropriately (Fig. 5), although, on occasion, interpretations were at odds with sample type. Notably, HMW VWF multimer reduced samples V3 and V4 were occasionally identified as 'normal/not VWD', and samples V5 and V6 as 'type 2' and 'type 1' VWD respectively. Such 'errors' in interpretation, as related to laboratory identification/exclusion of VWD have been previously reported [16–19], inclusive of genuine VWD samples, and are consistent with occasional 'misdiagnoses' of VWD. These events may be due to transcription error, limitations in VWF test panels employed, and/or misinterpretations of test data (which is instead consistent with sample type).

The current study, like the earlier small study [9], has utilized a propriety process that employed *N*-acetycysteine (NAC) to achieve stepwise reduction in HMW VWF multimers. This in vitro process may mimic a natural in vivo process, described but incompletely characterized, that permits reduction of VWF in the absence of ADAMTS-13 [21], and also possibly assists formation of VWF complexes at a thrombus by a process of self-association [22]. NAC and 'ADAMTS-13 alternative' in vivo VWF processing activities may also have potential therapeutic benefit in thrombotic thrombocytopenic purpura (TTP), with clinical trials in process [23–25]. Should NAC or 'analogous' therapies become utilized in TTP, then laboratories may need to monitor such treatment, and the current study may provide additional context for this.

Naturally, our study has several limitations. First, all assessed samples were artificially generated and did not reflect genuine VWD patient samples. Nevertheless, study findings reflect positively and suggest such samples can be used as 'VWD-mimics', at least for EQA and cross-laboratory evaluations. Study findings are also consistent with previous reports, including observations with genuine cases of VWD (e.g., [9,15–19]). Importantly, logistical and ethical barriers hinder sample utilization from individuals with specific VWD types for large cross-laboratory studies. Moreover, specifically engineered material can be purpose generated (e.g., samples V5 and V6 had similar low levels of VWF:Ag, but differed according to VWF activity levels), and this would assist to control for interpretation errors based on differing VWF:Ag levels. Furthermore, a recent multi-laboratory study utilizing genuine VWD samples reported similar findings to ours [26]. However, our participants reflect heterogeneous expertise in VWD diagnostics, indicative of standard diagnostic practice, whereas the study of Lee et al. generally comprised 'expert/reference' laboratories [26]. Still, a follow up study reflecting 'expert/reference laboratories' with contemporary VWF activity assays may be useful to confirm our findings. Also, a commutability study co-assessing artificial samples with genuine VWD samples could be performed, albeit on a smaller scale to manage patient donations. Irrespective, participant interpretations in our study were largely consistent with sample type, and error-rates not unlike those previously reported in similar cross laboratory evaluations using genuine VWD samples [16–19,26].

In conclusion, we report a large international cross-laboratory evaluation of VWF testing using different VWF assays, and test samples showing sequential reduction in HMW VWF multimers, as well as samples reflecting type 1 or 2A VWD patterns. VWF 'activity' assays showed differential sensitivity to HMW VWF multimer reduction, even within the same assay category (e.g., VWF:CB). Furthermore, assay CVs ranged from 7 to 24% in the normal pooled plasma, demonstrating imprecision among different methods. Lastly, all VWF 'activity' assays contributed to differentiation of type 1 and 2 VWD patterns and enabled effective identification of reduction in HMW VWF multimers in the type 2 sample. We believe these findings hold significance for clinical practice, including diagnosis and therapy management for VWD (congenital and acquired) and TTP. Findings might also be relevant to identification of AVWS, particularly as associated with cardiac abnormalities and/or implantable devices [8,27], especially given that identification of HMW loss in these cases may be difficult with some assays.

Disclosure

The authors state that they have no interest that might be perceived as posing a conflict or bias.

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Author contributions

EJF: conceived, designed and coordinated the study, prepared study samples, undertook preliminary testing of samples, participated in method validation, undertook data analysis and wrote the original manuscript. RB: responsible for arranging and overseeing the logistics of the study from the RCPAQAP, arranged for sample lyophilizing, contributed to data analysis. SM: Co-ordinated sample testing at the ICPMR. MJH, PM, IJ, TW: contributed to data analysis and responsible for arranging and overseeing the logistics of the study from the perspective of ECAT/NEQAS. All co-authors: contributed to manuscript revision and have approved the final manuscript for publication.

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Peer reviewed **ORIGINAL ARTICLE**

EVALUATION OF A COST-EFFECTIVE ADAMTS13 ANTIGEN ASSAY

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ABSTRACT

Thrombotic thrombocytopenic purpura (TTP) is life-threatening and is characterised by platelet deposition in the microvasculature with thrombus formation in particular organs. This results in thrombocytopenia, microangiopathic haemolytic anaemia, kidney failure and neurological symptoms. It is a rare disorder, but can occur in patients infected with the human immunodeficiency virus (HIV). ADAMTS13 is the 13th member of a disintegrin and metalloprotease with thrombospondin type motifs. Its deficiency causes TTP. Therefore, the measurement of the ADAMTS13 levels in plasma is vital in the diagnosis of TTP and also important in distinguishing it from other thrombotic microangiopathies (TMA's). Unfortunately, commercial ADAMTS13 antigen assays are expensive for healthcare service providers in developing countries. However, several antibodies and antibody pairs have been produced against ADAMTS13 and are commercially available. In this study, we evaluated an in-house ADAMTS13 antigen assay using two different commercial antibodies and compared the outcomes to that of a commercial ADAMTS13 antigen kit by using the plasma of 40 patients with possible HIV-associated TTP and 40 healthy subjects. The Intra- and inter-assay coefficients of variation were calculated as 8% and 7% respectively. The assay gave linear results between 0.78 to 12.5% ADAMTS13. The limit of detection was 0.2%, and the limit of quantification was 0.8%. The correlation of our assay compared to the commercial test kit was excellent, with a R^2 value of 0.9. In addition, the cost of our ADAMTS13 antigen assay was lower than that of the commercial ADAMTS13 antigen test kit. Our cost-effective in-house ADAMTS13 antigen test also produced reliable results. We therefore recommend that this assay be used to diagnose HIV-associated TTP.

KEYWORDS

ADAMTS13 antigen; human immunodeficiency virus (HIV); Thrombotic thrombocytopenic purpura (TTP)

INTRODUCTION

Thrombotic thrombocytopenic purpura (TTP) is a prothrombotic disorder that is characterised by microvascular platelet clumping, resulting in microangiopathic haemolytic anaemia, fragmented erythrocytes (schistocytes), consumptive thrombocytopenia, renal dysfunction and neurological symptoms.[1] Human immunodeficiency virus (HIV) infection precipitates TTP, probably by infecting vascular endothelial cells which results in dysfunction of these cells, thrombin generation and the consumption of the Von Willebrand factor protease, ADAMTS13.[2] The diagnosis of TTP and HIV-associated TTP remains based on clinical history, examination of the patient and the blood film.[1] Assays for measuring ADAMTS13 levels and its function in plasma help to confirm the diagnosis, differentiate TTP and HIV-associated TTP from other thrombotic microangiopathies (TMA's) and to monitor the efficacy of treatment. These assays are also helpful in the decision to begin additional or alternative therapy.[3] In a previous study, we suggested the use of ADAMTS13 levels as a confirmatory test for HIV-associated TTP. The measurement of ADAMTS13 activity using the FRET assay did not confirm the disease and auto-antibodies to ADAMTS13 were found in only 50% of patients with HIV-associated TTP.[4]

Although ADAMTS13 antigen kits are available, it is still an expensive test for small laboratories and for the patients from lower income countries. We have developed a cost-effective ELISA test, using commercial antibodies to ADAMTS13. We have also evaluated the technical performance of this assay with the use of plasma samples from 40 healthy subjects and 40 patients with suspectedHIV-associated TTP and compared it to a commercial test kit.

MATERIALS AND METHODS

Subjects

Peripheral blood from 40 healthy subjects and 40 patients with suspected HIV-associated TTP were collected into sodium citrate. The Health Sciences Research Ethics Committee of the University of the Free State approved this study in 2016 with ethics approval number: 92/2016.

Sample collection and preparation

Peripheral blood was collected into VacutainerTM tubes (BD Vacutainer Systems, Plymouth, UK) containing 0.105M sodium citrate with a ratio of 1:9 to blood. Platelet-poor plasma was prepared by centrifugation at 200xg for 20 minutes at room temperature and samples were stored in polypropylene tubes at -70°C until analysed. All tests were performed on the original aliquots that had not been previously thawed.

Laboratory tests

In-house assay:

A 96-well plate (Nunc) was coated for two days at 4°C with

50ng/ml of a mouse anti-human ADAMTS13 antibody (R&D Systems, USA; 100µl per well). After washing with PBS with 0.1% Tween-20 (Sigma, USA), 100µl of the patient's plasma, volunteer's plasma or standard plasma was added and incubated for 2 hours at 37°C. Plasma samples from patients and volunteers were diluted 1:10 in PBS/Tween-20 of which 100µl was added to two wells and anysed in duplicate. The first International standard for ADAMTS13 (NIBSC, UK) was used as a calibrator and added in the following dilutions: 100%, 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56%. A blank sample was also included and served as a negative control. After washing the plate four times with a microplate washer (VACUTEC, South Africa), 2µg/ml of a rabbit anti- human ADAMTS13 detection antibody (Santa Cruz, USA; 100µl per well) was added and incubated for 1 hour at room temperature. The plate washing was repeated four times and 200ng/ml of a goat anti-rabbit horseradish peroxidase (HRP) conjugated antibody (100µl per well) was added and incubated for 1 hour, at room temperature. The plate was further washed for another four times. The colour was developed for 20 minutes by adding a substrate solution ortho-phenylenediamine (0.05%) in 0.1M citrate-phosphate buffer, pH 5.0 containing 0.03% H_2O_2 (90µl per well). The reaction was then stopped by adding 100µl of 4M H_2SO_4. The optical density (OD490-630) of the solution on the plate was read in a Synergy HT spectrophotometer (Biotek, USA) and an eight-point standard curve with the known ADAMTS13 concentrations of the International Standard (Fig 1A) was drawn. The ADAMTS13 level for each patient and normal volunteer was read from the standard curve.

Commercial assay:
The Immubind ADAMTS13 antigen kit from Sekisui Diagnostics (USA) was used according to the manufacturer's instruction. Briefly, plasma and standard samples were added to an anti-ADAMTS13 antibody coated ELISA plate. After incubation, a horseradish peroxidase conjugated anti-ADAMTS13 antibody was added and the plate was coloured with a substrate for peroxidase. The same ADAMTS13 standard was used to compare the two assays.

Precision studies:
Precision studies were carried out according to the guidelines of the Clinical and Laboratory Standards Institute.[5] In order to determine the inter-assay precision, we repeat the assay eight times on the same patient's plasma in the same run using a patient with a low ADAMTS13 level (i.e. only one level was used). For the intra-assay precision, we used normal pooled plasma (combined from 20 persons) in 5 consecutive tests.

Normal reference range:
We determined the plasma levels of ADAMTS13 in 20 healthy males and 20 healthy females using our new assay and calculated the reference range as the mean ± two standard deviations (SD).

Test comparison:
We compared our method to the Imubind ADAMTS13 ELISA kit from Sekisui Diagnostics (MA, USA) by using plasma from 40 patients with possible TTP.

Statistical analysis

We calculated the reference intervals as the mean ± 2 SD and the Intra- and inter-assay precision by the mean, standard deviation (SD) and the coefficient of variation (CV) from the results of normal healthy volunteers. The precision goal for the Inter-assay and intra-assay was not to exceed 10% of the CV in the ELISA assay.[6] We calculated the limits of detection and quantification from the standard curve. The theoretical lower limits of detection (LLD) and the theoretical lower limit of quantification (LLQ) were then calculated as the minimum detectable concentration and reliable detection limits, respectively. We compared our assay to the commercial assay by the Bland-Altman and Deming regression plots using the 40 patient samples. We defined the commercial kit as the reference method and calculated the mean bias and 95% limits of agreement between both assays.

RESULTS

The standard curve for our ADAMTS13 antigen assay (Figure 1a and b) shows a good dose-response, with fast substrate colour development and steep upward linearity. The closeness of fit shows an r^2 value of > 0.99.

Table 1 summarises the technical performance of our method. The intra- and inter-assay CV's were 8.2% and 7.2% respectively. The reference range for the 40 standard samples varied

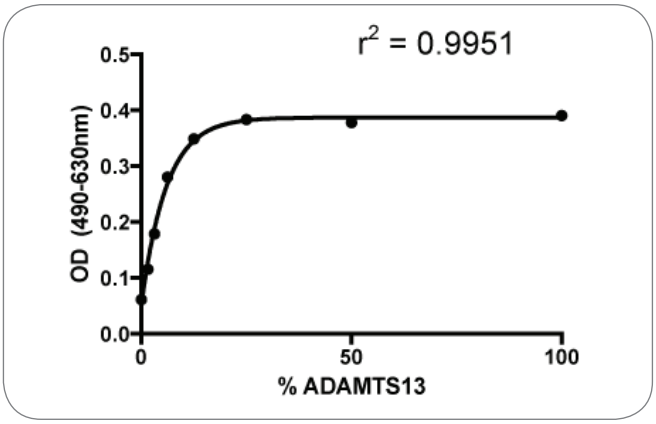

Figure 1a. Standard curve of our ADAMTS13 antigen assay. The first International Standard for ADAMTS13 from the NIBSC was used as a standard for ADAMTS13 concentrations ranging from 0% to 100% on the X-axis. Each point represents the mean of duplicate reading for a single representative experimental data set.

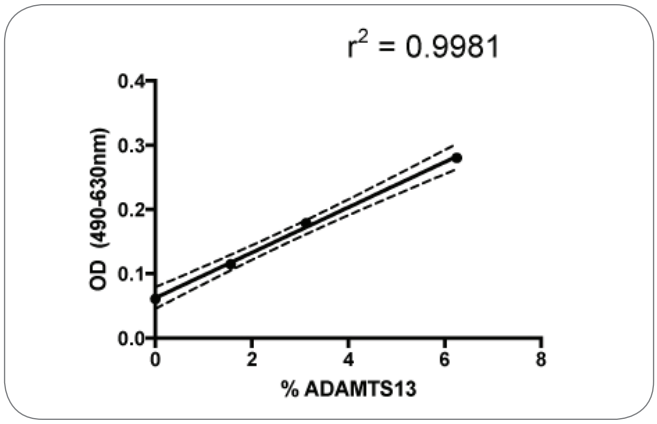

Figure 1b. Dilutional linearity fit of the assay with 95% confidence intervals. Dilutions range from 1.56% to 6.25% ADAMTS13.

Table 1. Acceptance criteria and performance characteristics of our ADAMTS13 antigen assay

VALIDATION PARAMETER	ACCEPTANCE CRITERIA	OBSERVED RESULTS		
		MEAN	SD	%CV
Intra-assay precision	CV ≤ 10 %	42.05	2.835	6.74
Inter-assay precision	CV ≤10 %	81.2	5.848	7.2
Limit of detection (LOD)		1.56% ADAMTS13		
Limit of quantification (LOQ)		1.56% ADAMTS13		
Dilution linearity • R2 • Slope • Y-Intercept • X-Intercept • range	0.99	**0.9981** 0,03523 ± 0,001089 0,0623 ± 0,003899 -1,769 1.56 – 6.25 % ADAMTS13		

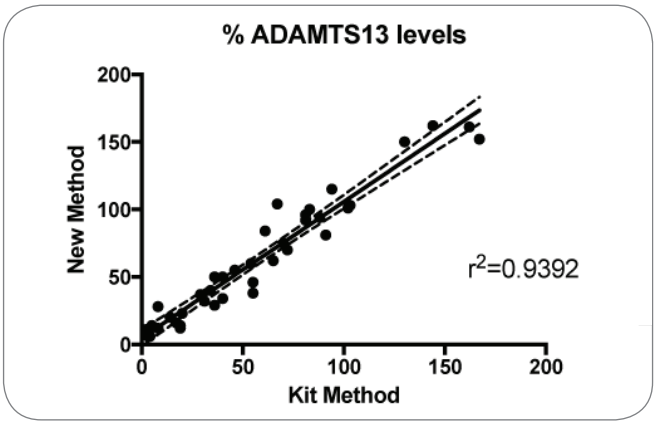

Figure 2a. Regression analysis with 95% confidence intervals comparing our method to that of the commercial assay using 40 patient samples.

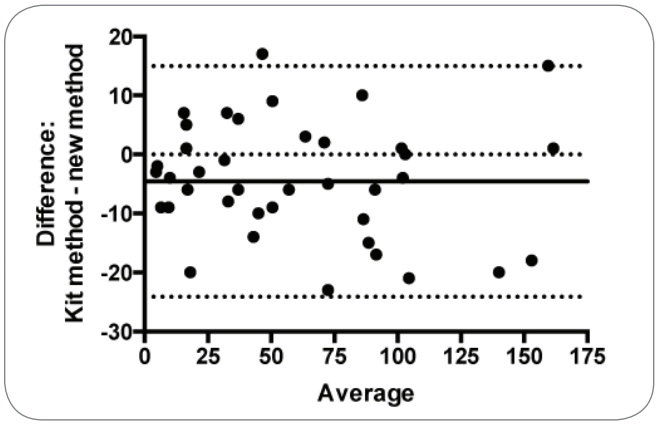

Figure 2b. Bland-Altman plot of the ADAMTS13 assay comparison. The mean relative bias is represented by a solid line, while the broken lines illustrate the 95% limits of agreement.

from 48% to 138% (93% ± 46%). The limit of detection and quantification was 1.56% ADAMTS13.

Figure 2a shows the Deming regression plot comparing our assay to the that of the commercial test kit. The two tests compared excellently with an r^2 of > 0.9 and a slope of almost 1. Figure 2b shows the Bland-Altman plot comparing the two methods. The bias was calculated as 4.575, while the 95% limits of agreement were between 24.12 and 14.97 difference.

DISCUSSION

Acute TTP is diagnosed if the plasma ADSAMTS13 level is found to be less than 10 percent.[7] However, it is difficult to distinguish between TTP and atypical haemolytic uraemic syndrome (aHUS) without an ADAMTS13 antigen assay. We have developed an in-house ELISA method to measure ADAMTS13 levels in plasma by using two different commercial antibodies which recognise epitopes on ADAMTS13 in a sandwich ELISA.

The purpose of the evaluation studies was to ensure that analytical methods can detect the corresponding analyte and and in addition provide repetitive and accurate results.[8] No other assays to date, have been validated against a commercial assay in current literature. In this assay, we measured the Intra and inter-assay precision, as well as the limit of detection and limit of quantification of our assay. The intra and inter-assay CV of the commercial kit method was found to be 5.2% and 7.2% respectively, which correlated well with the corresponding values of 6.7% and 7.2% of our assay. For viability It has been previously recommended that the CV's must be less than 10%.[5] Both the commercial assay and our assay performed well within these limits.

Regression analysis showed that both assays were linear when measuring ADAMTS13 in different dilutions of human plasma. The two assays were comparable in the measurement of ADAMTS13 in the plasma of patients with possible HIV-associated TTP.

The range of detection reported by the manufacturers of the commercial assay is from 2% up to 150% ADAMTS13. Our test's detection range was up to 100%. However, our detection range is appropriate for patients with possible HIV-associated TTP patients who are suspected to have very low levels of ADAMTS13.[6]

The lower limit of detection of our assay was 1.56%. This correlates well with that of the commercial test of 2%.

Compared to the cost of the commercial kit, our method was 90% less than the commercial kit. The commercial kit costs R500 per test minus the labour costs. If the labour costs were to be included, the assay costs would exceed that of the current medical aid rates. In comparison our assay costs R50 per test

minus the labour costs. Labour costs were designated to be the same for both assays.

Despite a good correlation, the Bland-Altman test showed a mean bias of 4.575. The Deming regression analysis of the two assays showed a slope of 1.009 ± 0.04166 in which the 95% confidence intervals included the slope of 1.0, This is well within the acceptable limits.

CONCLUSION

Our assay showed excellent inter- and intra-assay precision that can detect different ADAMTS13 levels in plasma up to 100%. We are therefore confident that our assay can successfully be used to diagnose patients with HIV-associated TTP since the results compare favourably to those of a commercial available kit.

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Research Paper

Tissue factor: A potent stimulator of Von Willebrand factor synthesis by human umbilical vein endothelial cells

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Abstract

Inflammation and dysfunction of endothelial cells are thought to be triggers for the secretion of Von Willebrand factor. The aim of this study was to examine the effects of the inflammatory cytokines interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor-alpha (TNF-α) and the coagulation factors, tissue factor and thrombin on the release and cleavage potential of ultra-large von Willebrand factor (ULVWF) and its cleavage protease by cultured human umbilical vein endothelial cells (HUVEC). HUVEC were treated with IL-6, IL-8, and TNF-α, tissue factor (TF) and thrombin, and combinations thereof for 24 hours under static conditions. The cells were then exposed to shear stress after which the VWF-propeptide levels and the VWF cleavage protease, ADAMTS13 content were measured. All treatments and their combinations, excluding IL-6, significantly stimulated the secretion of VWF from HUVEC. The VWF secretion from the HUVEC was stimulated most by the combination of TF with TNF-α. Slightly lower levels of ADAMTS13 secretion were found with all treatments. This may explain the thrombogenicity of patients with inflammation where extremely high VWF levels and slightly lower ADAMTS13 levels are present.

Key words: Endothelium, Von Willebrand factor, ADAMTS13, Inflammation.

Introduction

To date, extensive progress has been made in the knowledge of inflammation. It is now known that, pro-inflammatory mediators are released or produced from the surrounding tissue and cellular components such as mast cells after injury [1]. Under inflammatory conditions, the endothelium responds by regulating its own permeability and releases pro-inflammatory mediators such as cytokines. Among the pro-inflammatory cytokines, tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-8 (IL-8) have been implicated as the primary endogenous mediators of inflammation [2]. IL-6 up-regulates fibrinogen, tissue factor, Von Willebrand factor (VWF), and factor VIII levels [3,4]. IL-6, IL-8, and TNF-α were shown to have stimulatory effects on the endothelial cell release of ULVWF multimers, but not on the cleavage of ULVWF by ADAMTS13 [5]. Thrombin has also stimulatory effects on the endothelial cell release of ULVWF multimers [6]. Its effect on ADAMTS13 synthesis has not been studied. The stimulatory effect of TF on endothelial cells to release ULVWF and ADAMTS13 has also not been studied.

The increased amounts of VWF multimers due to entothelium stimulation with these cytokines, then might bind platelets to form thrombi in small vessels. A disintegrin-like metalloprotease with thrombospondin type I repeats-no 13 (ADAMTS13) released from endothelial cells cleaves the VWF multimers into smaller and less active forms. These VWF multimers mediate the initial adhesion of activated platelets, the first step in thrombus formation. This process may be affected by the amount of VWF and ADAMTS13 secreted [7].

Ultimately, the increased VWF levels and the decreased ADAMTS13 levels contribute to the development of thrombotic and inflammatory diseases, such as sepsis, antiphospholipid syndrome,

atherosclerosis, systemic schlerosis, diabetes and thrombotic thrombocytopenic purpura (TTP) [8-11]. The extremely high VWF levels and slightly lower ADAMTS13 levels in these patients are not completely understood.

This study examines the effects of the most commonly present inflammatory cytokines (IL6, IL8 and TNF-α) and coagulation factors (tissue factor and thrombin) and especially combinations thereof on the secretion of VWF and ADAMTS13 by cultured HUVEC. For optimal VWF-propeptide and ADAMTS13 secretion, we used sub-confluent and fourth passage cells [12-14]. This might enable us to determine the highest contributing factors to the extremely high VWF levels and slightly lower ADAMTS13 levels in patients with thrombotic disorders.

Materials and Methods

Procedure

Cultured human umbilical vein endothelial cells (HUVEC) were stimulated with cytokines IL-6 (100 ng/mL), IL-8, (100 ng/mL) and TNF-α (100 ng/mL) and also with coagulation factors, thrombin (2 IUn/mL) and TF (662ng/mL) and combinations of these compounds. The combinations include that of IL-8 and thrombin, IL-8 and TF, TNF-α and thrombin and TNF-α and TF. The control for the model was untreated cells for each treated sample.

Endothelial cell culture

HUVEC cell line C-003-5C (Invitrogen, USA) were maintained in round 22.1cm^2 fibronectin (10ng/mL, Invitrogen, USA) coated tissue culture dishes at a concentration of 1.25 x 10^4 cells/mL with a volume of 5 ml per culture dish. The cells were cultured in Medium 200 supplemented with Low Serum Growth Supplement (LSGS, Invitrogen) containing foetal bovine serum (FBS), hydrocortisone, human epidermal growth factor, basic fibroblast growth factor and heparin in a humidified 37°C, 5% CO_2 / 95% air cell culture incubator. We cultured the HUVEC in the flasks for approximately 3 days until the confluency was estimated to be 80%. Sub-culturing was done with Trypsin/EDTA (0.025% Trypsin / 0.01% EDTA solution) at room temperature for 3 minutes whereafter the action of Trypsin/EDTA was blocked by 3 mL of Trypsin Neutralizer solution (Invitrogen, USA). Only 4th-passaged HUVEC were used for all experiments. HUVEC were used, since it is a major endothelial cell type that produces ADAMTS13 in humans and it is readily available and the results are comparable [5, 12-16]. Human umbilical vein endothelial cells are also capable of expressing VWF and also carry ADAMTS13, which makes it a viable option for VWF research [17, 18].

Cell culture treatments under shear stress

The 4th-passaged HUVEC were grown until confluent on all tissue culture dishes. Thereafter, the old medium was discarded and the dishes prepared for treatment. The HUVEC were stimulated with inflammatory cytokines, IL-6, IL-8 and TNF-α (Invitrogen, USA) and also with human TF (Innovin, Siemens, South Africa) and bovine thrombin (Beckman Coulter, South Africa). This was done by incubating the cells for 24 hrs with the different compounds and combinations of the cytokines respectively before applying shear stress. Six culture flasks were used for each stimulant, three for the control (untreated) and three for the treatment (treated). The final concentration for each treatment was prepared in 5 mL of supplemented Medium 200. The following final concentrations were used for the inflammatory cytokines: 0 ng/mL as control, and 100 ng/mL as treatment. For thrombin, 0 IUnits/mL as control, and 2 IUnits/mL as treatment and for tissue factor 0 ng/mL as control, and 662 µl/mL as treatment were used. The same concentrations of the stimulants were used for the combinations as for the different compounds alone. The cytokine concentrations were based on the experiments done by Bernardo *et al.* (2004) and Cao *et al.* (2008) who used the same concentrations [5, 7]. The coagulation factor concentrations were determined by a previous non-published dose-response study.

After the treatment period, the control and stimulated dishes were carefully removed from the incubator, and placed onto a ROTEM orbital shaker for 1 hour to generate a wall shear stress of about 2.5 dynes/cm^2. It is known that under shear stress conditions, VWF becomes more susceptible to proteolysis by ADAMTS13. This shear stress is necessary for HUVEC to exposed VWF to proteolysis [19-20]. The concentration of the treatment cytokines and coagulation factors were assumed to stay constant during the treatment and rotation process, since evaporation was negligible. Lastly, the flasks were removed from the ROTEM and the perfusate collected, aliquoted and stored at -80°C until the measurements were performed.

ADAMTS13 levels

Due to the expected low ADAMTS13 levels, the ADAMTS13 release was detected using a sodium dodecyl-sulphide – poly-acrylamide gel-electro-phorsis (SDS-PAGE), followed by Western-blot detection. The SDS-PAGE was prepared and performed using a 12% separating gel and a 4% stacking gel. Following SDS-PAGE, the gel was

blotted onto a PVDF membrane (Thermo Scientific, USA). The PVDF membrane first blocked with 2% skimmed milk powder in TBS-0.1% Tween-20 for 1 hour at room temperature and washed 6 times with TBS-0.1% Tween-20. The membrane was then incubated for 2 hours at room temperature in a 1:100 dilution of a rabbit polyclonal IgG antibody against human ADAMTS13 (Santa Cruz Biotechnology, CA, USA). After washing again, it was incubated for 1 hour at room temperature with a polyclonal goat anti-rabbit antibody conjugated with horseradish peroxidise (HRP) (1:2,000 dilution, Santa Cruz Biotechnology, CA, USA) to detect the presence of ADAMTS-13. Equal volumes of ECL Western-blot detection reagent 1 and 2 (AEC Amersham, UK) were mixed and poured onto the membrane for 1 minute whereafter the membrane was sealed with plastic film and exposed to an X-ray film for 1 to 10 minutes in the dark. Finally, the film was developed in an automated film developer (Kodak, CA, USA) and the picture was scanned into a computer. The strength of the signal of ADAMTS13 was quantified by densitometric analysis using the ImageJ software (Thermo Scientific, USA). itometric analysis of ADAMTS13 content was done and the percentage difference from the control was calculated by dividing the difference in density for each treated sample from the density for the control by the density of the control sample.

VWF-propeptide levels

VWF-propeptide levels in the perfusates were determined using an in-house ELISA. In short, a 96-well ELISA plate was coated overnight at 4°C with a monoclonal antibody against the VWF-propeptide (CLB-Pro 35, Euro-Immune, Germany, 1:100 dilution in PBS, 100 µl per well). The plate was blocked with 4% bovine serum albumin (BSA) in PBS (200 µl/well) for 2 hours at room temperature and washed four times with PBS/0.1%Tween-20. The perfusates were added in duplicate (100 µl/well) and incubated for 2 hours at 37°C. An HRP-conjugated monoclonal antibody against VWF-propeptide (CLB-Pro 14.3, Euro-immune, Germany) was added in a 1:100 dilution after another wash step, and incubated for 1 hour at room temperature in order to detect the concentration of VWF-propeptide in the perfusate. We used OPD (50 mg.L-1) as the substrate for HRP. The WHO's (World Health Organisation) 6th FVIII/VWF standard was used as the standard against which the perfusates were measured. The results were expressed as percentage difference from the control samples. Thus (VWF-propeptide level of stimulated sample minus VWF-propeptide level of control) / VWF-propeptide level of control * 100.

Statistics

It is important to notice that the whole experiment was done three times. Thus for each stimulant we culture 6 flasks, three control and three treated flasks, and each perfusate was measured in duplicate for the ADAMTS13 and VWF-propeptide levels. The percentage increase or decrease from the control samples was calculated and all the experimental data were presented as mean ± SD (standard deviation). The unpaired 2-tailed Student t-test was used to test for differences and P- values less than 0.05 ($P<0.05$) were considered as statistically significant.

Results

Figure 1 shows the densitometic analysis of the ADAMTS13 content in the stimulated samples. This is expressed as percentage difference from the control samples. The original Western-blot picture is shown in Figure 2. An overall decrease in density of the stimulated samples compared to that of the controls is clearly visible.

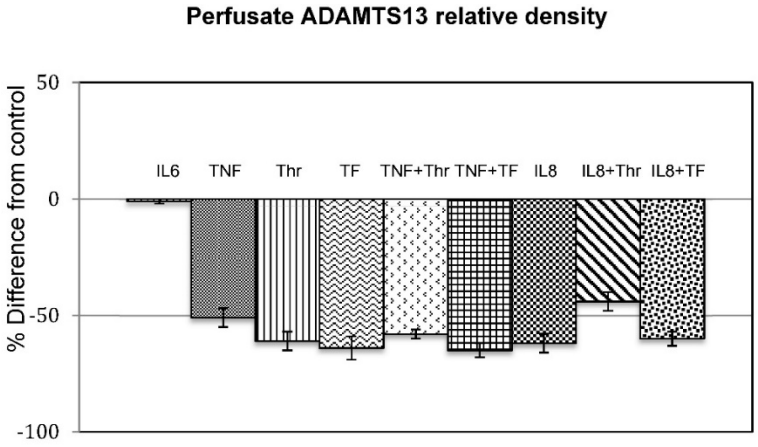

Figure 1. Effect of cytokines (IL6. and TNF), coagulation factors (Thr and TF) and combinations thereoff (TNF+Thr, TNF+TF) on ADAMTS13 levels in the perfusates. The bar graphs shows the mean % difference from the control of the 3 experiments done in duplicate (n=3) and the error bars indicates the standard deviation.

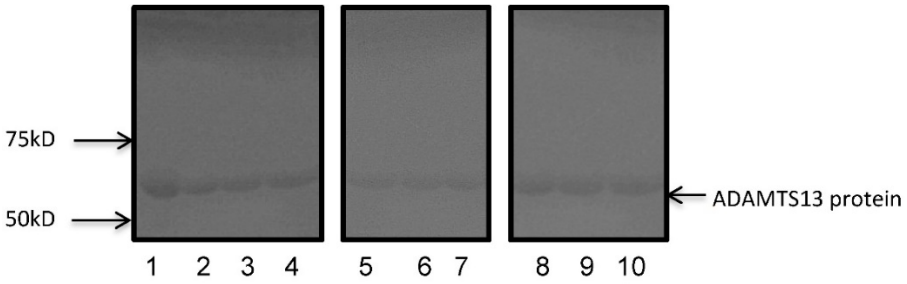

Figure 2. Western Blot indicating the presence of the ADAMTS13 protein in all samples. The positions of the molecular markers are shown on the left side. For illustration purposes, only some of the samples are shown. Not stimulated sample (lane 1), IL6 (lane 2), IL8 (lane 3), TNFα (lane 4), thrombin (lane 5), tissue factor (lane 6), IL8 + thrombin (lane 7), TNFα + thrombin (lane 8), IL8 + tissue factor (9) and TNFα + tissue factor (lane 10).

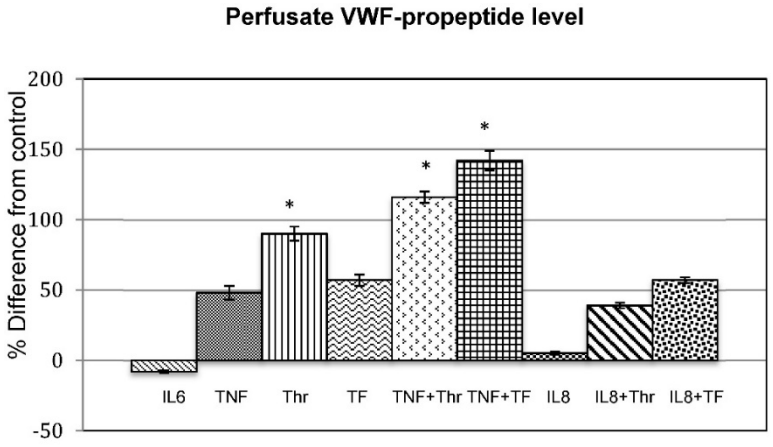

Figure 3. Effect of cytokines (IL6, IL8 and TNF), coagulation factors (Thr and TF) and combinations thereof (TNF+Thr, TNF+TF, IL8+Thr and IL8+TF) on VWF-propeptide levels. The bar graphs shows the mean % difference from the control of the 3 experiments done in duplicate and the error bars indicates the standard deviation. The * indicates statistic significance ($p<0.05$).

The percentage difference of all stimulated samples, except for IL-6 to that of the control samples ranged between a ratio of 30 to 60%. Although this was not a quantitative measurement, it nevertheless showed that the ADAMTS13 secretion was decreased when endothelial cells were stimulated with IL-8, TNF-α, thrombin and TF and combinations of TF and thrombin with the cytokines (IL-6 had no effect).

In summary, endothelial cells (HUVEC) stimulated with cytokines and coagulation factors secreted more VWF and less ADAMTS13.

The VWF-propeptide levels rather than VWF antigen levels were measured in the perfusate, since it provides a more accurate measurement of VWF synthesis [21]. Figure 3 shows the mean percentage change from the controls, while table 1 provides the original data of the VWF-propeptide levels in the control and treated samples.

VWF-propeptide were secreted by all treatments, except for IL-6. The amount of VWF-propeptide was secreted from the lowest to the highest values by the following treatments: IL-8, IL-8+thrombin, TNF-α, thrombin, TNF-α, TNF-α+thrombin, tissue factor, IL-8+tissue factor, and with the highest increase seen at TNF-α+tissue factor treatment. Treatments with thrombin, TNF-α and thrombin and TNF-α and tissue factor shows a significant increase in VWF propeptide levels compared to that of the control.

Table 1. Effect of cytokines (IL6, IL8 and TNF), coagulation factors (Thr and TF) and combinations thereoff (TNF+Thr, TNF+TF, IL8+The and IL8+TF) on VWF-propeptide levels (%).

Stimulant	% VWF-propeptide of Control (mean ± SD) (n=6)	% VWF-propeptide of Treated (mean ± SD) (n=6)	Percentage increase from control (mean ± SD)
IL-6	56.5 ± 1	52 ± 1	(-)8 ± 1
TNF	54 ± 1.5	104.5 ± 2.5	48 ± 5
Thr	73.5 ± 4	140 ± 5	90 ± 5 *
TF	47.5 ± 2	109.5 ± 4.5	57 ± 4
TNF + Thr	64.5 ± 2	139 ± 5	116 ± 6 *
TNF + TF	54 ± 2	130.5 ± 5	142 ± 4 *
IL-8	21 ± 1	23.5 ± 1.5	5 ± 1
IL-8+ Thr	27 ± 1	44.5 ± 2	39 ± 2
IL-8 + TF	20 ± 3	30.5 ± 2	53 ± 6

The results are given as the mean of 3 experiments in duplicate ± 1 standard deviation (n=6). The * indicates statistic significance at $p<0.05$.

The levels of secreted VWF-propeptide, except for IL-6 which cause a decrease, increased in the following order from low to high: IL-8, IL-8+thrombin, TNF-α, thrombin, TNF-α, TNF-α+thrombin, tissue factor, IL-8+tissue factor, and

with the highest increase the TNF-α+tissue factor treatment. Only stimulation with Thrombin, TNF-α+thrombin and TNF-α+tissue factor increased the VWF-propeptide levels significantly.

Discussion

We hypothesized that the combination of certain inflammatory cytokines and coagulation factors that are released during inflammation may stimulate the synthesis and release of VWF and simultaneously slightly decrease the synthesis of ADAMTS13. This might result in a thrombotic state where excessive amounts of VWF-platelet strings cannot be degradated and might occlude small vessels.

To test this hypothesis, we determined the effects of 3 inflammatory cytokines, interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor-α (TNF-α), which are released during the early stages of systemic inflammation on the secretion of VWF and its cleaving protease ADAMTS13 in human primary HUVEC. This study presents the first *in vitro* evidence on the effects of thrombin and tissue factor, combined with inflammatory cytokines, on VWF synthesis and cleavage in HUVEC.

Since VWF becomes more susceptible to proteolysis by ADAMTS13 during shear stress, we used a fluid shear stress of 2.5 dyne/cm^2 for a small time period to ensure the expression of the VWF-propeptide and ADAMTS13 [20, 21]. The VWF-propeptide levels were measured instead of the VWF antigen levels, since it is a more accurate measurement of VWF secretion [21].

IL-6 was the only cytokine that did not affect VWF-propeptide and ADAMTS13 secretion (Table 1, Figure 1). However, this was expected because HUVEC do not have receptors for IL-6 [22, 23]. This result confirmed the findings of Bernardo *et al.* (2004), who reported that IL-6 alone does not stimulate the VWF synthesis from HUVEC [5]. However, IL-6 in complex with its receptor on other endothelial cells induced a small but significant release of VWF [5].

The VWF-propeptide levels increased when HUVEC was stimulated with TNF-α (Table 1). This supports the results of other similar studies [5, 16, 24]. The precise mechanism through which TNF-α induces this increase is unknown [25]. In accordance with Cao *et al.* (2008), which found lower ADAMTS-13 mRNA levels after 24-hour treatment with 10 ng/mL TNF-α, we also found that TNF-α induced a decreased ADAMTS13 release [7].

IL-8 did not stimulate the secretion of VWF by HUVEC. It however induced a decrease in HUVEC ADAMTS13 levels (Fig. 1). To date, no studies have been done on the effect of IL-8 treatment on ADAMTS13 release.

We found no evident change in the HUVEC ADAMTS13 release upon stimulation with TF. This correlates with studies where shiga toxin was used to stimulate TF expression. Cells treated with shiga toxin also did not increase or decrease the ADAMTS13 levels [26, 27].

This is also the first time where the effect of tissue factor was tested on VWF secretion in HUVEC cells. Tissue factor increased VWF secretion markedly. The highest increase was when HUVEC was stimulated by the combination of tissue factor and TNF- α (Table 1). It is however not clear if TF can be linked to increased VWF-propeptide levels *in vivo* [28-30]. However, in HIV where TF levels are increased, it is possible that the constant stimulation of VWF release by tissue factor might contribute to the extremely high levels of VWF in these patients [31].

Our results with IL-8 and TNF-α-stimulation were consistent with a study done by Bernardo et al., (2004) who indicated a similar effect of IL-8 and TNF-α on VWF synthesis by endothelial cells [5].

We found total opposite results with ADAMTS13 compared to VWF-propeptide in the perfusates. IL-8, TNF-α, tissue factor, IL-8+tissue factor and TNF-α+tissue factor decreased the levels of ADAMTS13 (Fig. 1). Although the results with the different stimulation regimes were not significantly different, the decrease with all stimulation regimes were substantial. The affected decrease in release of ADAMTS13 in human umbilical endothelial cells by IL-8, TNF-α, thrombin and tissue factor and their combined effects, may offer a logical explanation of how systemic inflammation and/or infection might trigger a thrombotic condition.

We suggest that certain inflammatory cytokines and coagulation factors that are released during pathological conditions may affect the balance between the quantity of VWF multimers and ADAMTS13 released from endothelial cells. More VWF and VWF-propeptide might be secreted and less ADAMTS13. As a result, the excessive amounts of VWF, with lesser amounts of the VWF cleavage protease, ADAMTS13, might cause obstructions of platelet-VWF plugs in smaller vessels, leading to thrombosis [27, 29-31]. This should be investigated further in more detail.

Conclusion

We investigated the effect of inflammatory and thrombotic stimuli and the combination thereof on the release of VWF and its cleavage protease ADAMTS13 by human umbilical vein endothelial cells (HUVEC). Tissue factor and especially the combination of TF and TNF-α was the most potent stimulator of von Willebrand factor secretion by HUVEC. Thus, the

results in this study may provide a link between inflammation and thrombosis, which may also be of therapeutic importance. It may also help to understand the mechanisms that lead to thrombotic disorders with increased VWF levels and lower ADAMTS13 levels.

Acknowledgement

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Competing Interests

The authors have declared that no competing interest exists.

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Tissue Engineered Small Vessel Conduits – The Anti-Thrombotic Effect of Re-Endothelialization of Decellularized Baboon Arteries: A Preliminary Experimental Study

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Data Interpretation D
Manuscript Preparation E
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Background: The use of decellularized biological scaffolds for the reconstruction of small-diameter vascular grafts remains a challenge in tissue engineering. Thrombogenicity is an important cause of obstruction in these vessels due to decellularization. Seeding of the decellularized vascular constructs with endothelial cells is therefore a prerequisite for the prevention of thrombosis. The aim of this study was to seed decellularized baboon arteries with endothelial cells and to compare the thrombogenicity to that of decellularized arteries after circulation of blood.

Material/Methods: Carotid, radial, and femoral arteries (12 arteries in total) were harvested from 2 *Papio ursinus* baboons. Ten arteries were decellularized. Normal morphology was confirmed in the control vessels. The effect of re-endothelialization was studied in the vessel scaffolds using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Decellularization resulted in vessel scaffolds with well-preserved extracellular matrix and intact basal membranes. Six of the decellularized vessel scaffolds were seeded with viable human umbilical vein endothelial cells (HUVEC). Luminal endothelialization was established after 7 days in a bioreactor and SEM confirmed confluency. Two control, 4 decellularized, and 6 decellularized re-endothelialized vessel scaffolds were studied in an *in vitro* flow chamber using baboon blood.

Results: The decellularized arteries showed an absence of endothelial lining, and an intact basement membrane. The seeding process produced a complete endothelial layer on the surfaces of the arteries. After perfusion with whole blood, no thrombi were formed in the control arteries and re-endothelialized vessels. Widespread platelet activation and adhesion occurred in the decellularized vessels despite a relatively intact basal membrane.

Conclusions: This study supports the development of re-endothelialized tissue engineered small-vessel conduits.

MeSH Keywords: Blood Coagulation • Blood Vessel Prosthesis • Endothelial Cells • Tissue Engineering • Tissue Scaffolds

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Background

Vascular diseases are responsible for more than 25% of all deaths worldwide [1]. Therapies for vascular diseases often require bypassing or replacement of the diseased vessels with vascular grafts. However, many patients do not have healthy vessels available for grafting due to pre-existing vascular conditions, size mismatch, or available autograft conduits [2,3]. Currently, arteries, such as the aorta or the iliac arteries, are reconstructed using synthetic grafts that are made of expanded polytetrafluoroethylene (ePTFE) or Dacron. These synthetic grafts are also used for reconstruction of small-diameter arteries; however, the patency rates are not favorable because of thrombogenicity and limited re-endothelialization capacity *in vivo* [4]. Some autologous endothelial cell seeding grafts have been implanted with promising results even in very small (4 mm)-diameter grafts that were clinically used [4,5]. The disadvantages, however, were still the use of foreign materials, as well as complicated production and waiting periods of at least 4–6 weeks, for use of these grafts [4,5].

Thus, there is still a worldwide shortage of small-diameter (<6 mm) conduits with sufficient patency rates that can be used to bypass or replace small peripheral diseased arteries [2,3,6]. Autologous arteries are still the criterion standard for vascular replacement due to their inherent physiological properties [7].

Tissue engineered vessels can potentially be used to replace diseased and damaged native blood vessels [8]. Decellularized biological scaffold material from both xenograft and allograft origin can be used in constructing tissues and organs to restore or establish normal function [4,6,7], aiming to develop living autologous grafts with the capacity for growth, repair, and remodelling. Decellularized allograft tissue can also attenuate immune response-related degeneration as a result of chronic rejection by recipients [4]. However, thrombogenicity remains a major concern, as decellularized arteries have no endothelial lining, thus exposing collagen fibers to circulating blood. This direct exposure of collagen results in thrombosis due to platelet adhesion and activation in circulating blood [9]. The absence of an endothelial lining is also associated with accelerated vessel calcification and degeneration [10].

Surface coating of small-diameter grafts with angiogenic growth factors has some promise but does not solve the problem completely due to the inability to form a monolayer [11]. Recently, a more promising approach for construction of small-diameter vascular grafts is the re-endothelialization of decellularized vascular constructs with autologous vascular endothelial cells before implantation [1,10]. Other recent investigations have shown that decellularized scaffolds have no negative effect on cell seeding [2]. The endothelial layer incorporates many of the anti-thrombogenic properties of blood vessels. However, endothelialization of vascular grafts has been limited due to the cost and availability of reagents, and because it is difficult for endothelial cells to stay attached to the scaffold [11].

The aim of the present study was to re-endothelialize small-diameter (<6 mm) decellularized baboon arteries using cultured HUVECs. Additionally, the re-endothelialized arterial scaffolds were perfused with baboon blood at high shear stress and compared to those of fresh baboon arteries.

Baboon models possess similar hemostatic characteristics to humans. Their coagulation system and platelet behavior closely resembles that of humans, whereas other animal species such as dogs, sheep, and pigs do not. Baboon vascular endothelial cell growth characteristics are also thought to be similar to that of humans. Furthermore, they share about 98% homology to human genes, possess similar protein structures to humans, and reflect the anatomical, physiological, and behavioral makeup of humans [3,12,13].

Material and Methods

All experiments were performed in accordance with the "Principles of the Laboratory Animal Care" prepared by the National Society of Medical Research, and the "Guide for Care and Use of Laboratory Animals" developed by the US National Institutes of Health, (NIH, revised 1996) and the South African laws for animal welfare. Ethics approval for this study was obtained from the Interfaculty Animal Ethics Committee of the Faculty of Health Sciences of the University of the Free State (NR 17/2014). Two male baboons (*Papio ursinus*) weighing between 10 and 15 kg were anaesthetized with 0.01 ml/kg Rompun (Bayer, Johannesburg, South Africa) and 10 mg/kg Ketamine hydrochloride (SigmaAldich, Johannesburg, South Africa) and euthanized with an overdose of potassium chloride (Bayer, Johannesburg, South Africa). Heparin (17 IU/kg, Pfizer, Johannesburg, South Africa) was given before euthanasia. Twelve medium-sized arteries with diameter smaller than 6 mm and a minimum length of 6 cm were harvested from different regions of the 2 sacrificed baboon bodies. Six arteries were collected from each baboon. The main source of blood vessels was from arteries branching from the aorta: the right (R) and left (L) carotid, R and L radial, and R and L femoral arteries. Ten of the baboon arteries were decellularized. Two femoral arteries were used as controls. Six decellularized arteries (2 from each group) were seeded with HUVECs (Clonestics™ HUVEC systems, Lonza Walkersville, Inc., MD, USA). The remaining 2 decellularized baboon arteries were not seeded. HUVECs were used because they are a readily available cell source for tissue engineering without sacrificing intact vascular tissues. Many other studies have successfully used them for seeding vascular grafts [9]. Furthermore, they are cost-effective and yield large quantities.

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Decellularization

Ten baboon arteries (carotid (n=4), radial (n=4) and femoral (n=2)) were washed with sterile phosphate-buffered saline (PBS, pH 7.4; Invitrogen, Carlsbad, CA) to remove residual blood clots. Decellularization was accomplished using the acid- and detergent-based method [4]. Arteries were decellularized using a combination of 1% sodium deoxycholic acid, 0.05% sodium dodecyl sulphate, and 0.05% triton-X100 at 37°C. Extensive rinsing steps with saline followed this.

A control sample of a circumferential 0.5 cm resection from all explanted arteries was obtained for histology, scanning electron microscopy (SEM), and transmission electron microscopy (TEM) analysis before and after decellularization. We then stored the arteries at 4°C under sterile conditions in PBS containing penicillin (200 U/ml), streptomycin (200 µg/ml), amphotericin B (10 µg/ml), ciprofloxacin (50 µg/ml), and cefuroxime (750 µg/ml) (ScienCell Research Laboratories, Carlsbad, CA).

Cell culture and seeding

Laboratory procedures

HUVECs were cultured according to the manufacturer's instructions (Clonestics™ HUVEC systems; Lonza Walkersville, Inc., MD, USA) in a laminar flow cabinet under sterile conditions. The HUVECs were passaged only 3 times for the purpose of this study, to retain the unique function of endothelial cells [14]. To optimize cell culturing, the morphology of the cells was assessed for homogenous cobblestone morphology present throughout the culture, but no pictures were taken of the cells. Cell viability and proliferation rate were determined by the MTT assay (MTT Cell Viability and Proliferation Assay Kit, ScienCell, Carlsbad, CA).

First, the luminal surfaces of the 6 decellularized arteries (carotid (n=2), radial (n=2), and femoral (n=2)) were coated with a solution of 10 µg/ml fibronectin (Human plasma fibronectin, Gibco®, Grand Island, NY) in 1 ml PBS. The arteries were then incubated at 37°C for 45 min to allow the fibronectin to bind onto the ECM. After that, we aspirated the unbound fibronectin and washed the arteries twice with sterile PBS. The decellularized coated arteries were immediately seeded using a bioreactor filled with endothelial growth medium (Clonestics™ EGM-2 Bullet kit medium, Lonza Walkersville, Inc., MD) and kept in a 5% CO_2/air atmosphere incubator at 98% humidity at 37°C, as previously described [15]. The pH level was maintained at 7.4 by modulating the CO_2 supply. The static environment provided a low shear stress environment during the seeding process. The artery constructs were then washed twice with PBS and supplemented with penicillin and streptomycin to limit any contamination on the vascular grafts.

A density of 2.5×10^3 endothelial cells/cm^2 were used to seed the freshly coated graft surfaces. The EC culture was suspended into the sutured graft within the bioreactor and the air was removed. After substituting ECs, the bioreactor was placed into a biostrabilizor (Biegler Medizinelektronik GmbH, Mauerbach, Austria) to perform a standardized EC [16]. The bioreactor was rotated to expose the entire luminal surface of the arteries to achieve optimal attachment conditions. After 3 h of seeding, the artery constructs were rinsed with PBS to remove non-adherent cells. The arterial grafts were then maintained in fresh culture medium overnight at 37°C in a 5% CO_2 incubator to allow ECs to grow onto the arteries. This was performed for an additional 7 days, while changing the medium every 48 h. We cut small circular pieces (0.5 cm) from each seeded artery after days 1 and 7 of the seeding procedure to verify the success of endothelialization using SEM analysis.

Cell viability on the scaffold was determined with the same MTT assay kit (MTT Cell Viability and Proliferation Assay Kit, ScienCell, Carlsbad, CA). Two small circular pieces (0.5 cm) from each seeded artery were cut; the cells were trypsinized and then cultured in a 24-well plate for 24 h. HUVECs from the original cell culture were used as a positive control. Only viable cells were counted.

Perfusion study

Blood samples (50 ml) were collected from 4 healthy baboons in 3.2% sodium citrate and used within 4 h for *in vitro* perfusion. An *in vitro* flow chamber connected to a peristaltic pump was used to provide a closed system, which delivers laminar flow to the arteries. The luminal surfaces of 2 normal, 4 decellularized, and 4 seeded arteries were first washed (2×5 min) with 50 ml of PBS supplemented with penicillin and streptomycin. Then, 50 ml of blood was circulated for 2 h at 37°C with a pressure of 120/80 mmHg through the sutured baboon artery. Calcium chloride (0.6 M) was added to the blood at the time perfusion was started. After perfusion, the vessels were washed once for 5 min with PBS. Small circular pieces (0.5 cm) were cut for SEM and TEM analysis. SEM and TEM analysis were done to assess the morphological differences of arteries before and after the decellularization, re-endothelialization, and the perfusion experiment. TEM analysis was done according to standard diagnostic methods that are widely used for diagnostic purposes. TEM showed the presence and condition of the basement membrane on the luminal surface of the decellularized arteries. Although histology studies were done as well, this is not included in this report, since the SEM and TEM analysis shows more detail.

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Electron microscopy

Scanning electron microscopy

Each vascular graft tissue sample for SEM was prepared by the Centre for Microscopy at the University of the Free State. All samples were fixed in 2.5% glutaraldehyde (Merck, Johannesburg, South Africa). Tissue specimens were dried using the critical point method (Tousimis critical point dryer, Rockville, MD, USA, ethanol dehydration, and carbon dioxide drying gas) and were metallized using gold (BIO-RAD, Microscience Division Coating System, London, UK; Au/Ar sputter coating @ 50-60 nm). Evaluations were performed with a Shimadzu SSX 550 scanning electron microscope (Kyoto, Japan, with integral imaging). The surface area of each specimen was examined and photographed in different positions. SEM micrographs were used to assess endothelial integrity and to evaluate the quality of the extracellular basal membrane.

Transmission electron microscopy

Vascular graft samples were fixed in 3.0% glutaraldehyde overnight, post-fixated in Palade's osmium tetroxide, and dehydrated in a graded acetone series [15]. Dehydrated samples were impregnated/embedded in epoxy to facilitate the making of ultra-thin sections for the TEM evaluation. Ultra-thin sections were cut from the sample embedded in the epoxy using an ultra-microtome (Leica Ultracut UC7, Vienna, Austria). After sectioning the samples, they were stained with uranyl acetate and lead citrate. Sections of the leaflet samples were evaluated by using a transmission electron microscope (CM100, FEI, The Netherlands) and photographed using an Olympus Soft Imaging System Megaview III digital camera with Soft Imaging System digital image analysis and documentation software (Olympus, Tokyo, Japan).

Results

Decellularization efficacy

Transmission electron microscopy analysis

TEM examination of normal baboon arteries showed a normal endothelial lining (Figure 1A) with an intact basement membrane. In a decellularized artery (Figure 1B), the endothelial monolayer was absent. The decellularized artery contained cellular debris and cellular components.

Scanning electron microscopy analysis

SEM clearly showed differences between normal and decellularized arteries (Figure 2). The normal artery had a smooth surface, indicating that there are EC on the luminal surface. The decellularized artery had an intact basal membrane with limited areas with exposed collagen fibers, proving the absence of an endothelial lining.

HUVECs culture

Although not shown, HUVECs had the typical cobblestone morphology of ECs in growing cultures. Their cell viability in cultures exceeded 90% (Table 1).

Seeding

Scanning electron microscopy (SEM) analysis of seeded arteries

The decellularized arteries supported re-endothelialization (Figure 3). Endothelial cells adhered to the decellularized artery direct after seeding (Figure 3A). Figure 3B shows the proliferation and migration of the ECs on the decellularized artery after 1 day of seeding (indicated by the red arrows), forming an almost confluent monolayer. The ECs had formed an almost confluent monolayer in the middle section of the arterial construct. Seven days after seeding, a complete endothelial layer formed on the surfaces of the arteries (Figure 3C).

Viability of seeded HUVECs

Seeded HUVECs showed increased mitochondrial activity with an increased number of cells (Figure 4).

Perfusion

Scanning electron microscopy of perfused arteries

Figure 5 represents SEM images of a decellularized artery (a), a normal artery (b), and a seeded decellularized artery (c) after perfusion with whole blood. The normal artery and the seeded decellularized arteries were devoid of thrombi on their luminal surfaces. There were, however, areas on both with few isolated spots of platelets adhesion. This might be due to possible damage to the endothelial layer or mishandling of the arteries during the seeding process. However, the decellularized arteries (a) had more platelet adhesion and activation on the ECM after perfusion with whole blood, indicating that the decellularized scaffold promotes thrombosis.

Discussion

The decellularized arterial scaffolds contained some cellular debris but no cells were present, as shown by TEM and SEM (Figures 1, 2). SEM further confirmed the absence of an

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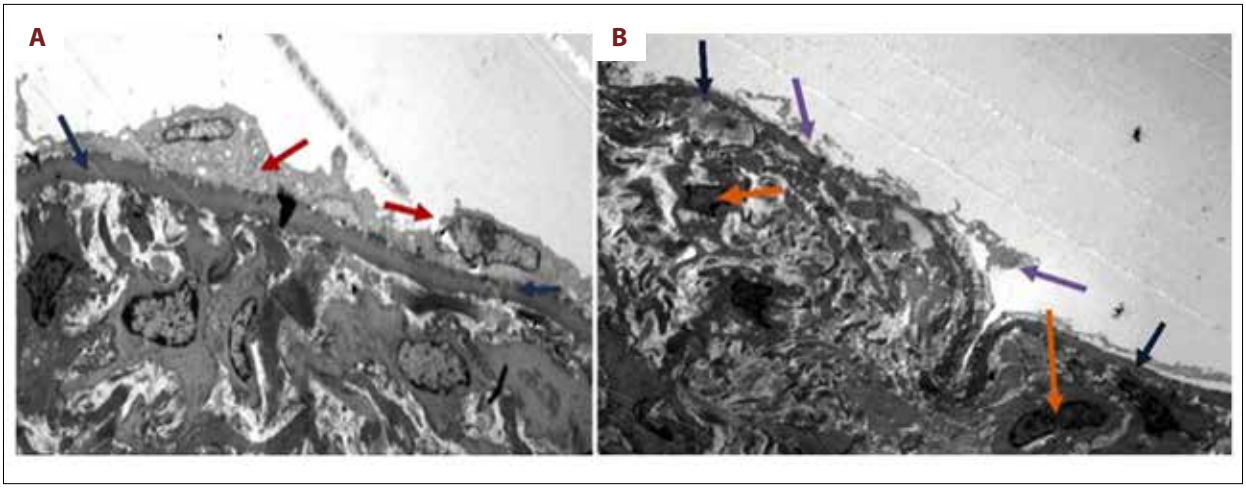

Figure 1. TEM images of a normal artery (**A**) and a decellularized artery (**B**). Both a and b were taken at 1250× magnification. a shows an endothelial monolayer of a normal blood vessel indicated by the red arrows, with an intact basement membrane indicated by the blue arrows (n=2). (**B**) Shows the missing endothelial layer of a decellularized artery. There was cellular debris on the intimal surface (purple arrows) and ruptured remaining cellular material (orange arrows) throughout the matrix scaffold. It also has an intact basement membrane (dark blue arrows) (n=4).

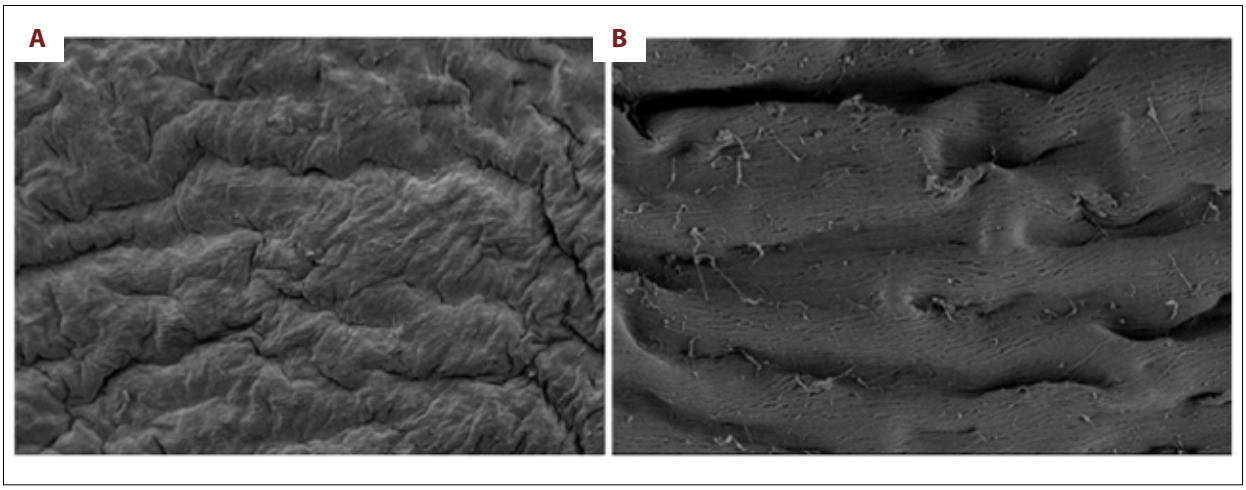

Figure 2. SEM images of a normal baboon artery (**A**) (n=2) and a de-endothelialized baboon artery (**B**) (n=4). (Images at 1000× magnification).

Table 1. Total cell counts and percentage cell viability of the primary culture, after the first passage and after the second passage. Values are expressed as mean (n=2). The total cell count increased with each passage. The percentage viability of the HUVEC cells remained high in the primary culture and increased with each passage.

	Primary culture	First passage	Second passage
Total cell count * 10^6	0.97	1.99	2.75
% Viability	94	96	97

endothelial lining. It is important to note that the basement membrane was intact after decellurization (Figure 1). This is an important prerequisite to re-endothelialization because it modulates cell-matrix interactions by supporting cell adhesion, migration, and proliferation during development and regeneration [6]. Furthermore, a preserved extracellular matrix contributes to maintaining a non-thrombotic environment in graft material if used in bypass surgery [2]. The decellurization process needs to be refined since cellular debris could still be observed. It is, however, important not to damage the basal membrane.

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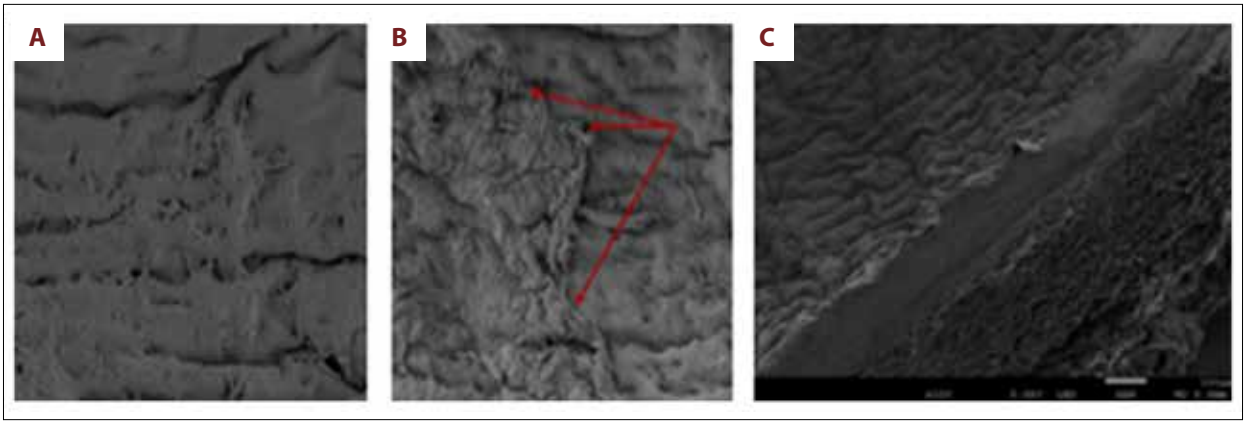

Figure 3. SEM images of a seeded decellularized artery. Attached ECs are visible on the scaffold after seeding (**A**: 1000x magnification, n=3). After day 1, ECs started to form a confluent monolayer and appeared to be migrating towards the direction of the red arrows in image **B** (**B**: 450× magnification, n=3). Notice the smooth surface on the seeded area of the decellularized scaffold compared to the rough unseeded area of the artery in image (**B**). After 7 days of seeding, image (**C**) indicates a complete endothelial layer on the surface of the arterial sections (magnification 100×, n=3). Notice the smooth surface of the endothelium. The internal elastic lamina separating the tunica intima from the tunica media could be observed from the cross-section. This confirmed the presence of the endothelium.

In vitro seeding techniques using cultured autologous cells are required to endothelialize smaller vascular constructs before implantation, since *in vivo* repopulation of smaller decellularized conduits causes thrombosis [15]. We used HUVECs to successfully re-endothelialize the de-endothelialized baboon arteries (Figure 3) because HUVECs are mostly used to obtain ECs for seeding vascular grafts surfaces [11]. Other sources of ECs include endothelial progenitor cells derived from peripheral blood, bone marrow, and umbilical cord vein ECs [5]. We used HUVECs cell cultures because they are readily available commercially. They can also be easily cultured under optimal conditions with a good growth potential. EC cultures were viable and had excellent proliferation capabilities (Table 1).

The decellularized baboon arterial grafts were successfully seeded with HUVECs. The seeded grafts were endothelialized along their entire length from day 1 (Figure 3B), and a confluent monolayer of ECs was observed after 7 days (Figure 3C). Although a slight breakage of the endothelial layer occurred due to twisting during sectioning, a confluent monolayer could still be observed.

Baboon vessels were used because the hemostatic mechanisms closely resemble that of humans, whereas other animal species such as dogs, sheep, and pigs do not. Their vascular EC growth characteristics are also thought to be similar to that of humans. They also share about 98% homology to human genes and possess similar protein structures [17]. Furthermore, the baboon immunological and coagulation system is closest to that of humans. Baboons show similar fibrinogen level and thrombin time to that of humans. FVIII activity is also similar to humans, and FVIII antigen cross-reacts with

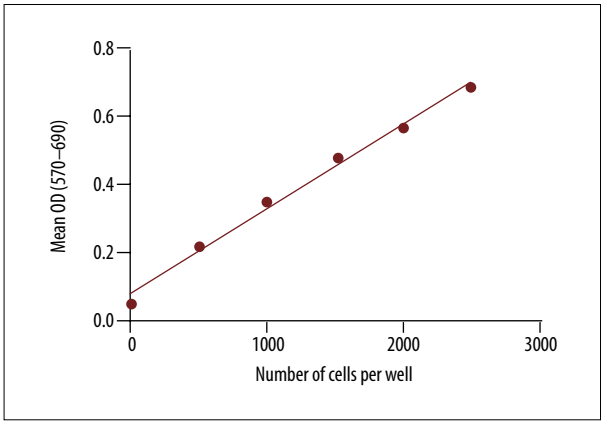

Figure 4. MTT assay of seeded HUVECs after 24-h cell culturing from trypsinized small circular pieces (0.5 cm) of the seeded arteries (n=2). The seeded HUVECs showed increased mitochondrial activity with increased number of cells.

human factor VIII antibodies. Response of baboon platelets to collagen ristocetin is similar to humans; however, response to ADP and EPI is slightly reduced and response to arachidonic acid is slightly increased.

The aim of this study was to determine if re-endothelialized, decellularized biological scaffolds could reduce thrombogenicity when compared to the decellularized scaffolds. Decellularized scaffolds promote thrombosis formation due to the absence of an endothelial lining responsible for maintaining an anti-thrombotic surface [17,18]. The perfusion studies support this finding, as unseeded decellularized arteries showed widespread platelet activation during the perfusion experiment

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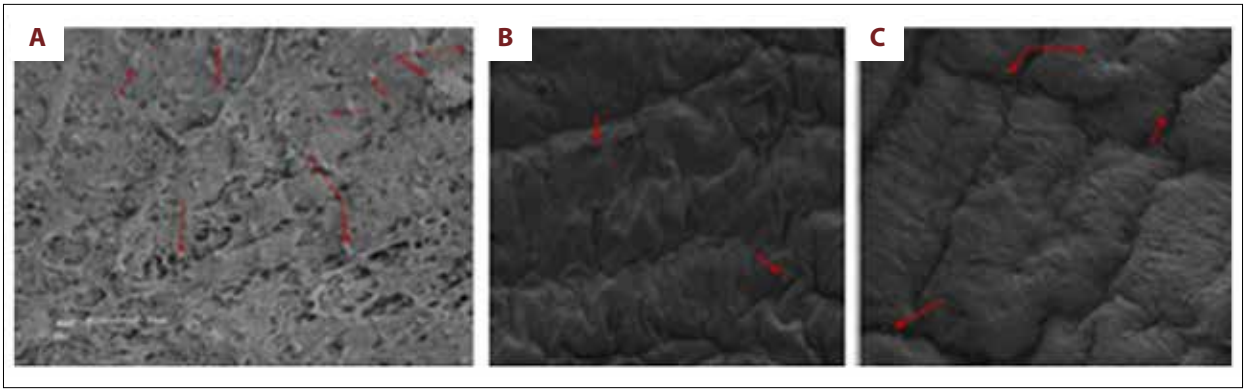

Figure 5. SEM results of decellularized (**A**), normal (**B**), and seeded (**C**) arteries after perfusion with whole blood. Image (**A**) (2000× magnification) demonstrates platelet adhesion and activation on the surface of a decellularized artery (red arrows) (n=4). (**B, C**) (450× magnification) represent normal (n=2) and seeded (n=4) arteries, respectively. There were few locations (red arrows) of minimal platelet adhesion.

with baboon blood (Figure 5). The seeded arterial grafts and the healthy artery, on the other hand, showed no thrombus formation. However, there were areas where few isolated platelets adhered to the surface. This might have been due to damaged ECs, thus exposing collagen fibers or the underlying basal membrane in those areas. A similar result of platelet adhesion on areas where the EC lining that was not intact is found in the literature [11,17]. In addition, exposed collagen following vascular injury leads to immediate activation of platelets through the activation of the coagulation pathway to seal the wound. It is therefore not surprising that the exposed fibrous structures of decellularized arteries would promote platelet activation.

It is important to note that perfusion did not dislodge the seeded endothelial cells (Figure 5). Endothelialization of vascular grafts is limited by the inability of endothelial cells to remain attached to the scaffold after exposure to flow [1]. The seeding process used in this study was clearly effective in creating an intact endothelial cell lining that withstood high shear stress during whole blood perfusion. It is also encouraging that endothelial viability could be demonstrated after the perfusion study (Figure 5).

Study limitations

Limitations of the present study include the small number of arteries that were seeded. Since this study was done to prove that baboon arteries can be seeded with human endothelial cells, we did not use many arteries. A follow-up study would be worthwhile with a large number of small arteries, in which autologous endothelial cells or progenitor cells with increased growth potential will be isolated from blood and seeded onto these arteries.

Future Prospects and Conclusions

HUVECs were successfully seeded on decellularized baboon arteries. The decellularization did not alter the morphology of the extracellular matrix of the arteries and, importantly, the basal membrane remained intact. Endothelialization clearly prevented thrombus formation on the decellularized arterial scaffold surfaces after perfusion with whole blood at high shear rate. Importantly, the 2-h perfusion did not damage the seeded endothelial cells, and these results were similar to those obtained in the perfused control. It is therefore possible to speculate that the findings in a primate model can be extrapolated to humans, and this topic warrants further investigation.

Conflict of Interest

None.

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The use of an algorithm for the laboratory diagnosis of von Willebrand disease

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ABSTRACT

Von Willebrand Disease (vWD) is a bleeding disorder caused by either quantitative (type 1 and 3) or qualitative (type 2) defects of von Willebrand factor (vWF). No single test is available that provides appropriate information about the various functions of vWF and the laboratory diagnosis of vWD is based on a panel of tests that includes the measurement of factor VIII coagulant activity (VIIIC), vWF antigen (vWF:Ag), vWF activity as measured by ristocetin cofactor activity (vWFR:Co), vWF multimer analysis, ristocetin induced platelet agglutination (RIPA), the factor VIII binding assay of plasma vWF and the bleeding time. Due to the heterogeneity of vWF defects and the variables that interfere with vWF levels, a correct diagnosis of types and subtypes may sometimes be difficult but is very important for therapy. Furthermore, the Ristocetin Cofactor test and the RIPA test are based on platelet agglutination in reaction with the non physiological antibiotic, ristocetin. These tests also have low sensitivity and are difficult to standardise. Therefore, several analyses (tests) are required to diagnose vWD and it is important to take the pitfalls that these tests are subject to in consideration in the diagnosis of vWD.

In this article, the laboratory diagnosis of vWD is presented on patients with type 1, 2A, 2B and 2M vWD. The diagnosis is done by using an algorithm that is proposed by the guidelines for diagnosis and treatment of vWD in Italy. The pitfalls in this diagnosis of vWD are outlined by 4 other patients.

Introduction

Von Willebrand Disease (vWD) is a bleeding disorder caused by a quantitative or qualitative defect of vWF. vWF is a high molecular weight glycoprotein that plays an essential part in the early phases of haemostasis by promoting platelet adhesion to the sub endothelium and platelet aggregation under high shear stress conditions [1]. vWF is also the carrier of factor VIII in plasma and a deficiency or abnormality of vWF also results in an impairment of blood coagulation. By the non covalent interaction between vWF and factor VIII, factor VIII is protected against binding to membrane surfaces and to proteolytic attack by a variety of serine proteases, including activated protein C [2]. In the majority of cases, vWD is a congenital disease that is inherited in an autosomal dominant fashion. Patients with vWD may have a mild, moderate or severe bleeding tendency since childhood, usually proportional to the degree of the vWF defect. Inherited vWD has been subdivided into three types that reflect its pathophysiology. Types 1 and 3 vWD reflect respectively, the partial or virtually complete deficiency of vWF. Type 2 vWD is a defect that is subdivided into 4 subtypes (2A, 2B, 2M and 2N). Type 2A refers to variants with decreased platelet dependent function and is associated with the absence of high molecular weight multimers. Type 2B refers to variants with increased affinity for platelet glycoprotein 1b. Type 2M refers to variants with decreased platelet dependent function not caused by the absence of high molecular weight multimers and Type 2N to variants with markedly decreased affinity for factor VIII.

The spectrum and severity of vWD is wide, ranging from few, doubtful haemorrhagic symptoms to severe life threatening bleeding episodes. This is due not only to the heterogeneous vWF gene which may impair its haemostatic function, but also to the influence exerted by other genes (e.g. those for ABO blood groups) [3]. In addition many acquired conditions, either physiologic (stress, pregnancy) or pathologic (inflammation), can induce fluctuations in vWF levels [3]. This, highly variable clinical picture and the presence of many different defects in the vWF molecule, complicate the diagnosis of vWD [3].

The guidelines for diagnosis and treatment of VWD in Italy propose the use of an algorithm (Fig 2). We adopted these guidelines in our Haematology Clinic and tested the usefulness thereof.

Materials and Methods

Subjects

Patients referred to the Haematology Clinic with a history of a bleeding tendency were screened for von Willebrand Disease.

Sample collection

Blood samples were collected into two Vacutainer tubes containing 0.105M sodium citrate in a ratio of 1:9 with blood. Platelet poor plasma was prepared by centrifugation of whole blood at 2000 g for 20 minutes at room temperature. Samples were stored in polypropylene tubes at 70°C until analysed. All tests (except the vWF multimer test) were done on original aliquots that were not previously thawed.

Screening tests

The platelet count was determined on the TECHNICON H1 blood cell analyser (Bayer Diagnostics, Germany). A bleeding time, prothrombin time (PT) and activated partial thromboplastin time (APTT) were determined on each patient with the STart 4 coagulation monitor, (Diagnostica Stago, Asnières, France). Furthermore, the blood group of each patient was determined. Plasma factor VIII levels were also determined on the ACL coagulation analyser (Instrumentation Laboratories, Italy).

VWF antigen (vWF:AG)

The vWF concentration in plasma was measured with an enzyme linked immuno adsorbent assay (ELISA).

Ristocetin cofactor assay (vWF:RCO)

This assay is done with the ristocetin cofactor assay kit from Helena Laboratories (France). Formalin fixed washed platelets do not agglutinate in the presence of the antibiotic ristocetin unless normal plasma is added as a source of vWF. The agglutination follows a dose response curve that is dependent on the amount of plasma vWF added. The test was done on the Monitor IV Plus platelet aggregometer (Helena Laboratories, France). A standard curve of calibrated human plasma is used as the standard against which the patient's plasma is measured.

Ristocetin induced platelet agglutination (RIPA)

RIPA is measured by mixing different concentrations of ristocetin ranging from 0.2 to 2 mg/ml with increments of 0.1 mg/ml with the patient's platelet rich plasma (PRP) in an aggregometer. The results are expressed as the concentrations of ristocetin (mg/ml) able to induce 30% agglutination. Types 2A and 2M vWD show a low response to ristocetin, i.e. ristocetin concentrations more than 1.2 mg/ml are needed to induce 30% agglutination. An important exception is type 2B vWD in which there is a hyper responsiveness to ristocetin, due to a higher than normal affinity of vWF to platelet GP1b [5]. In these patients low ristocetin concentrations (0.2 to 0.8 mg/ml) are needed to induce 30% agglutination.

vWF multimers

The multimeric structure of vWF in plasma was determined by a highly sensitive and rapid method described by Krizek and Rick in 2000 [6]. This method utilises submerged horizontal agarose gel electrophoresis, followed by transfer of the vWF onto a polyvinylidine fluoride membrane, and immuno localisation and luminographic visualisation of the vWF multimer pattern. This method distinguishes type 1 from type 2A and 2B vWD. Type 2A shows a total absence of the high and intermediate molecular weight multimers and type 2B shows an absence of only the high molecular weight multimers. All the other types or subtypes show a multimer pattern similar to that of normal plasma. Advantages to this method include rapid processing, simplicity of gel preparation, high sensitivity to low concentrations of vWF and elimination of radioactivity. Figure 1 shows multimer patterns of normal plasma, type1, and 2A and 2B vWD.

Factor VIII binding assay

The capacity of plasma vWF to bind exogenous FVIII was measured with an ELISA. A micro plate (Maxisorp, Nunc, Denmark) was coated by incubation for 2 days at 4°C with 2 μg/ml of rabbit polyclonal antihuman vWF (Dako, Denmark). After washing with Tris 50 mmol/l NaCl 100 mmol/l, pH 8.0 (TBS) buffer containing 0.1% bovine serum albumin (BSA) and 0.05% Tween, the wells were saturated with TBS containing 3% BSA. Then 100 μl of serial dilutions of plasma from patients and normal pooled plasma were added and incubated overnight at 4°C. Each patient sample was tested

in six serial dilutions, the first being adjusted to 5% vWF antigen level. After removal of endogenous FVIII using 350 mmol/l $CaCl_2$ (10 min, twice), 70mU of recombinant FVIII were added to each well. After incubation for 2 hours at 37°C and washing, bound FVIII was quantified using 1 μg/ml of peroxidase conjugated sheep polyclonal antihuman FVIII (Kordia, The Netherlands). After washing, immobilised VWF was measured using 0.1μg.ml of peroxidase conjugated rabbit polyclonal anti humanVWF (Dako). The colour was developed by addition of OPD and the OD was read at 490nm. Two reference curves were established in parallel, one for the quantification of immobilised vWF and one for the quantification of bound rFVIII by diluting normal pooled plasma. For each plasma dilution, the val ues of bound rFVIII were plotted against the amount of immobilised vWF. The slopes of the obtained regression lines reflect binding capacity of vWF to FVIII.

Results

Diagnosis of vWD

The algorithm used for the laboratory diagnosis of vWD is outlined in Figure 2. A proportional reduction of both vWF:Ag and vWF:RCo with a RCo/Ag ratio >0.7 suggests type 1 vWD. If the RCo/Ag ratio <0.7, type 2 is diag nosed. Type 2B vWD can be identified in cases of an enhanced RIPA (<0.8mg/ml) while type 2A and 2M in a low RIPA (>1.2mg/ml). Multimeric analysis in plasma is necessary to distinguish between type 2A vWD (lack of largest and intermediate multimers) and type 2M vWD (all the multimers are present as in normal plasma). In type 1 vWD the ratio between factor VIII and vWF:Ag is always >1. When this ratio <1, type 2N vWD is suspected and this type of vWD can be confirmed by performing a factor VIII binding assay.

The results of patients who have been diagnosed with the different types and subtypes of vWD are given in table 1. Patient 1 has low vWF:Ag and vWF: RCo values with a RCo/Ag ratio of 0.74, therefore >0.7, which sug gests type 1 vWD. The RIPA is normal and high molecular weight multimers are present. The ratio between factor VIII and vWF: Ag is also >1. Patient 2 has a very low RCo value with a RCo/Ag ratio of 0.44. This suggests type 2 vWD. The RIPA was decreased (30% agglutination occurs at 1.5 mg/ml ris tocetin) and the high and intermediate vWF multimers were absent, which indicates type 2A vWD. The multimeric pattern of this patient is showed in figure 1. Patient 3 also has a RCo/Ag ratio <0.7 which suggest type 2 vWD. The RIPA is however increased (30% agglutination occurs at 0.6 mg/ml ris tocetin) and the high molecular weight multimers are absent. This indicates a type 2B vWD. Patient 4 is also a type 2vWD, since the RCo/Ag ratio is 0.55. The RIPA is decreased and the multimer pattern is normal. This patient is therefore diagnosed as a type 2M vWD.

The results of 4 patients where the diagnosis of the types and subtypes of von Willebrand disease are not clear are outlined in table 2. Patient 5 has an RCo/Ag ratio <0.7 which suggest type 2 vWD. The multimers were normal and suggested a type 2B, but the RIPA, however, was normal. Other mem bers of this patient's family were diagnosed with type 1 vWD. The RCo value (or vWF:Ag) could be wrong in this case. In patient 6 the RCo value was almost twice as much as that of the VWF:Ag. This indicates a "super" functional vWF that does not exist. All the other results of this patient were normal. Patient 7 indicated a type 2 vWD, because of a RiCo/Ag ratio <0.7. The FVIII/Ag ratio was also discrepant and indicated a type 2N vWD. The RIPA was also normal that fits in with a type 2N vWD. The HMW multimers , however, were absent, which indicates a type 2A vWD. Therefore the RIPA and/or the factor VIII level seem to be wrong in this case. Patient 8 also indi cates type 2 vWD since the RiCo/Ag ratio <0.7. The RIPA is however nor mal and the HMW multimers were present. This indicates on the type 2M vWD, but the RIPA cannot be explained.

Discussion

We have outlined a systematic way to diagnose vWD that is recommended by the International Society on Thrombosis and Haemostasis. It is important to note that the RCo/Ag ratio is necessary to distinguish between type 1 and type 2 vWD. The RCo test however has a poor sensitivity (50%) (Scott et al., 1991), is difficult to standardise (Casonato et al., 1999), and lack physi ological analogue. It is however still the standard method for measuring vWF activity that is approved by the Standardization Committee of the International Society on Thrombosis and Haemostasis (ISTH). The low sen sitivity of the RCo assay makes diagnosis of vWD difficult as seen in patients 5 and 6. In patient 5 the family history and the results of the other tests indicate a type 1 vWD, but the RCo value was not proportionate to the Ag level. In patient 6 the RCo value was twice as much as the Ag level that indicates an over active vWF that does not exist in vWD patients.

The RIPA test is necessary to distinguish between type 2B and type 2A vWD and the multimer pattern will indicate type 2A or 2M vWD. It is also important to do the RIPA test with ristocetin concentrations ranging from 0.2 mg/ml to 2 mg/ml with increments of 0.1 mg/ml. This will indicate at which concentrations maximum agglutination value of 30% occur. The RIPA test that is currently used by most laboratories in South Africa is done with only 3 ristocetin concentrations (0.5, 1.0 and 1.25 mg/ml). It is sometimes diffi cult to determine if the RIPA is enhanced or reduced on only 3 values. This was seen in patients 7 and 8 where the RIPA seems normal, but the HMW multimers were absent in patient 7 and in patient 8, all other results indicate a type 2M vWD. Laffan et al. (2004) also found the RIPA normal in patients with an RCo value of less than 30%. And they found the RIPA only decreased in severe forms of vWD.

Multimeric analysis in plasma is necessary to distinguish between type 2A vWD (lack of the largest and intermediate multimers) and type 2M vWD (all multimers present). Our method includes rapid processing, simplicity of gel preparation, high sensitivity to low concentrations of vWF and elimination of radioactivity.

Type 2N vWD can be suspected in case of discrepant values of factor VIII. Thus, in case of factor VIII levels lower than the vWF:Ag, diagnosis of type 2N vWD should be confirmed by the factor VIII binding assay.

Since vWD is such a complex diseases to diagnose, this systematic diag nosis process makes the diagnosis of vWD more accurate, which is very important for the treatment of the disease. There are however pitfalls in this diagnosis process that are due to the limitations in sensitivity, reproducibili ty and interlaboratory variability of the agglutination based RCo and RIPA tests (Favaloro et al., 1999).

Another functional assay that more laboratories are starting to use over the last 7 years is the collagen binding assay (CBA) of vWF (Favaloro et al., 2004). The CBA is based on the ability of the HMW multimers of vWF to preferentially bind collagen. This is an ELISA based assay where dilutions of the patient's plasma is added to a collagen coated ELISA plate and the amount of bound vWF evaluated using an anti HRP conjugated vWF anti body. The values are expressed in U/dl, considering the optical density observed in the normal pooled plasma dilution as 100. This assay has been shown to be sensitive in the discrimination of Type 1 and types 2A and 2B vWD (Favaloro & Koutts, 1997). Casonato et al., 2001 demonstrated that the CBA was consistently more sensitive to large and intermediate vWF mul timer representation than the RCo assay, since none of type 1 vWD patients studied showed CBA more decreased than the Ag levels. That was not the case with the RCo assay. The decreased values of the CBA in type 2A and 2B patients were more consistent than that of the RCo assay. The CBA is however insensitive to type 2M vWD patients.

In conclusion, it is crucial to use this systematic way to diagnose vWD. The CBA should be included as a useful diagnostic test in the profile of vWD diagnosis to counteract the pitfalls in the diagnosis of this disease.

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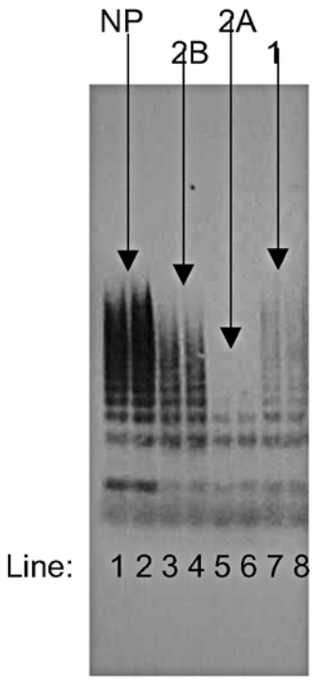

Figure 1
The multimeric structure of vWF in normal plasma (NP) (lines1 and 2) Type 2B vWD (lines 3 and 4), Type 2A vWD (lines 5 and 6) and type 1vWD (lines 7 and 8).

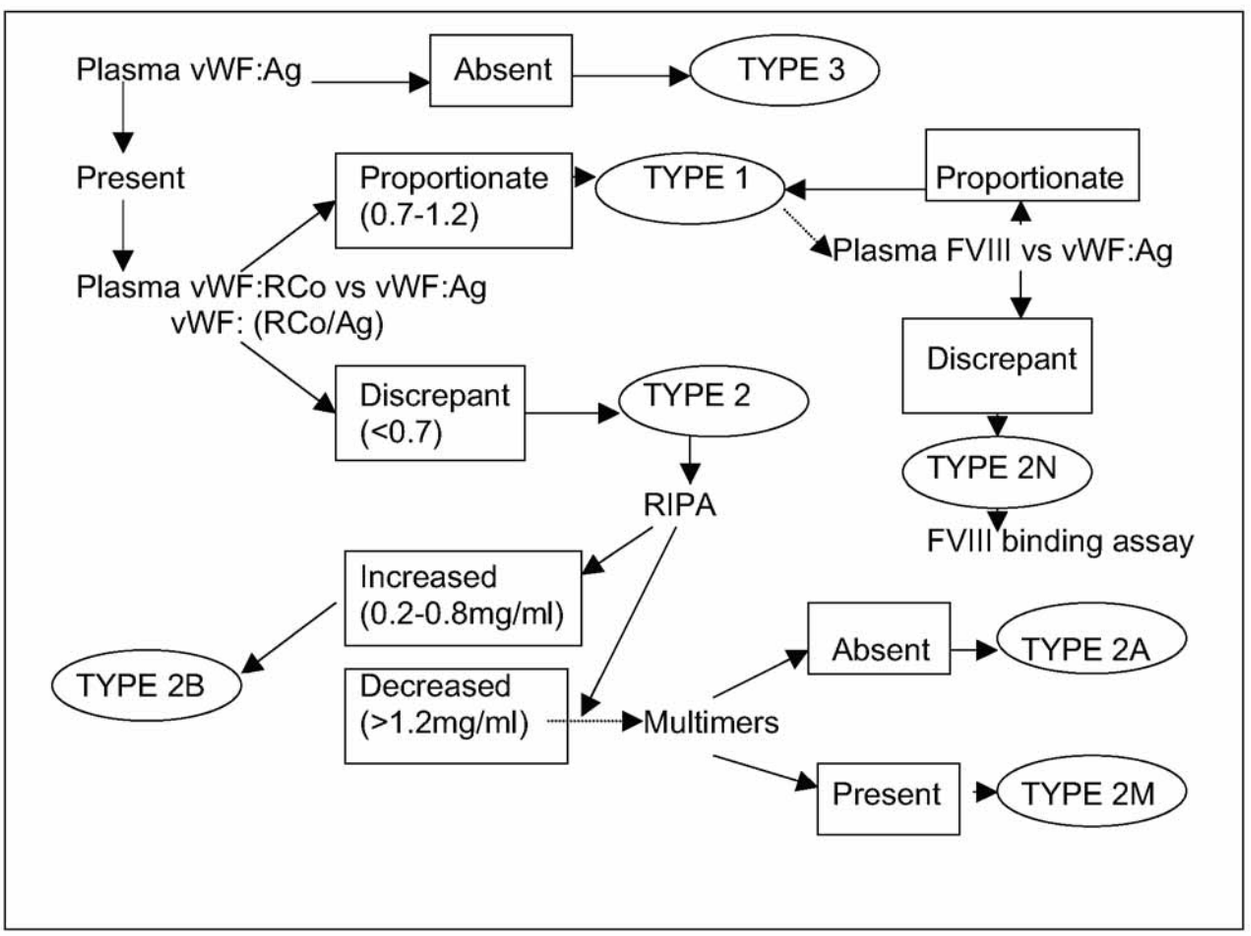

Figure 2
Algorithm for the laboratory diagnosis of vWD

TABLE 1
Laboratory results of 4 patients with different type and subtypes of von Willebrand disease.

	Patients			
Tests	1	2	3	4
Factor VIII	123	57	49	77
vWF:Ag	54	54	42	82
vWF: RCo	40	24	22	45
RCo/Ag ratio	0.74	0.44	0.52	0.55
RIPA (Ristocetin conc.)	Normal (1.1mg/ml)	Decreased (1.5mg/ml)	Increased (0.6mg/ml)	Decreased (1.3mg/ml)
HMW Multimers	Present	Absent	Partially absent	Present
Diagnosed Type	Type 1	Type 2A	Type 2B	Type 2M

TABLE 2

Laboratory results of 4 patients where the diagnosis of the types and subtypes of von Willebrand disease are not clear.

	Patients			
Tests	**5**	**6**	**7**	**8**
Factor VIII	60	55	56	110
vWF:Ag	56	63	116	79
vWF: RCo	30	104	46	15
RCo/Ag ratio	0.53	1.65	0.4	0.18
RIPA (Ristocetin conc.)	Normal 1 mg/ml	Normal 1 mg/ml	Normal 0.9 mg/ml	Normal 1 mg/ml
HMW Multimers	Present	Present	Absent	Present
Diagnosed Type	Type 2M or 1?	Type 1?	Type 2A?	Type 2M?

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Laboratory Diagnosis of von Willebrand Disease

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Abstract

von Willebrand disease (VWD) is a bleeding disorder caused by either quantitative (type 1 and 3) or qualitative (type 2) defects of von Willebrand factor (VWF). No single available test provides appropriate information about the various functions of VWF, and the laboratory diagnosis of VWD is based on a panel of tests, including the measurement of factor VIII coagulant activity (FVIIIC), VWF antigen levels (VWF:Ag), VWF activity as measured by the ristocetin co-factor activity (VWF:RCo), the collagen-binding activity of VWF (VWF:CB), VWF multimer analysis, ristocetin-induced platelet agglutination (RIPA), the factor-VIII-binding assay of plasma VWF and VWF propeptide levels. Due to the heterogeneity of VWF defects and the variables that interfere with VWF levels, a correct diagnosis of types and subtypes may sometimes be difficult, but is very important for therapy. Furthermore, the RCo assay and the RIPA test are based on platelet agglutination in reaction with the non-physiological antibiotic ristocetin. These tests also have low sensitivity and are difficult to standardise. Therefore, several analyses (tests) are required to diagnose VWD and it is important to be aware of the pitfalls to which these tests are subjected in terms of the diagnosis. In this article, the laboratory diagnosis of patients with type 1, 2A, 2B, 2M, 2N and 3 VWD will be explained by using a modified algorithm that was first proposed by the guidelines for diagnosis and treatment of VWD in Italy.

Keywords

von Willebrand factor, von Willebrand disease, classification, subtypes

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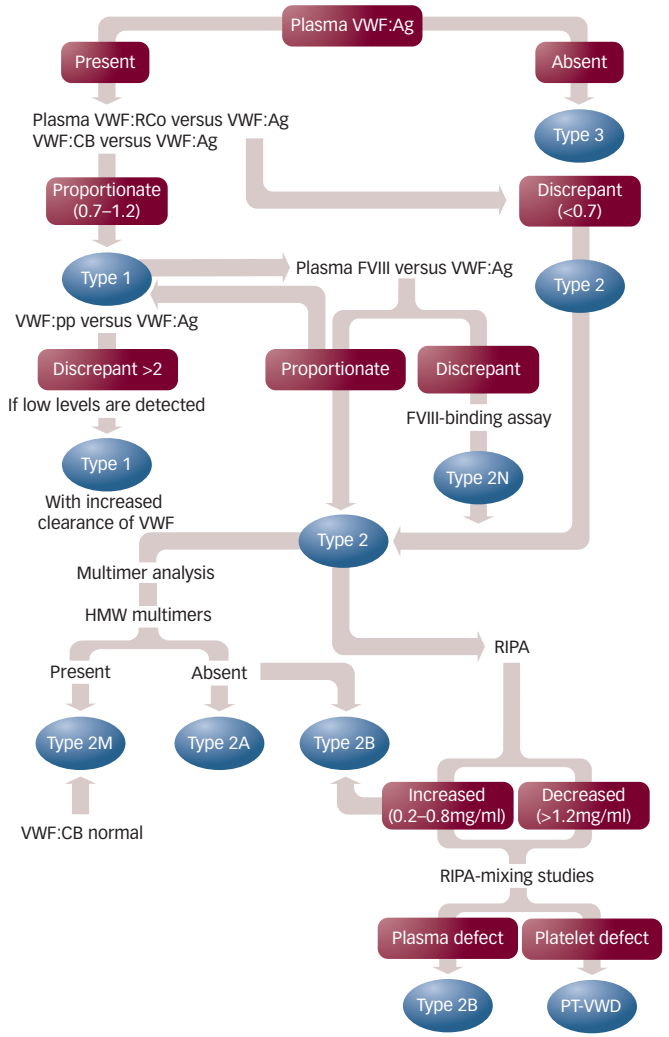
von Willebrand disease (VWD) is a bleeding disorder caused by quantitative or qualitative defects of von Willebrand factor (VWF). VWF is a high-molecular-weight (HMW) glycoprotein that plays an essential part in the early phases of haemostasis by promoting platelet adhesion to the subendothelium and platelet aggregation under high shear stress conditions.[1] VWF is also the carrier of factor VIII (FVIII) in plasma, and a deficiency or abnormality of VWF also results in an impairment of blood coagulation. By the non-covalent interaction between VWF and FVIII, FVIII is protected against binding to membrane surfaces and to proteolytic attack by a variety of serine proteases, including activated protein C.[2] In the majority of cases, VWD is a congenital disease that is inherited in an autosomal-dominant fashion. Patients with VWD may have had a mild, moderate or severe bleeding tendency since childhood, usually proportional to the degree of the VWF defect. Inherited VWD has been classified into three types that reflect its pathophysiology. Type 1 VWD is characterised by partial quantitative deficiency of VWF. It is the most common type, with a prevalence of about 80%. The mechanisms involved include reduced synthesis and secretion of VWF or increased clearance of VWF from plasma.[3] The treatment is simple, as 1-deamino-8-D-arginine vasopressin (DDAVP) causes the release of structurally normal VWF from endothelial stores. However, in patients with increased clearance of VWF, DDAVP treatment would not be effective as VWF in plasma is cleared very quickly from the circulation. Therefore, it is important to diagnose patients with an increased clearance rate of VWF.

Type 3 VWD is the least common subtype and reflects a virtually complete absence of VWF. Again, DDAVP treatment is not effective in type 3 VWD. The current treatment of choice for type 3 VWD, as well as most type 2 VWD, is transfusional therapy with plasma-derived FVIII/VWF concentrates.[3]

Type 2 VWD is a quantitative defect that is subdivided into four subtypes (2A, 2B, 2M and 2N VWD). Type 2A refers to variants with decreased platelet-dependent function and is associated with the absence of HMW multimers; type 2B refers to variants with increased affinity for platelet glycoprotein 1bα; type 2M refers to variants with decreased platelet-dependent function not caused by the absence of HMW multimers; and type 2N refers to variants with markedly decreased affinity for FVIII. DDAVP treatment is not recommended in patients with type 2 VWD, as it would only increase the dysfunctional VWF.

Platelet-type VWD (PT-VWD) is a rare autosomal-dominant bleeding disorder. The genetic defect is in platelets rather than VWF, and the disease is characterised by abnormally high binding affinity of the platelets to the VWF, similar to type 2B VWD.[4] Therefore, most people with PT-VWD are misdiagnosed as type 2B VWD. However, these may require different therapeutic management, so discrimination is clinically important. The spectrum and severity of VWD is wide, ranging from a few doubtful haemorrhagic symptoms to severe life-threatening bleeding episodes. This is due not only to the heterogeneous VWF gene,

Figure 1: Algorithm for a Laboratory Diagnosis of von Willebrand Disease



VWF = von Willebrand factor; RCo = ristocetin co-factor; Ag = antigen; CB = collagen-binding; pp = propeptide; FVIII = factor VIII; HMW = high-molecular-weight; RIPA = ristocetin-induced platelet agglutination; PT = platelet type.

which may impair its haemostatic function, but also to the influence exerted by other genes (e.g. those for ABO blood groups).[6] In addition, many acquired conditions – either physiological (stress, pregnancy) or pathological (inflammation) – can induce fluctuations in VWF levels).[6] This highly variable clinical picture and the presence of many different defects in the VWF molecule complicate the diagnosis of VWD.[6] The guidelines for diagnosis and treatment of VWD in Italy[7] propose the use of an algorithm (see *Figure 1*). We adopted and modified these guidelines in our VWD testing facility.

Laboratory Diagnosis of von Willebrand Disease

Sample Collection

Blood samples must be collected into tubes containing 0.105M sodium citrate in a ratio of 1:9 with blood. Platelet-poor plasma (PPP) is prepared by centrifugation of whole blood at 2,000g for 20 minutes at room temperature. Samples must be stored immediately after centrifugation in polypropylene tubes at -70ºC until analysed. It is important to note that a cryoprecipitate may form if plasma samples are stored at temperatures over -70ºC. Cryoprecipitate contains large quantities of VWF, especially HMW multimers.[8] All tests must be performed on original aliquots that were not previously thawed, and plasma samples should be thawed to 37ºC before performing the diagnostic tests. Special care should be taken to ensure that no cryoprecipitate is present in the samples; therefore, it must be dissolved before the tests are performed as it will influence the results.[8]

Screening Tests

Screening tests for bleeding disorders include a platelet count, a bleeding time, prothrombin time (PT) and activated partial thromboplastin time (APTT), plasma FVIII levels and the blood group of a patient. These tests are usually carried out by a routine coagulation laboratory.

Laboratory Tests

The first line of tests includes the VWF concentration in plasma (VWF:Ag), the ristocetin co-factor assay (VWF:RCo) and the collagen binding assay (VWF:CB). VWF:Ag is measured with an enzyme-linked immunoabsorbent assay (ELISA). An ELISA plate is coated with a specific rabbit antihuman VWF antibody that captures the VWF to be measured. The plasma to be measured is added in 1:50 and 1:100 dilutions in blocking buffer. Afterwards, a rabbit antihuman VWF antibody conjugated to peroxidase is added. This antibody binds to the remaining free antigenic determinants of VWF, subsequently forming a 'sandwich'. The bound enzyme peroxidase is revealed by its activity in a pre-determined time on the substrate ortho-phenylenediamine (OPD) in the presence of hydrogen peroxide. After stopping the reaction with a strong acid, the intensity of the colour produced bears a direct relationship to the VWF concentration initially present in the plasma sample. A standard curve of calibrated human plasma is used as the standard against which the patient's plasma is measured.

The RCo assay is performed with formalin-fixed washed platelets in an aggregometer with software suited for the test. Formalin-fixed washed platelets can be obtained commercially or can be self-prepared from normal platelet-rich plasma (PRP). Washed platelets do not agglutinate in the presence of the antibiotic ristocetin unless normal plasma is added as a source of VWF. The agglutination follows a dose–response curve that is dependent on the amount of plasma VWF added. Usually, plasma concentrations of 1:2 and 1:4 in Tris-buffered saline (TBS) are used. A standard curve of calibrated human plasma is used as the standard against which the patient's plasma is measured. Another functional assay that more laboratories have been starting to use over the last seven years is the collagen-binding assay (CBA) of VWF.[9] The CBA is based on the ability of the HMW multimers of VWF that preferentially bind to collagen. This is an ELISA-based assay where dilutions of the patient's plasma (comparable to the VWF:Ag 1:50 and 1:100 dilutions) are added to a collagen-coated ELISA plate. The type of collagen seems to be important, but discordance still exists about which type of collagen to use (type 1,

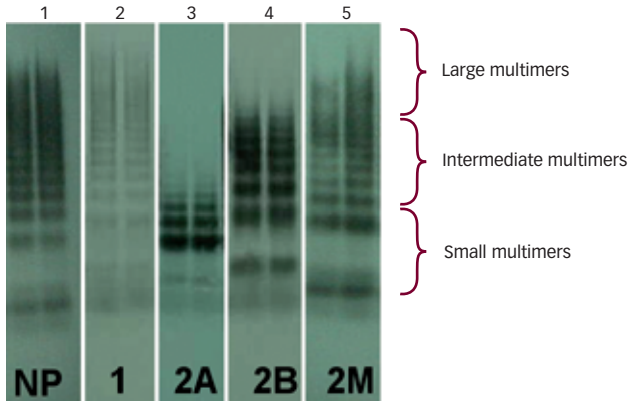
A functional assay that more laboratories have been starting to use over the last seven years is the collagen-binding assay of von Willebrand factor.

type 3 or a combination thereof).[10] The amount of bound VWF is determined by using an anti-horseradish peroxidase (HRP)-conjugated VWF antibody. The values are expressed in U/dl or per cent. A standard curve of calibrated human plasma is used as the standard against which the patient's plasma is measured. This assay has been shown to be sensitive in the discrimination of type 1, 2A and 2B VWD.[11] Casonato et al.[12] demonstrated that the CBA was consistently more sensitive to large- and intermediate-VWF multimer representation than the RCo assay. The decreased values of the CBA in type 2A and 2B patients are more consistent than that of the RCo assay. However, the CBA is insensitive to type 2M VWD patients.[13]

The ristocetin-induced platelet agglutination (RIPA) and the VWF multimer patterns are performed to diagnose the type 2 subtypes. RIPA is measured by mixing different concentrations of ristocetin ranging from 0.2 to 2mg/ml in increments of 0.1mg/ml with the patient's PRP in an aggregometer. The results are expressed as the concentration of ristocetin (mg/ml) able to induce 30% agglutination. RIPA-mixing studies are performed to distinguish between type 2B VWD and PT-VWD. In short, the patient's PRP and PRP from a control person are centrifuged and gently resuspended to 200x10^6 platelets per millilitre in the following manner: patient platelets/patient plasma; normal platelets/normal plasma; control platelets/patient plasma; and patient platelets/normal plasma. Different ristocetin concentrations are added to each of the platelet suspensions and the agglutination is measured in an aggregometer, similar to the RIPA assay.[5] PT-VWD is diagnosed when the RIPA-mixing studies confirmed a platelet origin. The multimeric structure of VWF in plasma is determined by a highly sensitive and rapid method originally described by Krizek and Rick in 2000.[14] This method utilises submerged horizontal agarose gel electrophoresis followed by transfer of the VWF onto a polyvinylidine fluoride membrane and immunolocalisation and luminographic visualisation of the VWF multimer pattern. This method distinguishes type 1 from type 2A and 2B VWD. The density of the high-, intermediate- and low-molecular-weight multimers of each multimer pattern is determined using a gel-documentation system.[15] VWF multimer patterns from normal plasma and type 1, 2A, 2B and 2M VWD is shown in *Figure 2* and the density graphs in *Figure 3*.

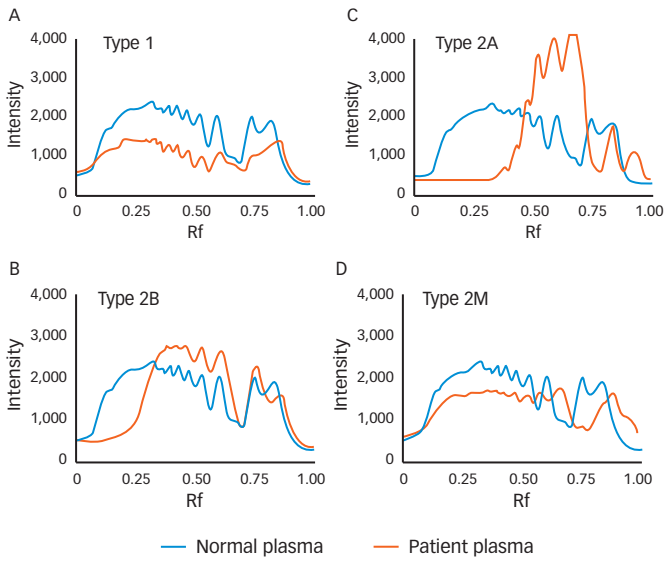
The capacity of plasma VWF to bind exogenous FVIII is measured with an ELISA. A microplate (Maxisorp, Nunc, Denmark) is coated by incubation for two days at 4°C with a rabbit polyclonal antihuman VWF. After washing with a TBS buffer of 0.1% bovine serum albumin (BSA) and 0.05% Tween, the wells are saturated with TBS containing 3% BSA. Next, 100µl of serial dilutions of plasma from patients and normal pooled plasma are added and incubated overnight at 4°C. Each patient sample is tested in six serial dilutions, the first adjusted to 5% VWF-antigen level. After removal of endogenous FVIII using 350mmol/l $CaCl_2$ (twice for 10 minutes), 70mU of recombinant FVIII is added to each well. After incubation for two hours at 37°C and washing, bound FVIII is quantified using 1µg/ml of peroxidase-conjugated sheep polyclonal antihuman FVIII. After washing, immobilised VWF is measured using a peroxidase-conjugated rabbit polyclonal antihuman VWF. The colour is developed by addition of OPD and the optical density read at 490nm. Two reference curves are established in parallel. One is for the quantification of immobilised VWF and the other one for the quantification of bound rFVIII. For each plasma dilution, the values of bound rFVIII are plotted against the amount of immobilised VWF. The slopes of the obtained regression lines reflect the binding capacity of VWF to FVIII.[16]

Figure 2: The Multimeric Structure of von Willebrand Factor in Normal Plasma and von Willebrand Disease Types 1, 2A, 2B and 2M



Source: Meiring et al., 2005.[15]

Figure 3: Densitometric Tracing of Lane Sets 2–5



The density of the different bands is plotted against the relative front (Rf value) of the different lanes.

The VWF propeptide (VWF:pp) level in the patient's plasma is also measured with an ELISA. Microtitre wells are coated with CLB-Pro 35 antibody and incubated with the plasma sample. Subsequently, the wells are washed and the bound propeptide-containing protein is detected with CLB-Pro 14.3 coupled to peroxidase. Calibrators for the VWF:propeptide assay are used to set up a standard curve against which the patient's plasma is measured.[17]

Diagnosis of von Willebrand Disease

The algorithm used for the laboratory diagnosis of VWD is outlined in *Figure 1*. A proportional reduction of both VWF:Ag and VWF:RCo with a RCo/Ag ratio >0.7 as well as a proportional reduction of both VWF:Ag and VWF:CB with a CB/Ag ratio >0.7 suggest type 1 VWD. If type 1 VWD is diagnosed, it is important to determine the clearance rate of VWF; consequently, the VWF:pp is performed. If the ratio between the VWF:pp and the VWF:Ag is more than two, an increased clearance rate of VWF is suspected in this patient.

If the RCo/Ag ratio and/or the CB/Ag ratio is <0.7, type 2 VWD is diagnosed. Type 2B VWD can be identified with an enhanced RIPA (<0.8mg/ml). Type 2B VWD is distinguished from a PT-VWD (pseudo-

VWD) by performing the RIPA-mixing studies. Type 2A and 2M may have a low RIPA (>1.2mg/ml). Multimeric analysis in plasma is necessary to distinguish between type 2A VWD (lack of largest and intermediate multimers) and type 2M VWD (all the multimers are present). The VWF:CB is usually normal in type 2M VWD due to the presence of the HMW multimers, except where a collagen-binding defect is diagnosed in patients with type 2M VWD. In type 1 VWD, the ratio between FVIII and VWF:Ag is always concordant. When this ratio is discrepant with a FVIII level of <20%, type 2N VWD is suspected, and this type of VWD can be confirmed by performing a FVIII-binding assay.

Conclusion

We have outlined a systematic way to diagnose VWD. This algorithm is also recommended by the International Society for Thrombosis and Haemostasis (ISTH). It is important to note that the RCo/Ag ratio and the CB/Ag ratio are necessary to distinguish between type 1 and type 2 VWD.

The CBA is extremely sensitive in the discrimination of type 1 and types 2A and 2B VWD.[11] In contrast, however, the RCo test has a poor sensitivity of 50%,[18] which is difficult to standardise,[19] and lacks a physiological analogue; however, it remains the standard method for measuring VWF activity approved by the Standardisation Committee of the ISTH. Casonato et al.[12] demonstrated that the VWF:CB was consistently more sensitive to large and intermediate VWF-multimer representation than the VWF:RCo. None of the type 1 VWD patients studied showed a greater decrease in CBA than in Ag levels, which was not the case with the RCo assay. The decreased values of the CBA in type 2A and 2B patients were more consistent than those of the RCo assay. The VWF:CB is normal in type 2M VWD patients due to the presence of the HMW multimers. This assists in the diagnosis of type 2M VWD.

The mechanisms involved in type 1 VWD include reduced synthesis and secretion of VWF or increased clearance of VWF from plasma.[20] In patients with increased clearance of VWF, the DDAVP treatment would not be effective, as VWF in plasma is cleared from the circulation very quickly. The ratio between the propeptide and the antigen levels is used as an indication of VWF clearance in these patients.[3]

The RIPA test is necessary to distinguish between type 2B and type 2A VWD. It is also important to carry out the RIPA test with ristocetin concentrations ranging from 0.2 to 2mg/ml in increments of 0.1mg/ml. This will ensure a range of concentrations to determine the maximum agglutination value of 30%. Currently, the RIPA test that is being used by most laboratories in developing countries is performed with only three ristocetin concentrations (0.5, 1 and 1.25mg/ml). It is sometimes difficult to determine whether the RIPA is enhanced or reduced on only three values. Laffan et al.[21] also found RIPA to be normal in patients with a VWF:RCo value of less than 30%. They found RIPA decreased only in severe forms of VWD. When RIPA is increased and type 2B is suspected, it is important to perform the RIPA-mixing studies to distinguish PT-VWD from type 2B VWF.

VWF multimeric analysis in plasma is necessary to distinguish between subtypes of type 2 VWD. Our method includes rapid processing, simplicity of gel preparation, high sensitivity to low concentrations of VWF and elimination of radioactivity.[15] Type 2N VWD can be suspected in case of discrepant values of FVIII. In typical type 2N cases, the FVIII level is usually less than 20%.[22] The diagnosis of type 2N VWD should be confirmed by the FVIII-binding assay. As VWD is such a complex disease to diagnose, this systematic approach makes the diagnosis of VWD more accurate, which is of vital importance for the treatment of the disease. However, there are pitfalls in this diagnostic process that are due to the limitations in sensitivity, reproducibility and inter-laboratory variability of the agglutination-based RCo and RIPA tests.[23]

In conclusion, it is crucial to use a systematic method to diagnose VWD. Each laboratory test only forms one piece of the diagnostic puzzle and therefore it is necessary to put all of the puzzle pieces together for the whole diagnostic picture to emerge. ■

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Laboratory Diagnosis and Management of von Willebrand Disease in South Africa

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ABSTRACT

Patients with von Willebrand disease (VWD) in South Africa are cared for in 17 Hemophilia Treatment Centers. The exact prevalence of the disease is uncertain, but 539 patients are annotated in registries. VWD patients are mostly diagnosed in the five largest academic centers, and the classification of the subtypes is performed by one of these, the VWD testing facility. An algorithm is used for the diagnosis of VWD. The distribution of subtypes diagnosed by the VWD reference center is 38%, 58%, and 4% for type 1, 2, and 3, respectively, and ~15% of plasma samples received are rejected due to poor storage and transport conditions. A novel single nucleotide polymorphism has been found in an African patient with type 2B VWD. From the type 1 VWD patients who were diagnosed by the VWD testing facility, 45% seem to have an increased VWF clearance phenotype with a propeptide-to-antigen ratio of 1.9 ± 0.3. VWD patients are treated with desmopressin, factor (F)VIII/VWF concentrate (Haemosolvate FVIII; National Bioproducts Institute, Durban, South Africa), and tranexamic acid. Haemosolvate FVIII contains a VWF antigen concentration of 167 ± 27 IU/mL, a ristocetin cofactor activity of 100 ± 29 IU/mL, a collagen binding activity of 99 ± 29 IU/mL, normal VWF multimers, and a FVIII concentration of 50 IU/mL. Not all patients with VWD are currently classified, and many VWD patients in South Africa are probably undiagnosed.

KEYWORDS: von Willebrand disease, classification, diagnosis, hemophilia treatment centers, South Africa

South Africa has a total land area of slightly more than 1.2-million km^2, making it roughly the same size as Nigeria, Angola, Mali, or Colombia. It measures some 1600 km from north to south, roughly the same from east to west, and has a population of 49 million.

Patients with bleeding diatheses in South Africa are cared for in 17 Hemophilia Treatment Centers (HTCs) distributed all over the country. The HTCs function in collaboration with the South African National Department of Health, the South African Haemophilia Foundation (the national members' organization), the Medical and Scientific Council of South Africa, and the National Haemophilia Nurses Committee to ensure optimal management of patients with bleeding disease, including von Willebrand disease (VWD).[1]

Hemophilia care data collected from 2004 to 2007 shows that > 2200 patients with bleeding diatheses were cared for in this period by 79 professionals in 17 HTCs. Of these patients, 59% had hemophilia A, 21%

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VWD, and 12% hemophilia B; the remainder had rare bleeding diatheses and thrombocytopathies.[1]

The exact prevalence of VWD in South Africa is uncertain. The central plateau of South Africa is relatively dry. Epistaxis in the general population is therefore frequent, and there is not a high index of suspicion of bleeding disorders. Because of lack of emphasis during training, menorrhagia is termed *functional* if there is no obvious gynecologic cause. The woman is then offered oral contraceptives if she is younger and a hysterectomy if she is older. The 2008 Global Survey of the World Federation of Hemophilia estimated there are 522 diagnosed patients with VWD in South Africa. The access-based Hemophilia Registry mentions 539 patients in South Africa.[2] African patients might be grossly underdiagnosed because only 3.9% of patients mentioned in the registry are of African origin. Women form 63.8% of patients identified with VWD in South Africa.[3]

VWD patients are mostly diagnosed in the five largest academic centers, and the classification of the subtypes is done by only one of these five centers, the VWD testing facility. This article concentrates on the VWD testing facility's findings regarding the statistics, diagnosis, and challenges in the laboratory diagnosis of VWD in South Africa.

LABORATORY DIAGNOSIS OF VON WILLEBRAND DISEASE

The following diagnostic tests are performed by the VWD testing facility: von Willebrand factor antigen (VWF:Ag), ristocetin cofactor (VWF:RCo), collagen binding activity of VWF (VWF:CB), VWF propeptide levels (VWF:pp), multimeric analysis of VWF, the factor (F)VIII binding assay of VWF, and mixing studies to identify platelet-type VWD (PT-VWD).

The VWD testing facility adopted and modified the guidelines for diagnosis and treatment of VWD in Italy according to the algorithm outlined in Fig. 1.[4,5]

A proportional reduction of both VWF:Ag and VWF:RCo with a RCo:Ag ratio >0.7 as well as a proportional reduction of both VWF:Ag and VWF:CB with a CB:Ag ratio >0.7 suggest type 1 VWD if the VWF:Ag level is low ($<45\%$). If type 1 VWD is diagnosed, it is important to determine the clearance rate of VWF. The VWF:pp is then performed. If the ratio between the VWF:pp and the VWF:Ag is >2, an increased clearance rate of VWF is suspected for that patient.

If the RCo:Ag ratio and/or the CB:Ag ratio is <0.7, type 2 VWD is diagnosed. Type 2B VWD can be identified with of an enhanced ristocetin-induced platelet agglutination (RIPA) (response with <0.8 mg/mL). Type 2B VWD is distinguished from a PT-VWD (pseudo-VWD) by performing the RIPA mixing studies. Type 2A and 2M typically have reduced RIPA (response only at >1.2 mg/mL). Multimeric analysis in plasma is necessary to distinguish between type 2A VWD (lack of largest and intermediate multimers) and type 2M VWD (all the multimers are present). The VWF:CB is usually normal in type 2M VWD due to the presence of high molecular weight multimers, except where a collagen binding defect is diagnosed in patients with type 2M VWD. In type 1 VWD the ratio between factor VIII and VWF:Ag is always concordant. When this ratio is discordant with a FVIII level $<20\%$, type 2N VWD is suspected, and this type of VWD can be confirmed by performing a factor VIII binding assay.

Genotypic data are only obtained for patients with a functional abnormality of VWD. In an ongoing study we have searched for mutations in exon 28 of the VWF gene in five patients with functional defects of VWF to set up the method for genetic analysis of VWD. We used two patients with type 2M, two with type 2B, and one with type 2A VWD in this study. The whole exon 28 was analyzed in four specific fragments, using polymerase chain reaction with primers that mismatch the pseudogene. The mutations were identified by automatic sequencing of the different fragments. The following polymorphisms were detected. A silent single nucleotide polymorphism (SNP) 4641T/C in all five patients, the SNP 4141A/G in three patients, a silent SNP 3795G/A in one patient, and a new silent SNP 4923G/A in a patient from the African population. It is important to note that no polymorphisms in exon 28 were previously reported from African populations.

The VWFpp levels are only performed on type 1 VWD patients. We have found that 45% of our type 1 VWD patients seem to have an increased VWF clearance phenotype with a pp:Ag ratio of 1.9 ± 0.3. Our normal range for the pp:Ag ratio of normal subjects is 1.3 ± 0.24.

VON WILLEBRAND DISEASE DIAGNOSTIC STATISTICS

The VWD testing facility is situated in Bloemfontein, the legislative capital of South Africa, in the central part of the country. The academic complex in Bloemfontein serves patients from the Free State and the Northern Cape provinces with a total population of $\sim$4 million. The VWD testing facility, however, receives patient samples from all over the country for diagnosis but especially for the classification of VWD.

Table 1 outlines the relative proportion of the various VWD types diagnosed by the VWD reference center. A total of 250 patients were included in this data set.

Because the VWD testing facility receives mostly VWD samples to be classified and not to be diagnosed,

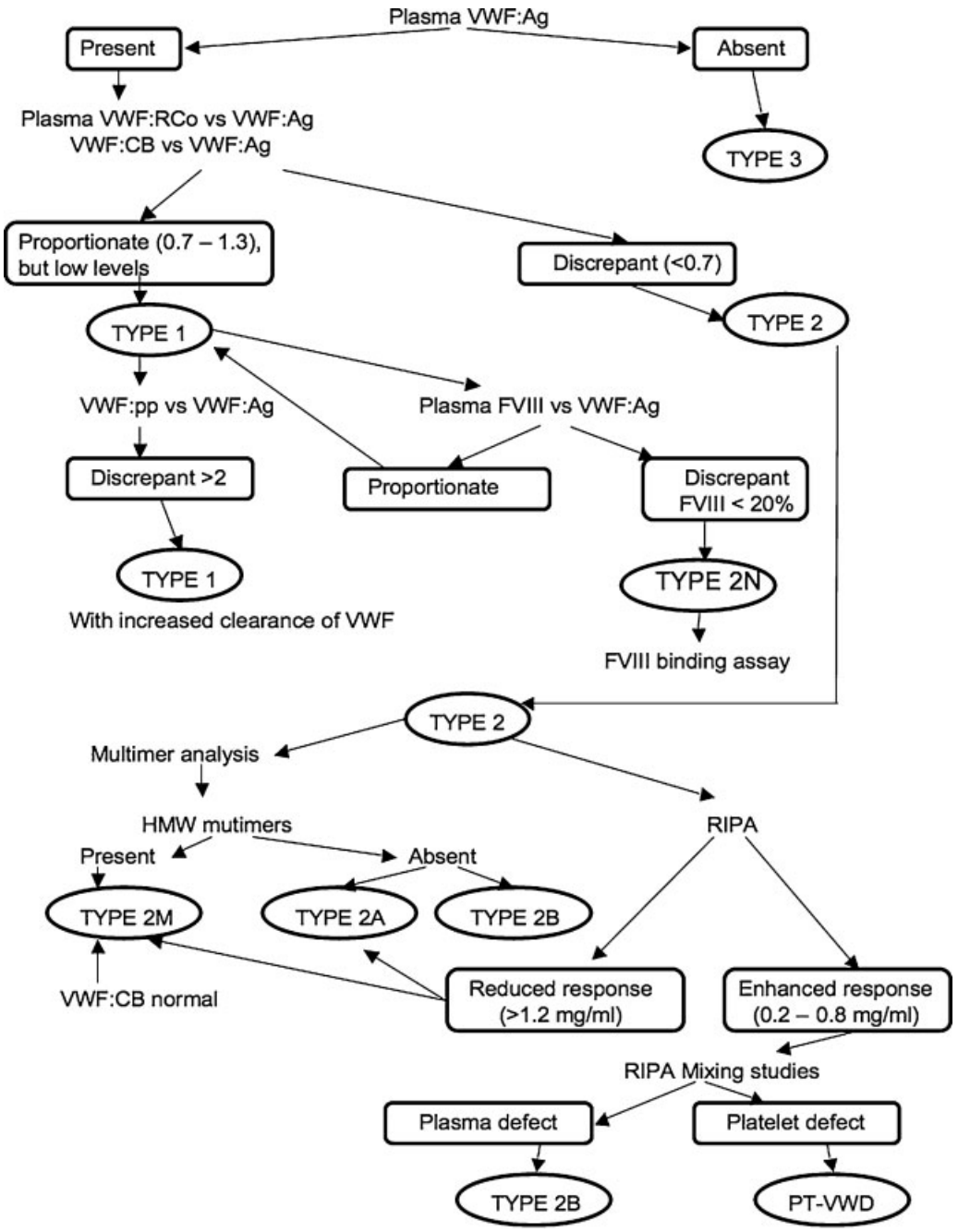

Figure 1 Algorithm for the laboratory diagnosis of von Willebrand disease (VWD) used in our center. FVIII, factor VIII; PT-VWD, platelet-type VWD; RIPA, ristocetin-induced platelet agglutination; VWF:Ag, von Willebrand factor antigen; VWF:CB, VWF collagen binding; VWFpp, VWF propeptide; VWF:RCo, VWF ristocetin cofactor.

the distribution of subtypes diagnosed is 38%, 58%, and 4% for types 1, 2, and 3, respectively. From the type 2 VWD patients, 23% were diagnosed with type 2A, 22% with type 2B, 13% with type 2M, and none with type 2N. No patients with PT-VWD have so far been diagnosed, probably due to the unavailability of platelets from these patients, because most of the type 2 VWD samples are referrals (i.e., transported plasma) from larger HTCs in the country.

CHALLENGES IN THE LABORATORY DIAGNOSIS OF VON WILLEBRAND DISEASE

Samples must be stored immediately after centrifugation in polypropylene tubes at – 70°C until analyzed.[5] It is thus important to note that a cryoprecipitate might form if plasma samples are stored at temperatures warmer than – 70°C. Cryoprecipitates contain large quantities of VWF and especially high molecular weight

Table 1 Relative Proportion of the Various von Willebrand Disease Types

Subtype of VWD	Percentage (Number) of Total Patients Diagnosed*
Mild type 1	30.7% (75)
Moderate to severe type 1	8.2% (20)
Type 2A	22.5% (55)
Type 2B	23.4% (57)
Type 2M	13.1% (32)
Type 2N	0% (0)
Type 3	2.1% (5)
Platelet type	0% (0)
Total	100% (244)

*Total number of patients = 250.
VWD, von Willebrand disease.

Table 2 Test Results of a Normal Plasma Sample Stored at − 20°C in a Domestic Chest Freezer with No Frost-Free Facility versus When Stored at − 70°C for 3 Weeks before Testing

Tests	Stored at − 20°C	Stored at − 70°C
VWF:Ag	71	74
VWF:CB	0	87
VWF:RCo	0	73
Multimer patten	LMW multimers absent	LMW multimers present

VWF:Ag, von Willebrand factor antigen; VWF:CB, VWF collagen binding; VWF:RCo, VWF ristocetin cofactor; LMW, low molecular weight.
See Fig. 2 for multimer patterns.

multimers.[6] All tests therefore must be done on original aliquots that were not previously thawed, and plasma samples should be thawed at 37°C before performing diagnostic tests. Special care should be taken to ensure that no cryoprecipitate is present in the samples. Therefore it must be dissolved before the tests are performed; otherwise it will influence the results.[6]

The VWD testing facility receives plasma samples for subtyping of VWD from most HTCs countrywide. About 15% of samples received are rejected due to poor plasma storage and transport conditions. Samples are now rejected if unfrozen upon arrival or if sent refrigerated only. This decision was made after performing a study where the VWF levels, activity, and multimer distribution were measured on plasma samples following different storage conditions. Normal plasma samples were exposed to different storage conditions and time intervals. We found that the VWF:Ag, VWF:CB, and VWF:RCo results remain normal after storage at − 70°C. The multimer patterns also remain normal. However, after storage at − 20°C in a household chest freezer (not frost free), both the functional assays showed a decreased activity of VWF, and the multimer analysis showed an absence of the high molecular weight multimers in some samples. The multimer pattern thus stays stable at − 70°C but not at − 20°C. Table 2 shows the results of one such normal plasma sample, and Fig. 2 shows the associated multimer pattern. Many laboratories in South Africa use household chest freezers. We also found that even when a plasma sample is thawed and frozen up to five times, the multimer pattern stays normal when stored at − 70°C. Fig. 3 shows the results of a sample that was frozen five times at − 70°C.

TREATMENT OF VON WILLEBRAND DISEASE IN SOUTH AFRICA

VWD patients are treated with desmopressin (DDAVP), FVIII/VWF concentrate, tranexamic acid, and oral contraceptives. The choice of medication depends on the severity of the bleeding or the type of surgical or dental intervention. DDAVP is usually the first product of choice for treatment of new patients with VWD.

The FVIII/VWF concentrate used in South Africa is Haemosolvate FVIII (National Bioproducts Institute, Durban, South Africa). Haemosolvate Factor VIII is an intermediate purity factor VIII concentrate, currently used for the treatment of hemophilia A and VWD. In a previous study we determined the concentration and activity of VWF in Haemosolvate Factor VIII. We received 32 batches of the concentrate from the National Bioproducts Institute in Pinetown, South Africa, and performed the VWF:Ag assay to determine the VWF levels. The functional activity of VWF was determined by performing the VWF:RCo and VWF:CB assays. We also determined the FVIII levels and the multimeric analysis of VWF in these concentrates. For all the tests, we needed to dilute the concentration

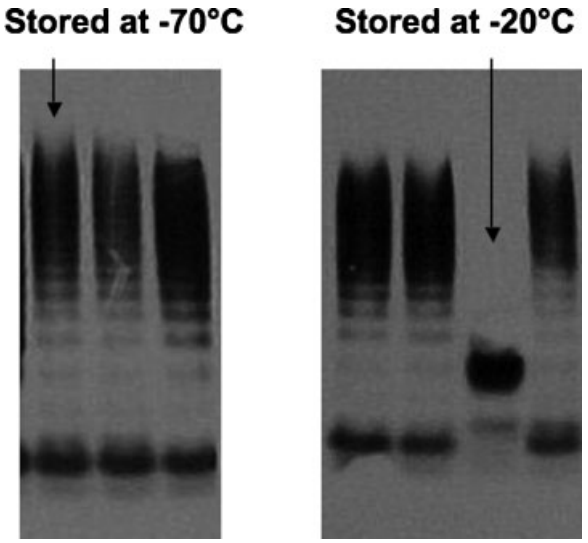

Figure 2 Multimer patterns of a normal plasma sample stored at − 70°C versus when stored in a domestic chest freezer at − 20°C for 3 weeks.

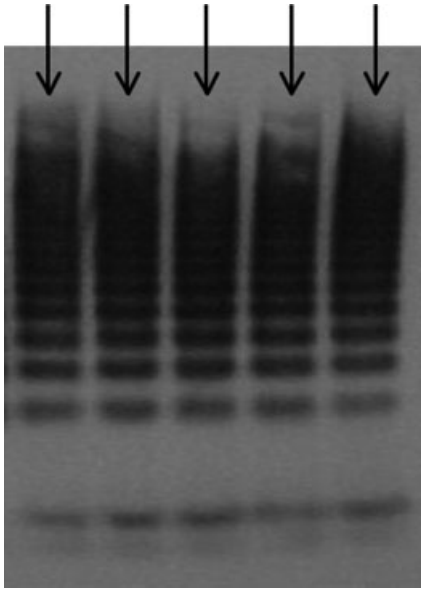

Figure 3 Multimer patterns of a normal plasma sample that was repeatedly thawed and frozen five times at – 70°C.

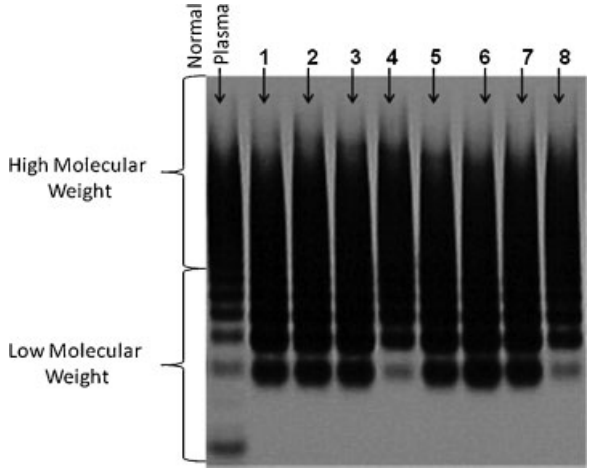

Figure 4 von Willebrand factor multimer patterns of eight batches of Haemosolvate FVIII.

extensively. The VWF:Ag concentration of all batches had a mean value of 167 ± 27 IU/mL, a VWF:RCo activity of 100 ± 29 IU/mL, a VWF:CB activity of 99 ± 29 IU/mL, and a factor VIII concentration of 50 IU/mL. The multimeric analysis showed a normal multimer pattern as seen in Fig. 4. We thus found that the VWF levels and activities in Haemosolvate Factor VIII are more than twice that of the FVIII level. This is now taken into account when this product is administered to patients for the treatment of VWD.

The antifibrinolytic drug tranexamic acid is often given as treatment for mucocutaneous bleedings, if necessary in combination with DDAVP or Haemosolvate Factor VIII. It is also given before and after surgical or dental procedures.

CONCLUSION

Patients with VWD in South Africa are cared for in 17 HTCs distributed throughout the country. These patients are treated according to international guidelines and with the VWF/FVIII concentrate produced and used for VWD in South Africa, Haemosolvate FVIII, a highly active VWF concentrate. The diagnosis of VWD is done mostly in only the five largest academic centers. Except for the VWD testing facility, all these centers diagnose VWD mostly by the VWF:Ag and the VWF:RCo or automated VWF activity assays. A discrepancy between these two tests would indicate type 2 VWD. The further typing of VWD is done only by the VWD testing facility, situated in Bloemfontein. However, only a very limited number of patient samples are referred to the VWD testing facility from the large academic centers. This is mostly due to expensive shipping costs. The inherent limitations in sensitivity, reproducibility, and interlaboratory variability of the agglutination-based VWF:RCo and RIPA tests are well known.[7] Thus, given the limited tests applied in most centers, many VWD patients in South Africa might be misdiagnosed or remain undiagnosed.

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Original Article

Challenges in the laboratory diagnosis and management of von Willebrand disease in South Africa

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Background: South African von Willebrand disease (VWD) care is challenged by the lack of laboratory testing standardisation, lack of national clinical guidelines, limited undergraduate exposure to bleeding disorders and limited pharmacological resources. As the only reference testing facility performing VWD confirmatory testing, our experience in the diagnosis of the majority of VWD cases in South Africa has highlighted many areas where improvements are desperately needed.

Methods: We conductedd a retrospective audit of 250 VWD cases at our laboratory where we calculated the percentage misdiagnoses should only the von Willebrand factor (VWF) levels and the ristocetin cofactor activity assays be used.

Results: We have shown that performing a limited panel of VWD testing (VWF antigen and ristocetin cofactor activity only) could lead to the misdiagnosis of VWD in up to 77% of cases analysed at our laboratory. If the multimeric analysis was to be included in the diagnostic setup, together with the limited panel, still 20% of patients would be misdiagnosed. The underdiagnosis and under-reporting of VWD greatly underestimates the burden of the disease and the impact on the healthcare system.

Conclusions: Future advancements in VWD care in South Africa should be focused on correcting these shortcomings.

Keywords: Von Willebrand disease (VWD); diagnosis; haemophilia treatment centres (HTCs);South Africa

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Introduction

According to the South African Haemophilia Foundation, patients with bleeding diatheses in South Africa are cared for in 20 haemophilia treatment centres (HTCs) nationwide. The function of HTCs, in collaboration with the South African National Department of Health, the South African Haemophilia Foundation (the national members' organization) with its Medical and Scientific Advisory Council, and the National Haemophilia Nurses Committee, is to ensure optimal management of patients with bleeding disorders such as von Willebrand disease (VWD) (1).

The 2016 Global Survey of the World Federation of Haemophilia estimated that there are 632 patients diagnosed with VWD in South Africa; 375 female and 257 male. Of these patients, 431 have mild, 61 moderate and 42 severe VWD. Most of these patients are diagnosed between the ages of 14 and 44 years. VWD patients are predominantly diagnosed in five academic centres and a single central reference laboratory does the confirmatory testing that aids in the subclassification of VWD. This paper will concentrate

on the reference laboratory's findings regarding these statistics, as well as challenges in the laboratory diagnosis and management of VWD in South Africa.

Methods

Laboratory diagnosis and management of VWD

The VWD reference laboratory adopted and modified the guidelines for diagnosis and treatment of VWD in Italy (2,3) according to the algorithm outlined in *Figure 1*. In concordance with these guidelines the following diagnostic tests are offered at the reference laboratory: von Willebrand factor (VWF) antigen (VWF:Ag), ristocetin cofactor activity (VWF:RCo), collagen binding assay (VWF:CB), multimeric analysis, VWF propeptide levels (VWF:pp), factor VIII (FVIII) binding assay, ristocetin induced platelet agglutination (RIPA), and RIPA mixing studies to identify platelet type VWD (PT-VWD) (3).

A proportional reductixon of both VWF:Ag and VWF:RCo with a RCo/Ag ratio ≥0.7 as well as a proportional reduction of both VWF:Ag and VWF:CB with a CB/Ag ratio ≥0.7 suggests type 1 VWD. If type 1 VWD is diagnosed, the clearance rate of VWF is determined and the VWF:pp then performed. If the ratio between the VWF:pp and the VWF:Ag is more than 2, an increased clearance rate of VWF is suspected for this patient (i.e., type 1 Vicenza).

If the RCo/Ag ratio and/or the CB/Ag ratio is <0.7, then type 2 VWD is diagnosed. Type 2B VWD can be identified with an enhanced RIPA (response to <0.8 mg/mL ristocetin). Type 2B VWD is distinguished from a PT-VWD (pseudo VWD) by performing RIPA mixing studies. Type 2A and 2M might show a reduced RIPA (response only to >1.2 mg/mL ristocetin). Multimeric analysis in plasma is necessary to distinguish between type 2A VWD (lack of largest and intermediate multimers) and type 2M VWD (all multimers are present). The multimeric distribution pattern of type 2M VWD often differs from that of normal plasma in the lower density of high molecular weight multimers and the higher density of lower molecular weight multimers. The VWF:CB is usually normal in type 2M VWD due to the presence of the high molecular weight multimers, except where a collagen binding defect is diagnosed in such patients. In type 1 VWD, the ratio between FVIII level and VWF:Ag is always concordant. When this ratio is discrepant (i.e., FVIII/Ag <0.7) with a FVIII level of less than 20%, type 2N VWD is suspected and this subtype can be confirmed by performing a FVIII binding assay (3).

This VWD reference laboratory is also the only laboratory in the country that performs the VWF:CB, the mutimeric analysis of VWF, the VWF:pp and the VWF:FVIII binding assay (3).

The diagnostic screening tests for VWD include the VWF:Ag, WF:RCo, VWF:CB and the multimeric distribution of VWF. Confirmatory tests include RIPA and the VWF:FVIII binding assay. However, most other laboratories in South Africa, except for the reference laboratory, only perform the VWF:Ag and VWF:RCo assays (i.e., essentially screening for VWD).

Retrospective data of 250 VWD cases were gathered and the percentage misdiagnoses were calculated, should only the VWF:Ag and VWF:RCo assays be used. The results are shown in *Figure 2*.

The relative distribution of the various VWD subtypes diagnosed by the VWD reference laboratory from 2011 to 2016 is outlined in *Table 1*. Laboratory statistics, as well as challenges in the laboratory diagnosis and management of VWD in South Africa were determined by the reference laboratory. Information regarding the available treatment modalities for VWD and challenges thereoff were gathered from all heamophilia treatment centers nationwide.

Results

Challenges in the laboratory diagnosis of VWD

By performing only the two most popular tests (VWF:Ag and VWF:RCo), 6% of type 1 VWD patients would have been misdiagnosed as type 2 VWD; 13% of type 2A patients would be classified as type 1 and 77% as type 2B disease; 8% of type 2B VWD patients would be misdiagnosed as type 1 and 55% as type 2B disease; 28% of type 2M VWD patients would be misdiagnosed as type 1 and 48% as type 2A or 2B VWD; 1% of type 3 VWD will be misdiagnosed as type 2 VWD. Moreover, even if the multimeric analysis were to be included in this diagnostic setup (i.e., together with the two most popular tests), still 20% of patients would still be misdiagnosed.

As previously published, the vast distances between referral laboratories and the reference laboratory leads to thermal sample compromise and testing challenges. When samples are stored at temperatures warmer than −70 ℃, a cryoprecipite might be formed which greatly affects multimeric analysis (3). In the time period between 2012 and 2017, 8% of referred samples were rejected due to poor sample storage and transport conditions. The challenges

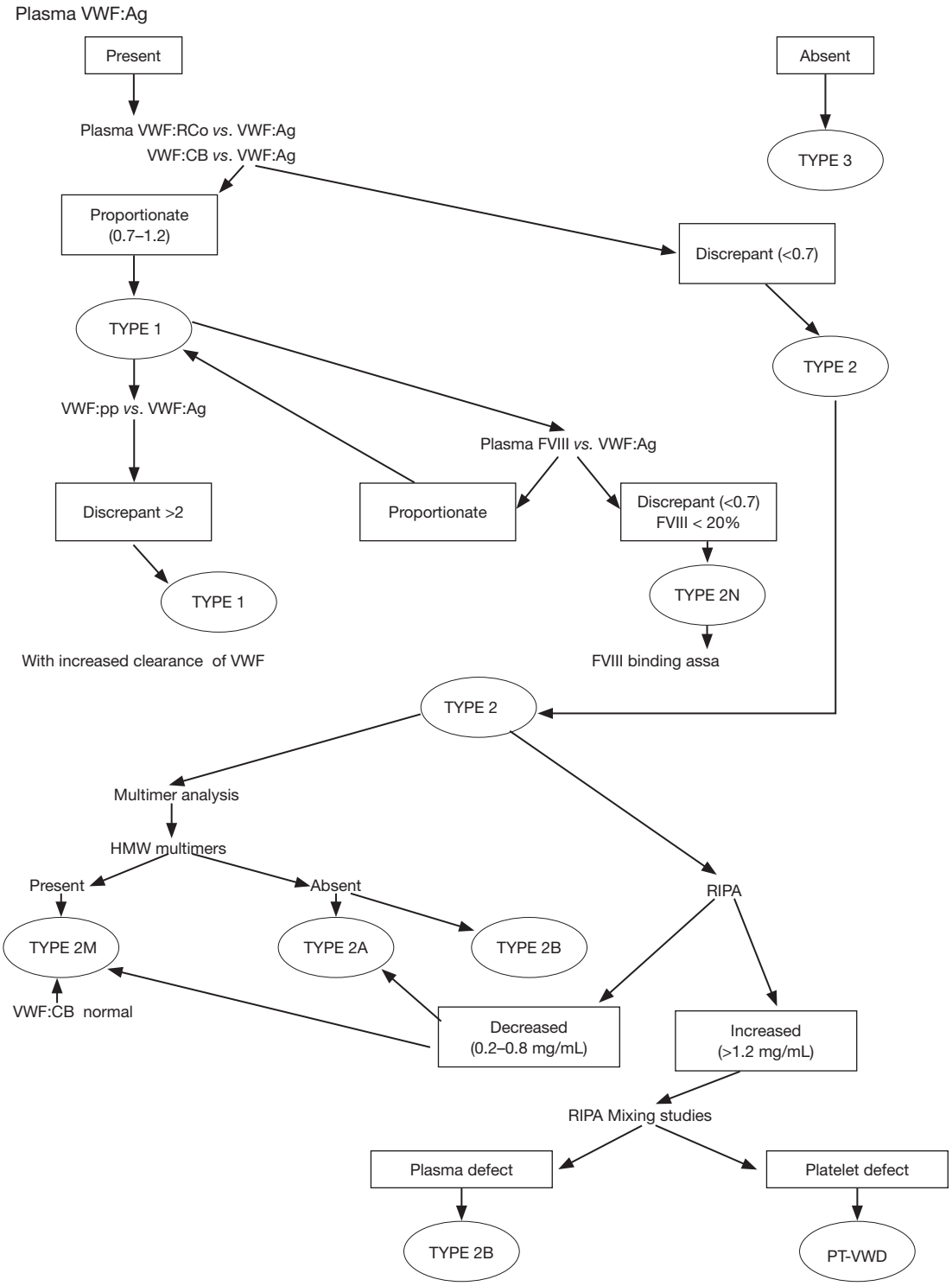

Figure 1 Algorithm for the laboratory diagnosis of VWD (3). VWD, von Willebrand disease; VWF, von Willebrand factor; HMW, high molecular weight; RIPA, ristocetin induced platelet agglutination.

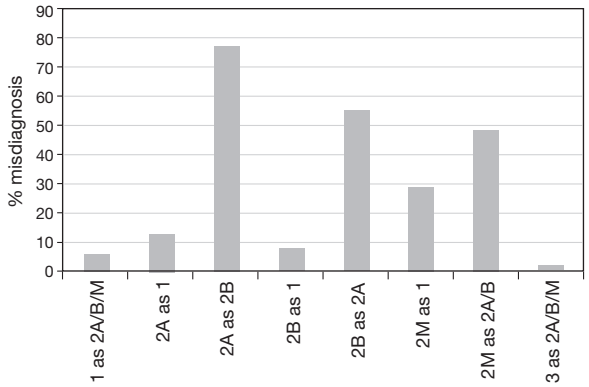

Figure 2 Percentage misdiagnosis of VWD subtyte, should only the VWF:Ag and VWF:RCo assays be used. VWD, von Willebrand disease; VWF, von Willebrand factor.

Table 1 Distribution of VWD subtypes diagnosed at the VWD reference laboratory

Subtype of VWD	Percentage [number] of total patients diagnosed, N=255
Mild type 1	23 [59]
Moderate to severe type 1	13 [33]
Type 2A	28 [73]
Type 2B	12 [30]
Type 2M	20 [50]
Type 2N	0 [0]
Type 3	4 [10]
Platelet type	0 [0]

VWD, von Willebrand disease.

posed by the geographically vast area of Southern Africa, as covered by the reference laboratory, and the previously described issues with specimen preparation, freezing and transport remain, and have furthermore been exacerbated by a general lack of dry ice for specimen transport (3).

Another challenge in the laboratory diagnosis of VWD, is the cost of the assays and the investigation. In the South African private medical sector, laboratory testing costs are covered by various medical aid schemes. However, the price that the medical aids are authorised to extend for these tests is often less than the costs of the latest test kits. Due to these cost constraints, the reference laboratory developed and validated in-house assays for more cost-effective VWD testing. These include the VWF:Ag and VWF:CB, the multimeric analysis and the propeptide assay (Meiring *et al.*, 2017 unpublished data). The VWF:RCo has challenges of its own, since the sensitivity is reduced at low levels (<15%) (4). It is thus of utmost importance to perform all diagnostic tests to prevent misdiagnosis of the disorder.

VWD diagnostic statistics

The VWD reference laboratory is situated in Bloemfontein, the legislative capital of South Africa, in the central part of the country. The academic complex in Bloemfontein serves patients from the Free State and the Northern Cape provinces (a geographically vast area) with a total population of about 4 million (5). The VWD reference laboratory, however, receives patient samples country-wide for both initial screening and diagnosis, but especially for the classification of VWD. The relative distribution of the various VWD subtypes diagnosed by the VWD reference laboratory from 2011 to 2016 is outlined in *Table 1*. The reference laboratory still receives mostly type 2 VWD samples to be sub-classified, as also mentioned in 2011 (3). Still no type 2N VWD patients have been diagnosed. The reason might be the improbability of an autosomal recessive disorder in our diverse population or the possibility of misdiagnoses of type 2N with haemophilia carriers by the HTCs.

Treatment of VWD in South Africa

Available treatment modalities include desmopressin (DDAVP), FVIII/VWF concentrates, antifibrinolytic drugs, topical therapies and hormonal treatment modalities for women. DDAVP is the treatment of choice in patients with type 1, 2A and 2M. Treatment with DDAVP is subject to a proven plasma response of FVIII levels and VWF:RCo activity.

Two intermediate purity FVIII/VWF concentrates are available in South Africa for the treatment of VWD: Haemosolvate® produced by the National Bioproducts Institute (NBI) from pooled fresh human plasma, and Virally Inactivated Factor VIII (VIAHF) produced by the Western Province Blood Transfusion Service (WPBTS) from small pools (5–6 bags) of cryoprecipitate. Both products are prepared from plasma obtained from voluntary non-remunerated donors after individual donation serologic and nucleic acid amplification and exclusion of human immunodeficiency virus (HIV), hepatitis B virus (HBV),

and hepatitis C virus (HCV) infection. Both undergo viral inactivation steps which inactivate HIV, HBV and HCV: Haemosolvate® using a solvent-detergent process, and VIAHF via 80 ℃ heating for 72 hours. Haemosolvate® or VIAHF are considered the treatment of choice when DDAVP is not indicated or ineffective, as both concentrates contain high molecular weight VWF multimers (6). In a previous study by our laboraotry, we have proven VWF:Ag levels in Haemosolvate® to be as high as double that of FVIII, and our centre is also involved in continuous quality control monitoring of this product (3).

Tranexamic acid (Cyclokapron®) is available in capsule and intravenous formulations. Recent studies confirm the effectiveness of prophylactic tranexamic acid in reducing the number of mild and moderate bleeding episodes (7,8); however, this has not yet been explored at our centre. The use of tranexamic acid on an on-demand basis with bleeding episodes, is advocated locally.

Hormonal treatments for women with VWD include combined oral contraceptives and hormonal intra-uterine devices, which are readily available in our country.

Available topical therapy is limited to fibrin glue (Thromboseel® and Tisseel®, South African National Blood Service and Adcock Ingram Critical Care).

Challenges in the treatment of VWD in South Africa

In South Africa, the route of administration of DDAVP is limited to a single DDAVP intravenous formulation which can also be administered subcutaneously. The intranasal preparation of DDAVP, although available in South Africa for the treatment of enuresis (0.1 mg/mL), is not sufficient in concentration for use in VWD, where a concentration of 1.5 mg/mL is quoted in international guidelines (6). This is a challenging limitation in paediatric VWD care where intranasal DDAVP would be a convenient choice.

Although intermediate purity FVIII/VWF concentrates are relatively freely available locally, no high purity VWF concentrate is currently available in South Africa. When used to treat VWD, FVIII/VWF concentrates can result in markedly elevated FVIII levels (disproportionate to the VWF levels achieved with treatment) which has been associated with thrombosis (9). A high purity VWF product could prevent such disproportionate increases in FVIII levels, further improving treatment safety (6).

The Medical and Scientific Advisory Council of the South African Haemophilia Foundation has issued guidelines for the treatment of haemophilia in South Africa (10), but no formal guidelines focusing on the management of VWD in South Africa have been published—a significant obstacle to safe, standardised care. Locally tailored guidelines similar to the United Kingdom Haemophilia Centre Doctors Organization guideline approved by the British Committee for Standards in Haematology (11) or the National Heart, Lung, and Blood Institute guidelines (6) should ideally be drafted on a national level. Guidance on issues specifically applicable to resource constrained environments like South Africa, such as prophylaxis, genetic testing, and management of surgical interventions, may be particularly useful. Such guidelines may also be of value to other developing countries, where the use of American and British guidelines may not be appropriate.

Discussion

The probable underdiagnosis of VWD in South Africa, may be a consequence of poor physician awareness of the disease, which may be a result of inadequate undergraduate medical training on the bleeding disorders in general, and VWD in particular. VWD as a specific disease entity receives only approximately 20 minutes of dedicated lecture time in the 5-year undergraduate medical curriculum at the University of the Free State.

This may be difficult to address in light of the heavy demands placed on undergraduate medical curricula to accommodate vast amounts of information from different disciplines, but may be amenable to interventions at post-graduate level. Training interventions targeted at interns and other junior doctors may be effective in improving practice standards, as was recently shown locally in transfusion medicine (12,13). Such training interventions should ideally also target physicians that are most likely to be faced with VWD patients at presentation, such as gynaecologists, paediatricians, otorhinolarynologists and general practitioners.

Misdiagnosis (now thought to possibly be a bigger problem than previously suspected) may be reduced by routinely using a more extensive testing profile, which is currently not standardised in South Africa. This reiterates the need for national guidelines on both the laboratory testing and treatment of VWD, which are currently lacking. Product issues (lack of suitable intranasal DDAVP preparations and high-purity VWF concentrates) remain a challenge as well as a general lack of physician awareness and limited undergraduate training, which may contribute to underdiagnosis of the disease.

Conclusions

The advancement of VWD care in South Africa, and perhaps many other developing countries, would likely benefit most from the establishment of national guidelines, targeted postgraduate training interventions and improved undergraduate medical training, an expanded treatment product repertoire and standardised laboratory testing.

Acknowledgements

None.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: As the entire study was based on pre-existing and anoymized samples, specific informed consent were deemed unneccessary according to local regulations.

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Blood Coagulation, Fibrinolysis and Cellular Haemostasis

2B or not 2B? Disparate discrimination of functional VWF discordance using different assay panels or methodologies may lead to success or failure in the early identification of type 2B VWD

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Summary

Laboratory proficiency in the identification of functional von Willebrand factor (VWF) discordance in type 2B von Willebrand disease (VWD) was assessed by external quality assurance surveys conducted by the RCPA Haematology QAP, and using six different type 2B VWD plasma samples (three historical and three previously unpublished) tested by up to 52 laboratories. For the three most recent samples, functional VWF discordance was either not identified in testing or by interpretation with misidentification as 'normal' or 'type 1 VWD', on average for 25.7% of test occasions when laboratories performed VWF:Ag and VWF:RCo as their primary VWF test panel, but somewhat fewer occasions (10.9%) for laboratories that incorporated VWF:CB as an additional functional VWF assay. VWF assay sub-methodologies also influenced the appropriate identification of samples as potentially type 2 VWD, and VWF functional discordance was more consistently identified when laboratories used (i) automated platelet agglutination for VWF:RCo compared to classical platelet aggregometry, (ii) in-house VWF:CB assays compared to commercial kit methods, and (iii) automated LIA-based 'VWF:Activity' assays compared to ELISA based assays. We conclude that: (i) laboratories are generally proficient in tests for VWD but interpretative diagnostic errors do occur; (ii) correct diagnosis is more likely when test panels are more comprehensive and include the VWF:CB; (iii) sub-methodology influences the appropriate identification of VWF functional discordance. On the basis of these findings, we provide a series of recommendations to enable the appropriate laboratory identification of VWD, in particular type 2B VWD.

Keywords

von Willebrand factor, VWF, von Willebrand's disorder, von Willebrand's disease, VWD, laboratory assessment, survey, haemostasis testing, diagnostic practice, quality control, quality assurance

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Introduction

von Willebrand's disorder/disease (VWD) is the most common inherited bleeding ailment, and is represented by a reduction and/or abnormal function of von Willebrand factor (VWF), an adhesive plasma protein essential for primary haemostasis (1–5). VWD is a heterogeneous disorder, subtyped using clinical and laboratory criteria, and diagnosis typically requires a comprehensive panel of tests (1–5). Although genetic testing may be undertaken for specific difficult cases or for eventual confirmation of a diagnosis (6–9), genetic testing is in fact rarely undertaken in practice. Testing for VWD is, in most laboratories, undertaken in a staged or step-wise process, with initial 'screening' using a von Willebrand factor (VWF) antigen (VWF:Ag) assay, together with factor VIII coagulant (FVIII:C) and one or more functional VWF assays (e.g. ristocetin cofactor [VWF:RCo], collagen binding [VWF:CB]) to determine the presence or absence of functional-VWF discordance (absent in type 1, but present in type 2 VWD). Dependent on the results of this initial testing, subsequent more extensive testing (e.g. ris-

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tocetin-induced platelet aggregation [RIPA], multimers, VWF:FVIII binding assay) may be performed, in particular to further classify type 2 VWD. Selection of an appropriate initial test panel, which includes appropriate functional VWF assays, is therefore critical for ensuring that further proper testing, or referral to expert VWD test laboratories or reference centres, will be undertaken.

Of primary importance, RIPA is recognised as the critical test for the correct identification of type 2B VWD but this test may only be performed, or referred onwards to a reference laboratory, if the initial VWF test panel identifies an abnormal finding, or at least identifies evidence of VWF functional discordance. Alternatively, the necessary RIPA testing may not be performed if initial test findings appear essentially 'normal' or are suggestive of (mild) type 1 VWD (VWF:Ag and functional VWF assay results are somewhat low but concordant). The findings of external quality assurance surveys conducted by the RCPA Haematology QAP are reported with a particular emphasis on diagnostic errors associated with testing for type 2B VWD, according to the methodology utilised.

Materials and methods

General

Survey samples were distributed to laboratories participating in the Special Haemostasis module of the RCPA Haematology QAP (10). Survey participant numbers have varied over the years, but are currently 55. These participants derive from a wide geographical area and primarily Australia (n=32) and New Zealand (n=6), but also Hong Kong (4), Malaysia (5), Singapore (1), India (1), South Africa (3), United Arab Emirates (2) and Sultanate of Oman (1). Sample processing instructions were provided, and laboratories (n=52 in 2006) were asked to perform all plasma-based tests that they normally used for laboratory identification (/diagnosis) of VWD (i.e. FVIII:C and specific VWF tests such as VWF:Ag, VWF:CB, VWF:RCo, VWF:Multimers, as locally available). Samples were blinded and identified only by a code ID.

Sample sets and sample testing

The results from six well-characterised type 2B samples provided over the last eight-year period were analysed. These 2B VWD samples were dispersed within a total of 47 samples dispatched over this period, the other samples including type 1, 3, 2A, 2N, 2M and normal samples (11–16; and unpublished data). The 2B VWD samples comprised three new (unpublished) samples dispatched in 2006, with findings compared to those of three other 2B VWD samples dispatched in earlier surveys. Details of the previously reported (11, 12) cases are only summarised here for comparison. All six 2B VWD patients had a significant personal history of bleeding problems, and all samples showed enhanced responsiveness to ristocetin (≤0.5 mg/ml) in a RIPA assay as requisite for identification of type 2B VWD (1). RIPA mixing studies (17) also confirmed that the enhanced RIPA responsiveness was plasma based for all (i.e. 2B VWD rather than pseudo/platelet-type-VWD).

For the new case set, sample 2006.1 was obtained from a 67-year-old female previously 'diagnosed' with 'thrombocytopenia', and baseline blood confirmed thrombocytopenia (Table 1), but showed normal routine coagulation test times. Sample 2006.2 from her 40-year-old daughter demonstrated mild thrombocytopenia (Table 1), with slightly prolonged APTT (38 seconds [sec]; normal reference range = 24–36 sec). Sample 2006.4 was from an unrelated 49-year-old female, and baseline blood demonstrated a borderline normal platelet count (Table 1), with normal coagulation test times. Baseline FVIII:C and VWF parameters for each patient are shown in Table 1, and VWF:Multimer results are shown in Figure 1. PFA-100® CTs were maximally prolonged (i.e. >250 sec) with both collagen/ADP and collagen/epinephrine cartridges for each patient. Additional details of the RCPA QAP's sample collection and processing procedures, and laboratory details for historical 2B VWD samples, are provided in previous reports (11, 12). All six patients showed loss of high-molecular-weight (HMW) VWF in multimer analysis, plus functional VWF discordance, although the level of HMW VWF loss and functional VWF discordance differed between patients (see *Discussion*). Extensive stability and homogeneity studies were performed on all samples provided to participants, and the samples detailed in this report passed stringent test stability/homogeneity criteria.

Evaluation of interpretative data

To help assess test panel diagnostic efficacy (i.e. the relative power for identification of VWD), laboratories were asked to provide numerical test data plus comment on the likelihood of VWD, as well as the particular subtype suggested by their own laboratory findings. The interpretative choices comprised: (i) normal/not VWD, (ii) equivocal (neither clearly normal nor VWD), (iii) type 1 VWD (mild/severe), (iv) type 2 VWD (further

Table 1: Baseline platelet counts, and phenotypic FVIII:C and VWF test data for the three unpublished type 2B VWD samples assessed in this report.

Sample ID	Platelet count (x10^9/L)	FVIII:C	VWF:Ag	VWF:RCo	VWF:CB	Ag/ RCo	Ag/ CB	RCo/ Ag	CB/ Ag
2006.1	42	62	71	40	39	1.8	1.8	0.6	0.6
2006.2	71	25	45	<10	20	5.0	2.3	0.2	0.4
2006.4	166	55	47	33	6	1.4	7.8	0.7	0.1
Normal reference range (NRR; 18)	150–400	45–180	45–200	35–350	50–250	0.5–1.5	0.5–1.5	>0.7	>0.7

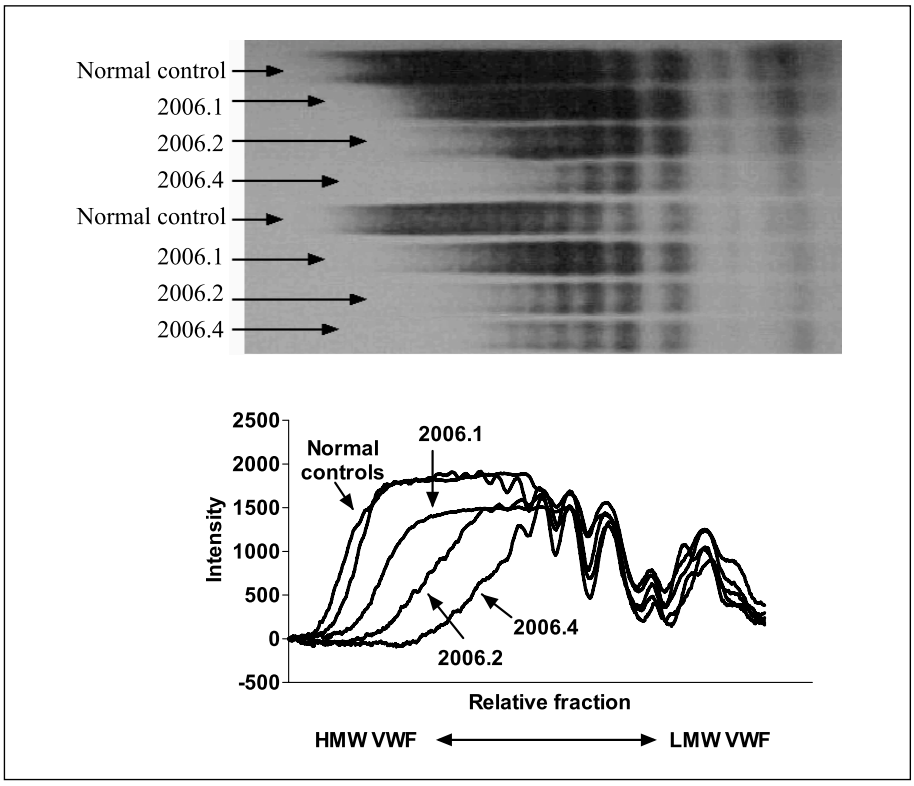

Figure 1: VWF:Multimer patterns for the new set of three type 2B VWD plasma samples detailed in the current report (shown in duplicate at differing intensities), together with a normal control sample and some resultant densitometry patterns. Note the relative loss of high-molecular-weight (HMW) VWF in each sample such that sample 2006.1 showed the least loss of HMW VWF, followed by sample 2006.2, and lastly sample 2006.4. LMW, low-molecular-weight.

subtyping as 2A, 2B, 2M, 2N was optional), or (v) type 3 VWD. In all surveys, participants could specify an alternative conclusion or else choose not to complete this section. This report focuses on 2B VWD samples and assesses the relative power of different laboratory 'VWD-diagnostic-screening' test panels in identifying these samples as VWD (or not), in determining whether a type 2 VWD type pattern was evident (e.g. evidence of VWF functional discordance), and whether such results would indicate, in normal laboratory and clinical practice, the need for further investigation (i.e. in particular for the current report, the need to refer patients to expert VWD test centres, or to perform RIPA to help identify type 2B VWD). An 'interpretive diagnostic error' was therefore defined to occur when 2B VWD samples were identified as 'normal', 'type 1' or 'type 3' VWD.

Evaluation of error rates

'Diagnostic error rates' were determined by calculating the proportion of 'interpretive (or diagnostic) errors' that occurred divided by the total number of diagnostic interpretations submitted for that sample and test group, expressed as a percentage, as previously outlined (15, 16).

Results

VWF test types and methodologies performed by laboratories

All participant laboratories currently perform FVIII:C testing, and also did so for previous surveys. In earlier surveys, all laboratories performed VWF:Ag assays, but currently one participant laboratory does not perform this assay (VWD screening panel is VWF:RCo plus FVIII:C). Current methodology for VWF:Ag is primarily by either immuno-turbimetric (or latex-immuno-assay; LIA) or by enzyme-linked-immunosorbent-assay (ELISA; Fig. 2). In earlier surveys, >90% of laboratories performed VWF:RCo assays, whereas currently <60% of laboratories still perform this assay. Thus, some laboratories have either abandoned this assay or have replaced VWF:RCo assays with alternative 'functional' assays (see later). Approximately equal numbers of current participants perform VWF:RCo by newer automated platelet agglutination assays or by 'classical' platelet aggregometry (Fig. 2). The number of laboratories performing VWF:CB has been more consistent throughout the surveys at close to 50%. Current methodology for VWF:CB is by ELISA, with most laboratories using a commercial kit (Life Therapeutics [previously Gradipore], Sydney, Australia) and fewer using in-house assays (Fig. 2). All in-house assays use type 1/3 collagen mixture products in the coating step, in line with local recommendations (19). 'VWF:Act' is a new category for the RCPA QAP and represents assays marketed by some manufacturers as 'VWF:activity' assays. These are monoclonal antibody (MAB) based assays (non-ristocetin-based and non-collagen-based) performed by either ELISA or LIA technology (Fig. 2). The former (ELISA) are marketed commercially by either Axis-Shield (Dundee, Scotland) or by Reaads Corgenix (distributed by Abacus Diagnostics, Brisbane, Australia), and are historically based on the work of Goodall et al. (20–22). The latter (LIA) represents a new assay available from Instrumentation Laboratory ('HemosIL von Willebrand Factor Activity' assay; Beckman-Coulter, Sydney Australia). Only three laboratories currently perform multimer analysis, although not rou-

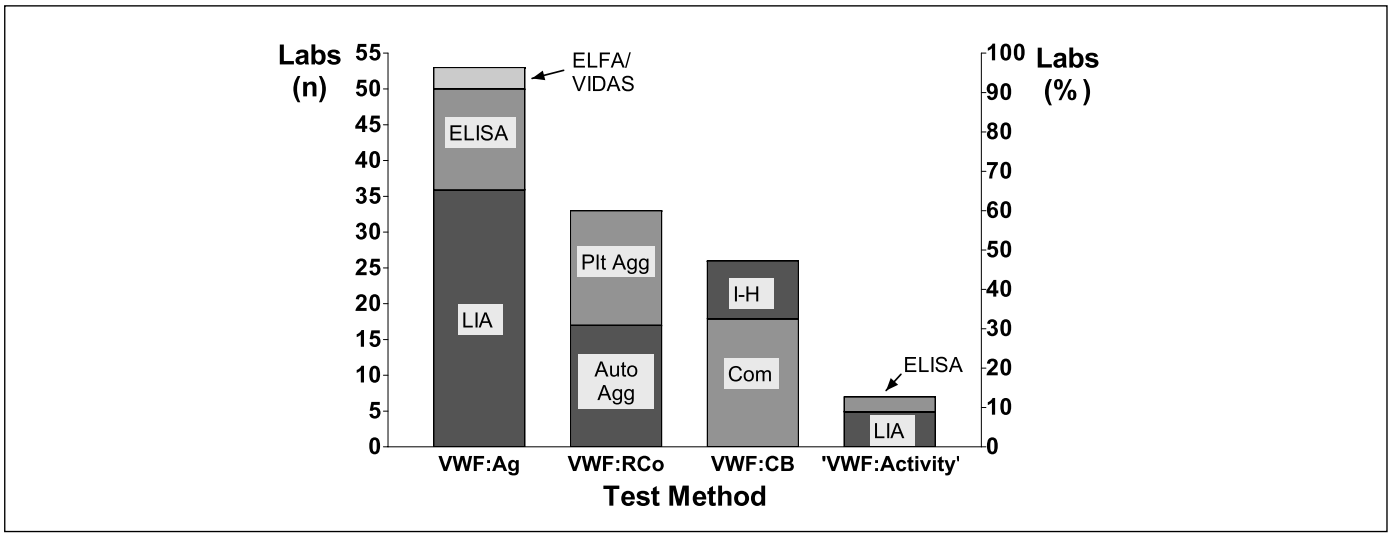

Figure 2: Summary of current relative sub-methodologies for VWF assays used by participants of the RCPA QAP. Number of laboratories ('labs'; left y-axis) or percentage of laboratories ('labs'; right y-axis) performing particular assay methods for VWF:Ag (LIA, ELISA or VIDAS/ELFA), VWF:RCo (Automated agglutination or classical platelet aggregometry), VWF:CB (in-house or commercial kit), or 'VWF:Activity' (LIA or ELISA). For VWF:Ag: a) LIA is primarily performed using reagents from Diagnostica Stago (Sydney, Australia; n=23), Dade-Behring (Sydney, Australia; n=6) or Instrumentation Laboratory (Beckman-Coulter, Sydney, Australia; n=6) and using instrumentation from the same companies [i.e. Diagnostica Stago (n=18), Dade-Behring (n=11), Instrumentation Laboratory/Beckman-Coulter (n=7)]; b) ELISA is performed using either commercial kits (Diagnostica Stago, n=2; Life Therapeutics, previously Gradipore, Sydney, Australia; n=4) or in-house methods (n=8) primarily using antibodies from Dako (Sydney, Australia); c) VIDAS instrumentation (ELFA [enzyme-linked fluorescent assay] technology; BioMerieux, Sydney, Australia) is used by only three laboratories. For VWF:RCo: a) 'agglutination' is primarily performed (n=18) using the von Willebrand reagent from Dade-Behring (Sydney, Australia) and various instrumentation [but primarily Dade-Behring (n=7), or Diagnostica Stago instruments (n=9)], and b) 'aggregometry' is performed using a wide variety of reagents and instrumentation.

tinely for all samples. The specific breakdown of sub-methodologies for VWF:Ag and VWF:RCo is detailed in Figure 2.

VWF test panels performed by laboratories

Some laboratories have a very limited test panel [e.g. only FVIII:C and VWF:RCo (n=1), or FVIII:C and VWF:Ag (n=10)]. However, around 80% of participant laboratories perform VWF:Ag, FVIII:C and at least one VWF functional assay (i.e. VWF:RCo, VWF:CB and/or VWF:Act). The actual VWF panel used by these laboratories varied widely as follows: VWF:Ag and VWF:RCo only = 11 (21.2%), VWF:Ag and VWF:CB only = 5 (9.6%), VWF:Ag and VWF:Act only = 1 (1.9%), VWF:Ag, VWF:RCo and VWF:CB = 16 (30.7%), VWF:Ag, VWF:RCo and VWF:Act = 2 (3.8%), VWF:Ag, VWF:CB and VWF:Act = 4 (11.5%), and VWF:Ag, VWF:RCo, VWF:CB and VWF:Act = 2 (3.8%).

Table 2: Summary of returned phenotypic data and diagnostic error rates for type 2B VWD samples assessed by the RCPA Haematology QAP.

Sample ID*	No. Labs **	Returned phenotypic data (median values):***								Correct diagnostic interpretations: n (%)****	Diagnostic interpretative error rates (%):*****		
		VWF: Ag	VWF: RCo	VWF: CB	'VWF: Act'	FVIII: C	Ag/ RCo	Ag/ CB	Ag/ 'Act'		Normal	'equivocal'	Type 1 VWD
1998.4	25	44.5	19.0	11.2	NA	55.5	4.1	4.2	NA	19 (76.0)	0	8.0	8.0
1998.8	25	36.2	15.5	7.2	NA	36.0	4.2	5.7	NA	16 (64.0)	0	0	24.0
2000.2	19	42.4	19.5	10.8	NA	55.1	3.4	6.7	NA	14 (73.7)	0	0	10.5
2006.1	52	70.0	31.0	32.0	24.0	64.0	2.4	2.1	2.8	31 (59.6)	11.5	9.6	9.6
2006.2	52	45.0	15.0	15.0	15.5	34.0	2.7	2.7	2.5	27 (51.9)	0	0	34.6
2006.4	52	28.0	8.0	9.0	13.5	25.0	3.4	2.9	2.2	38 (73.1)	0	0	11.5

* First three samples listed have been the subject of previous reports (11,12) and are listed here for comparative purposes only. Last three samples represent new 2B VWD sample data. ** Number of participant laboratories per survey has varied, but equalled 52 in 2006. *** Data shown as median values returned for each sample in each respective survey. **** Number (percent) of laboratories that indicated the possibility of type 2/2A/2B/2M VWD (thus fulfilling a need for further investigation by RIPA analysis). ***** Percentage of laboratories identifying the type 2B VWD samples as 'normal', 'equivocal', or 'type 1 VWD' respectively. NA = not available.

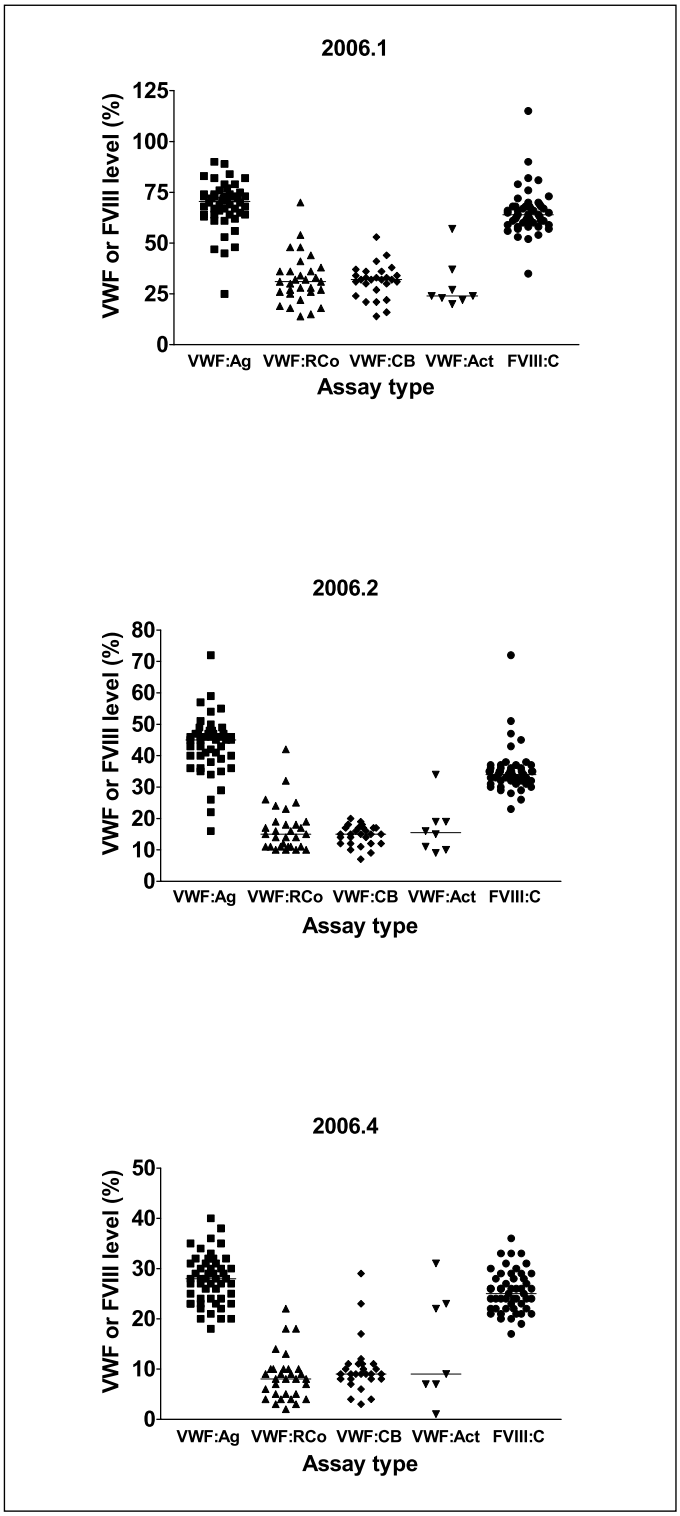

Figure 3: Scatterplot of VWF or FVIII:C level as reported by participants of the RCPA QAP for all assays assessed by the QAP, and namely VWF:Ag, VWF:RCo, VWF:CB, VWF:Act and FVIII:C, for the three new type 2B VWD plasma samples despatched in 2006 (see also Table 2). Data shown as percent of normal (y-axis), with 100% reflecting the theoretical yield of a large-pooled normal plasma. Small horizontal bars for each data set represent the median value.

Phenotypic data returned by survey participants

Data for the six 2B VWD samples is summarised in Table 2. As can been seen, all samples showed some evidence of functional VWF discordance with all functional VWF assays (i.e. VWF:Ag/VWF:functional ['Ag/fun'] assay ratios >2.0 for each case), although the degree of discordance varied between samples and between functional VWF methods. Three samples (1998.4, 2000.2, 2006.1) gave normal median values for FVIII:C (i.e. >50%), and one sample (2006.1) gave normal median values for VWF:Ag. In general, assay CVs were lowest for FVIII:C (usually <20%), followed by VWF:Ag (typically 10–25%), VWF:CB (typically 20–40%), and VWF:RCo (typically 25–50%). Assay CVs for the LIA based VWF:Act assay were generally comparable to these.

Detailed comparative phenotypic data for the three previously unpublished 2B VWD samples is provided in Figure 3. Detailed VWF assay ratio data for all samples are provided in Figures 4 and 5. This data is helpful in terms of interpreting error rates identified later. Most samples tended to show greater VWF functional discordance using the ratio of VWF:Ag/VWF:CB ('Ag/CB') or the reverse (VWF:CB/VWF:Ag or 'CB/Ag') compared to VWF:Ag/VWF:RCo ('Ag/RCo') or VWF:RCo/VWF:Ag ('RCo/Ag'). This was particularly evident for historical samples (1998.4, 1998.8, 2000.2; Fig. 4), but notably less evident with the new samples (2006.1, 2006.2, 2006.4; Fig. 5). Thus, for historical samples (Fig. 4), quite a few laboratories did not identify VWF functional discordance between VWF:Ag and VWF:RCo, whereas all but a few laboratories reported VWF functional discordance between VWF:Ag and VWF:CB. For the new samples (Fig. 5), a small number of laboratories did not identify VWF functional discordance using either approach, and there appeared less difference between VWF:RCo and VWF:CB data.

To assess additional methodological reasons for such discrepant findings, data was further analysed for the current data set according to sub-methodologies (Figs. 6 and 7). Users of VIDAS instrumentation (ELFA methodology) tended to report higher VWF:Ag data, and ELISA users tended to report the lowest data (Fig. 6A), although small VIDAS user numbers preclude definitive conclusions. Excluding obvious outliers, there was no obvious pattern of differential assay precision for VWF:Ag submethodologies. There was no consistent pattern of difference in VWF:RCo data between users of automated platelet agglutination and platelet aggregometry based assays, although there was some evidence of better precision with automated methods (Fig. 6B). There was no consistent pattern of difference or precision in VWF:CB data between users of commercial kit methods and in-house assays (Fig. 6C). For VWF:Act assays, results for users of ELISA methodology tended to be higher than those with LIA methodology (Fig. 6D), although small ELISA user numbers again precludes definitive conclusions. Any methodological differences noted above could lead to differences in identification of functional discordance, and this was observed in some cases (Fig. 7). Thus, for VWF:RCo, VWF functional discordance tended to be identified more often with automated platelet agglutination than with platelet aggregometry (Fig. 7A). For VWF:CB, there was a suggestion of better discordance observed for in-house assays compared to commercial kits (true for

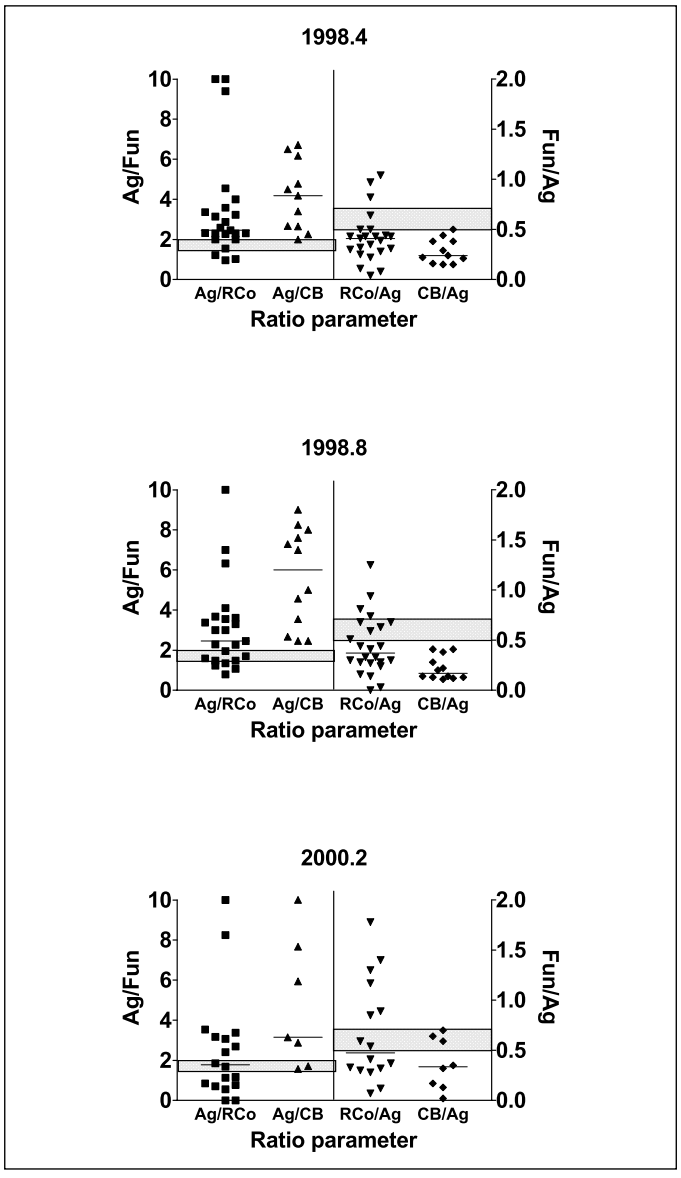

Figure 4: Scatterplot of VWF assay ratios using data for VWF assays as reported by participants of the RCPA QAP for VWF:Ag/VWF:function (left y-axis) or VWF:function/VWF:Ag (right y-axis), for the three historical type 2B VWD samples (1998.4, 1998.8, 2000.2) as per Table 2 and as in part previously reported (11,12). Data restricted to ratios of VWF:Ag/VWF:RCo ('Ag/RCo') and VWF:Ag/VWF:CB ('Ag/CB') or the reverse ratios (as preferred by some workers; 1) As this is the data available. Small horizontal bars for each data set represent the median value. Shaded horizontal bars in each figure represent the region of interest for cut-off for identification of functional VWF discordance. Current local cut-offs are 1.5 for Ag/Fun (or 0.7 for Fun/Ag; 18), but some workers use slightly different cut-off values (e.g. cut-off of 2.0; 1), so a range of 1.5–2.0 for Ag/Fun (or 0.5–0.7 for Fun/Ag) is shown in figures. Results above the shaded bar for Ag/Fun or below the bar for Fun/Ag would be acceptable evidence of functional VWF discordance (e.g. ?type 2A, 2B or 2M VWD) for further study, clarification and classification. Results below the shaded bar for Ag/Fun or above the bar for Fun/Ag would be acceptable evidence for lack of functional VWF discordance (e.g. normal or type 1 VWD depending on absolute VWF levels determined). Results within the shaded bar would represent equivocal findings, but would still suggest some follow up, including repeat testing and possible further extensive testing.

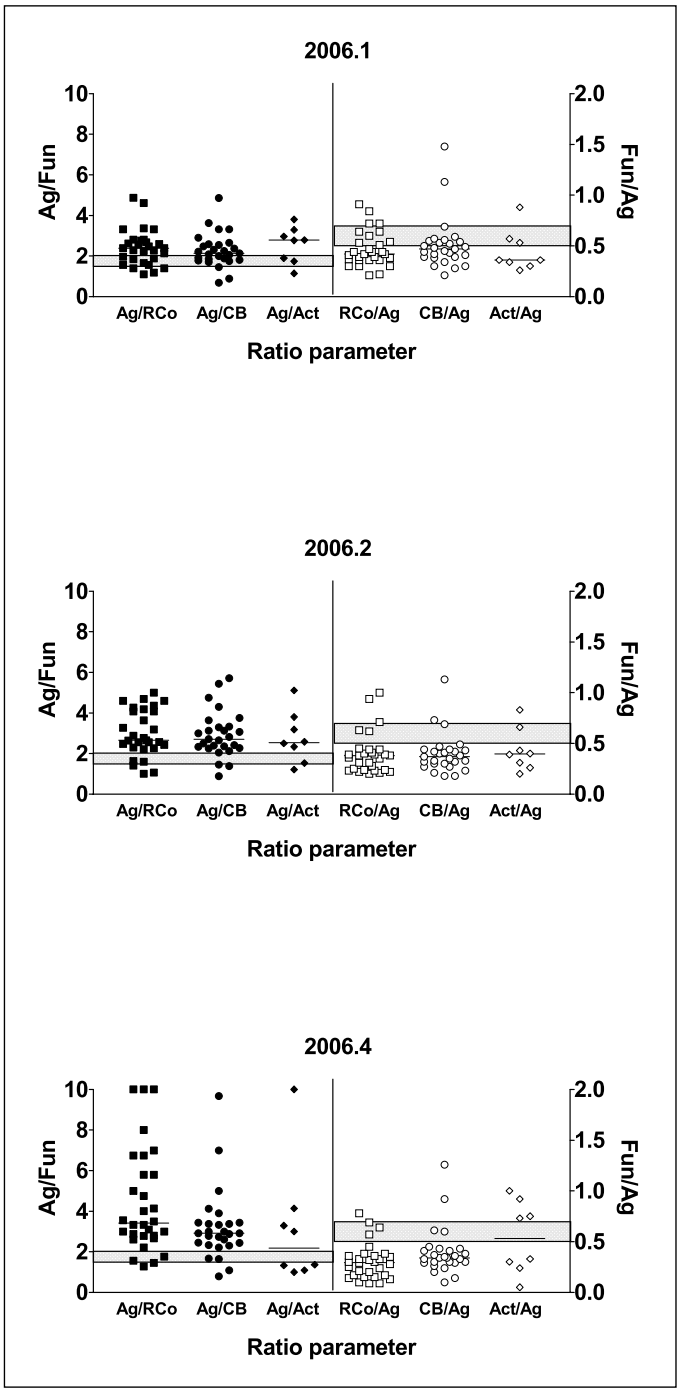

Figure 5: Scatterplot of VWF assay ratios using data for VWF assays as reported by participants of the RCPA QAP for VWF:Ag/VWF:function (left y-axis) or VWF:function/VWF:Ag (right y-axis), for the three new type 2B VWD samples (2006.1, 2006.2, 2006.4) as per Table 2 and as per current report. Data comprises ratios of VWF:Ag/VWF:RCo ('Ag/RCo'), VWF:Ag/VWF:CB ('Ag/CB') and VWF:Ag/VWF:'Act' (Ag/Act) or the reverse ratios (as preferred by some workers; see Fig. 4 legend). Small horizontal bars for each data set represent the median value. Shaded horizontal bars in each figure represent the region of interest for cut-off for identification of functional VWF discordance.

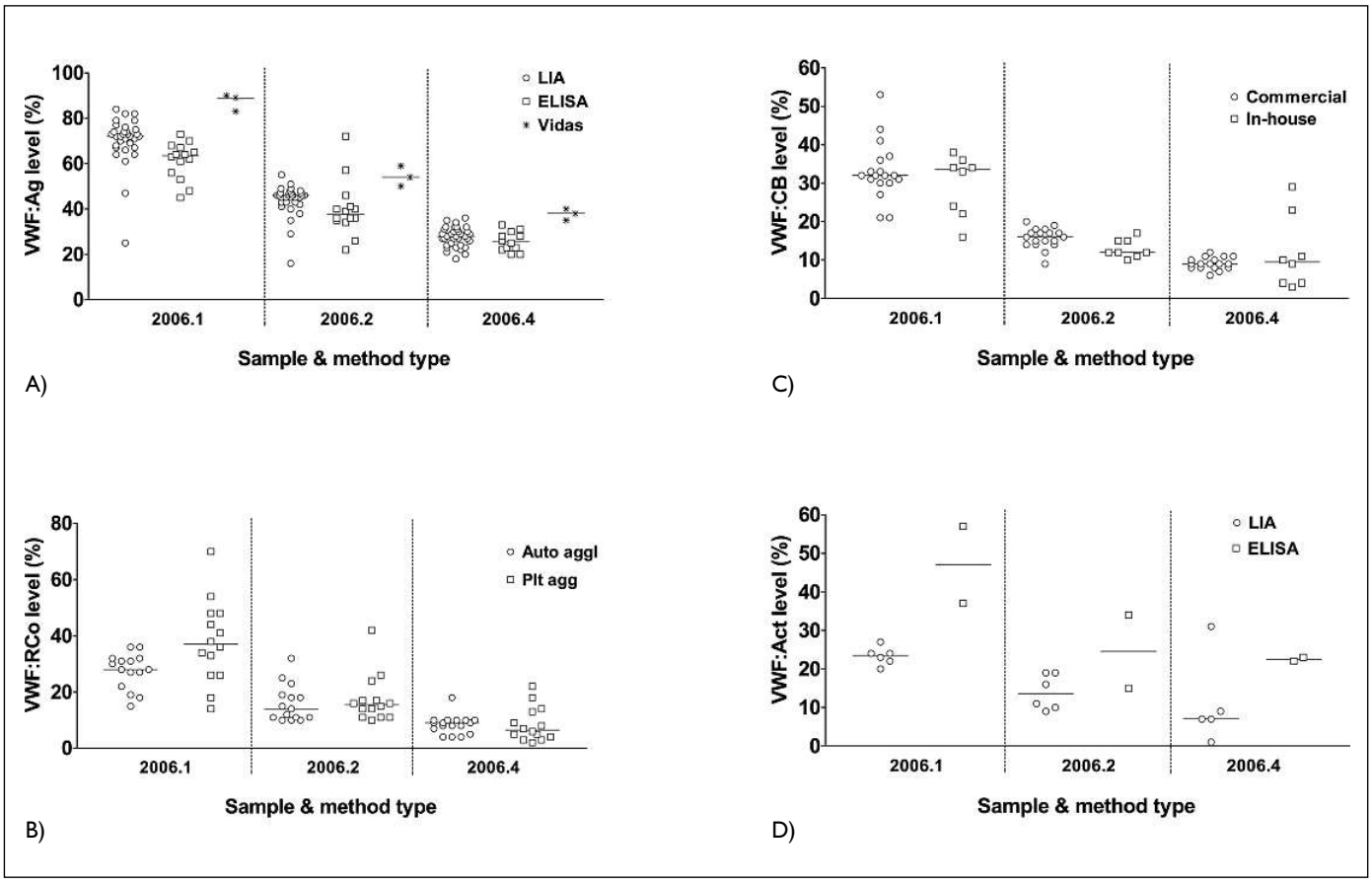

Figure 6: Scatterplot of VWF level as reported by participants of the RCPA QAP for all VWF assays assessed by the QAP, and namely VWF:Ag, VWF:RCo, VWF:CB, and VWF:Act, and shown by sub-methodology (viz: VWF:Ag = LIA, ELISA or VIDAS/ELFA; VWF:RCo = automated platelet agglutination or classical platelet aggregometry; VWFCB = commercial kit or in-house method; VWF:'Act' = LIA or ELISA). Data shown as percent of normal (y-axis), with 100% reflecting the theoretical yield of a large pooled normal plasma, for the three new type 2B VWD plasma samples despatched in 2006 (see also Fig. 3). Small horizontal bars for each data set represent the median value.

samples 2006.1 and 2006.2, but not 2006.3; Fig. 7B). For VWF:Act, there was a similar suggestion of better discordance observed for LIA based assays compared to ELISA-based kits (Fig. 7C).

Diagnostic interpretive error rates for type 2B VWD

This was assessed for the six samples with data summarised in Tables 2 and 3. There was a high error rate when laboratories only used a limited VWF test panel (i.e. VWF:Ag or VWF:RCo only). Indeed, an overall error rate of 76.9% misidentification as normal or type 1 VWD was obtained by these laboratories for the three most recent type 2B VWD samples when an attempt at interpretation was made. Such limited test panels obviously cannot identify functional VWF discordance. When laboratories used VWF:Ag and VWF:RCo as their VWF test panel, an error rate of 25.7% was identified (comprising nine identifications of 'normal' or type 1 VWD from 35 submitted responses; Table 3, Fig. 8). When laboratories added VWF:CB to the test panel of VWF:Ag and VWF:RCo, error rates fell to 10.9% (comprising 5 identifications of 'normal' or type 1 VWD from 46 submitted responses; Table 3, Fig. 8). Lower error rates were also associated with the VWF panel of VWF:Ag, VWF:CB and VWF:Act (8.3%, or 1/12 incorrect identifications). Interestingly, for VWF:Ag plus VWF:RCo performing laboratories, similar numbers of laboratories performed automated analysis by platelet agglutination or by platelet aggregometry, and automation did not protect laboratories from diagnostic identification errors (Fig. 8).

A high proportion of identification errors were related to laboratory interpretations rather than assay failures *per se*. Thus, for the misidentifications identified above, VWF functional assay discordance was actually obtained in many cases, but was not utilised by the laboratory in order to identify a possible type 2 VWD. In summary, for laboratories that misidentified the three new cases of type 2B VWD as normal or type 1 VWD, functional VWF assay discordance was actually obtained in 15/24 (62.5%) of test cases for VWF:RCo, 8/11 (72.7%) of test cases for VWF:CB, and 1/3 (33.3%) of test cases for VWF:Act. Although the split for VWF:RCo was fairly even between the two methodologies in terms of misidentification (ie automated agglutination vs. classical aggregation; Fig. 8), most cases of laboratory 'self-interpretation errors' belonged to users of automated aggluti-

nation (i.e. 6/7 [85.7%] test cases with misinterpretation errors actually showed evidence of VWF functional discordance). For VWF:CB, most cases of misidentification were in laboratories that utilised the commercial kit (i.e. 7/9 [77.8%]).

Discussion

In this report, focused on type 2B VWD identification, we have confirmed and extended the more general findings previously reported by us (11–16). In brief:

- (a) Approximately 20% of laboratories participating in the RCPA currently use a core screening panel of VWF:Ag and FVIII:C only, or in one case VWF:RCo and FVIII:C only, which are insufficient to identify a qualitative VWF disorder such as 2B VWD. Understandably, these limited test panels led to a high error rate of misidentification (76.9%) of these samples as either normal or type 1 VWD.
- (b) Addition of one functional VWF assay to VWF:Ag and FVIII:C reduces the misidentification rate, but the level of misidentifications is still significant. A VWF test panel comprising VWF:Ag plus VWF:RCo was associated with a misidentification error rate of over 25% (or one error for every four testing episodes; Fig. 8). Despite users of automated platelet agglutination tending to show better precision (Fig. 6B) and better evidence of functional VWF discordance (Fig. 7A), automation did not protect against VWD misidentification errors (Fig. 8). These findings are consistent with those previously reported by us for other type 2 VWD samples (15, 16).
- (c) Performance of an additional functional VWF assay (i.e. VWF:CB) within the test panel was associated with a much lower error rate (Fig. 8). These findings are also consistent with those previously reported for other samples (15, 16).
- (d) Particular functional VWF assay sub-methodologies tend to better identify VWF discordance (or possible type 2 VWD) than others (notably: (i) automated platelet agglutination for VWF:RCo compared to classical platelet aggregometry, (ii) in-house VWF:CB assays compared to commercial kit methods, and (iii) automated LIA-based 'VWF:Act' assays compared to ELISA-based assays). This is a novel finding for the current report.
- (e) Correct identification of functional VWF discordance (or possible type 2 VWD) differs according to the sample tested. For example, sample 2006.1 yielded normal median levels of VWF:Ag and FVIII:C (Table 1, Fig. 3), and many laboratories (Fig. 5) did not observe VWF functional discordance, irrespective of whether they used VWF:RCo, VWF:CB, or VWF:Act. This sample also showed the lowest loss of HMW VWF in multimer analysis (Fig. 1). Accordingly, this sample resulted in many cases of misidentification (Table 2) of 'normal' or 'mild type 1 VWD', as well as several reports of 'equivocal', and might represent a 'worse-case' scenario type 2B VWD sample. The donor (aged 67) had been incorrectly diagnosed for many years as simply 'thrombocytopenic', and the current report (where 15/44 laboratories failed to report a type 2 VWD pattern) may help explain why. Her daughter's results (sample 2006.2), yielded lower VWF and FVIII:C values (Table 2, Fig. 3), better evidence of functional VWF discordance (Table 1, Fig. 5), and a greater loss of HMW VWF (Fig. 1). There were no reports of 'equivocal' and no misinterpretations of 'normal' for this case, but 18/43 laboratories still misidentifed the sample as mild or severe type 1

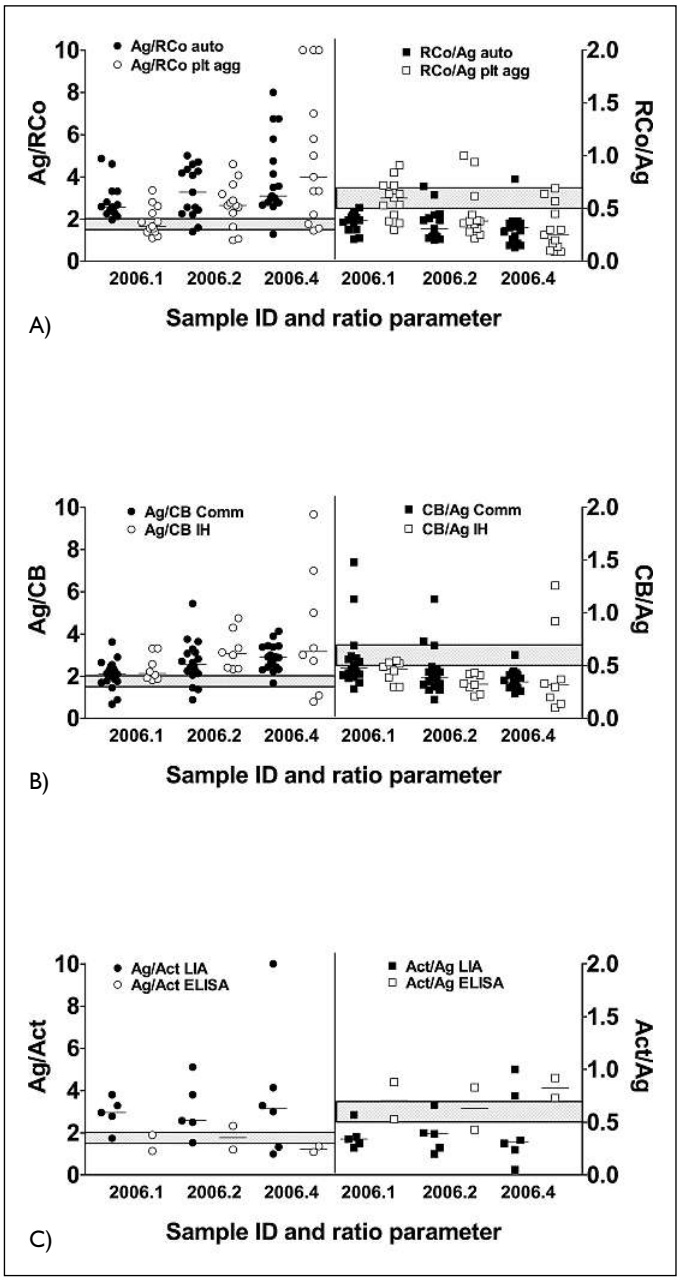

Figure 7: Scatterplot of VWF assay ratios using data for VWF assays as reported by participants of the RCPA QAP for VWF:Ag/VWF:function (left y-axis) or VWF:function/VWF:Ag (right y-axis), for the three new type 2B VWD samples (2006.1, 2006.2, 2006.4) as per Table 2 and as per current report, but with data separated according to sub-methodology as per Figure 6. Data comprises ratios of VWF:Ag/VWF:RCo ('Ag/RCo'), VWF:Ag/VWF:CB ('Ag/CB') and VWF:Ag/VWF:'Act' (Ag/Act) or the reverse ratios (as preferred by some workers; see Fig. 4 legend). Small horizontal bars for each data set represent the median value. Shaded horizontal bars in each figure represent the region of interest for cut-off for identification of functional VWF discordance.

Table 3: Summary of related VWF test panels versus diagnostic error rates for type 2B VWD samples assessed by the RCPA Haematology QAP.

Sample ID*	No. Labs **	'Incorrect' diagnostic interpretation	Number of incorrect diagnostic interpretations (n) according to the laboratory VWF test panel used***					
			Total	VWF:Ag & VWF:RCo	VWF:Ag, VWF:RCo & VWF:CB	VWF:Ag & VWF:CB	VWF:Ag or VWF:RCo only	Other VWF panel combinations
1998.4	25	'Equivocal' Type 1 VWD	2 2	2 1	1			
1998.8	25	Type 1 VWD	6	6				
2000.2	19	Type 1 VWD	2	1	1			
2006.1	52	'Equivocal' 'Normal' Type 1 VWD	5 6 5	4 2 1		1 1	4 2	1
2006.2	52	Type 1 VWD	18	6	3	3	5	1
2006.4	52	Type 1 VWD	6		2		2	2

* First three samples listed have been the subject of previous reports (11,12) and are listed here for comparative purposes only. Last three samples represent new 2B VWD sample data. ** Number of participant laboratories per survey has varied, but equalled 52 in 2006. *** Blank entries are equal to zero.

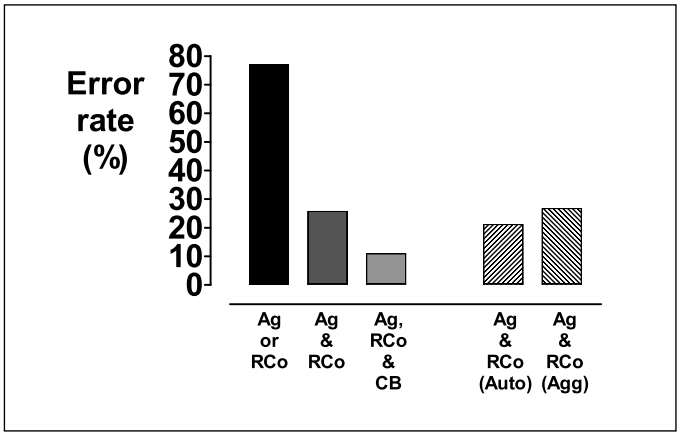

Figure 8: Misidentification error rates for various VWF assay panels. Data shown as composite percentage error (see *Methods*) for the new type 2B VWD samples when misidentified as either 'normal' or 'type 1 VWD'. Error rate (*y*-axis) shown separately for the following VWF test panels: VWF:Ag or VWF:RCo only (Ag or RCo), VWF:Ag plus VWF:RCo (Ag & RCo), VWF:Ag plus VWF:RCo plus VWF:CB (Ag & RCo & CB). Data for laboratories performing VWF:Ag plus VWF:RCo also shown according to sub-methodology (i.e. Ag & RCo (Auto) versus Ag & RCo (Agg)) (near equal numbers of laboratories).

VWD. Sample 2006.4 was the least problematic of the current sample set, with the fewest incorrect identifications. Of relevance, this sample showed the lowest levels of FVIII:C and VWF parameters (Table 2, Fig. 3), the best evidence of functional VWF discordance (Table 2, Fig. 5) and the greatest loss of HMW VWF (Fig. 1).

It was of some interest to note that many laboratories reported numerical data that showed evidence of VWF functional discordance, but then failed to identify the samples as probable type 2 VWD. This occurred more often in laboratories using the more limited VWF assay panels (e.g. VWF:Ag and VWF:RCo only). The two main likely explanations are that these laboratories either do not understand the significance of functional VWF discordance as suggesting type 2 VWD (i.e. educational issue) or else that these laboratories simply do not believe their own laboratory test data. We have previously reported that the VWF assay panel of VWF:Ag and VWF:RCo is associated with a higher rate of misidentification of normal or type 1 VWD samples as being type 2 than a more comprehensive panel including the VWF:CB (15, 16). That is, use of VWF:Ag and VWF:RCo is associated with a higher rate of false VWF functional discordance when type 1 VWD or normal samples are tested than test panels that incorporate the VWF:CB. Accordingly, it is likely that laboratories participating in the RCPA QAP simply place more faith on the discordance when identified using the more comprehensive combinations.

One significant novel finding in this report is the observed difference between the various so-called 'activity' assays ('VWF:Act'). Better VWF functional discordance was observed using LIA based 'VWF:activity' assays compared to ELISA based. Findings need to be tempered, however, by the small number of current users, and need to be confirmed in subsequent studies. VWF:CB and VWF:RCo assays have previously been shown to better identify VWF functional discordance than ELISA based 'VWF:Act' assays (23, 24), but good comparative studies between VWF:CB, VWF:RCo and LIA based 'VWF:Act' assays are lacking. Neither of the previous reports on this methodology (25, 26) adequately deal with identification of functional VWF discordance, nor are the comparisons with other functional VWF assays very comprehensive. Nevertheless, results from the current report do suggest some promise of clinical utility for this assay. The test system uses a MAB that binds to VWF at a functional site, but the premise that this would confer the assay some ability to measure VWF 'activity' (an argument previously forwarded by manufacturers of the ELISA based

MAB test systems [23, 24]) is still flawed. Nevertheless, it is possible that these tests show good correlation to the presence of HMW VWF, and that HMW VWF is preferentially 'captured' by this assay system, so further study will help clarify their place in VWD diagnostics. It also needs to be restated that neither of these VWF:Act assays (i.e. LIA or ELISA based) incorporate ristocetin or collagen within their test procedure. Thus, they would not be expected to behave identically to VWF:RCo nor VWF:CB assays. Two versions of true ristocetin-based ELISA VWF:RCo assays have been reported (27–29), but these have not yet been taken up by general diagnostic laboratories because the assays are complex, and reagents are not yet readily available; thus confirmatory studies are still lacking.

Our overall findings also need to be considered in view of recent literature related to type 2B VWD. Baronciani et al. (30) have found in a large retrospective case series that the VWF:CB better identifies functional VWF discordance than VWF:RCo, both for 2B VWD cases with and without a lack of HMW VWF. That is, there is some evidence that type 2B VWD reflects an inherent collagen-binding defect in addition to its case-dependent (or case-inconsistent) loss of HMW VWF. Thus, the lowered relative VWF:CB binding in type 2B VWD may reflect a combination of lowered plasma HMW VWF plus the presence of a specific VWF collagen-binding defect. In contrast, VWF:RCo may be less likely to show assay discordance, because the assay reflects a combination of reduced HMW VWF (leading to lower VWF:RCo) together with enhanced ristocetin binding (leading to potential 'inflation' of VWF:RCo). Interestingly, the ability of VWF:CB assays to better detect type 2B VWD by either mechanism (collagen binding defect or loss of HMW VWF) depends on the collagen source (type 1/3 mixture collagens work better than purified type 3 collagen [30]). Although consistent with previous published findings (4, 5, 19), it had always been assumed that the ability of type 1/3-mixture collagens to better identify 2B VWD was related solely to better capture of HMW VWF. In any case, such findings support our assertion regarding the added value of VWF:CB testing in VWD diagnostics, particularly when investigating the possibility of type 2 VWD, and preferentially using a type 1/3 mixture collagen based assay. This view is now supported by independent findings by many others (31–36). It is worth noting that in our geographic region, all in-house and commercial VWF:CB assays used by RCPA QAP participants appear to be based on type 1/3-mixture collagens. Nevertheless, in-house based VWF:CB assays appeared, in the current study, to more consistently identify VWF functional discordance in type 2B VWD than the commercial kit method. The company producing this kit (Life Therapeutics; previously Gradipore) has transferred manufacturing operations from Australia to the US, and it is possible that the current findings reflect some manufacturing process change. Alternatively, it might relate to the level of experience in laboratories, with 'in-house' assay users being the most experienced.

Of additional interest is the fall in relative utilisation of VWF:RCo assays. Currently, <60% of laboratories still perform this assay, whereas >90% of laboratories previously did so (11, 12). In contrast, performance of VWF:CB over the years in our geographic locality has been fairly consistent at around 50% (11–16). Many laboratories appear to be replacing their VWF:RCo assay, initially by replacing classical platelet aggregometry methods with automated methods, and more recently by replacing the assay itself with VWF:CB or VWF:Act assays, or otherwise abandoning functional VWF assays altogether. We do not support replacement of VWF:RCo with any other functional VWF assay (including VWF:CB), but emphasise that additional assays such as VWF:CB are complementary to VWF:RCo. Although, by direct comparison, optimised VWF:CB assays do tend to better identify functional discordance in type 2A and 2B VWD compared to VWF:RCo, and are also less likely to misidentify false functional discordance (e.g. type 1 VWD or normal samples as type 2 VWD [15, 16]), VWF:RCo is still required to identify some cases of type 2M VWD, and a dual functional VWF panel of VWF:RCo and VWF:CB is likely to be diagnostically more useful. Whether VWF:Act can replace VWF:RCo is simply unknown, although also unlikely – as yet, no good comparative studies are available, and it must be remembered that VWF:Act assays do not incorporate ristocetin into their methodology. We are also concerned by the move by some laboratories to abandon VWF functional assays altogether (10/52 laboratories participating in the RCPA QAP in 2006), since such assays are required to identify type 2 VWD, and to discriminate type 2 from other VWD subtypes. We suspect that this may be occurring, because functional VWF assays tend to be more technically demanding, time consuming and less precise than the simpler antigen assays. In addition, this may be a consequence of clinical pressures requesting 'stat' VWF testing, which can be accommodated using LIA testing for VWF:Ag, but less easily accommodated by functional VWF assays. The general move towards automation (i.e. performance of automated platelet agglutination assays rather than classical platelet aggregometry or automated VWF:Act based assays instead of VWF:RCo assays) is also of interest, as is the move towards LIA technology for VWF:Ag, at the expense of current ELISA technology and previous Laurell gel (EID) technology. This is also the experience of other external QAPs (37–39).

In conclusion, laboratories participating in the RCPA Haematology QAP were able in most cases to identify functional VWF discordance. However, in a substantial number of cases, functional VWF discordance was either not demonstrated, or when observed was not taken into account for the interpretation of possible VWD typing. We could also identify differential power depending on the VWF functional panel used (e.g. fewer errors when laboratories added the VWF:CB to the basic panel of VWF:Ag and VWF:RCo) or on the functional sub-methodology (e.g. better discordance observed by: users of LIA based VWF:Act assay compared to ELISA based, users of automated VWF:RCo compared to classical methods, users of in-house VWF:CB assays compared to commercial methods). We thus recommend that laboratories incorporate VWF:CB into their VWF test panel (using a type 1/3-collagen mixture-based assay) as this will better more consistently enable identification of functional VWF discordance present in type 2 VWD, or functional VWF concordance in type 1 VWD and normal samples, when compared to more limited testing. We do not support replacement of VWF:RCo assays with another functional VWF assay such as VWF:CB or VWF:Act assays, since these assays may miss some type 2M VWD. The role of VWF:Act assays in VWD

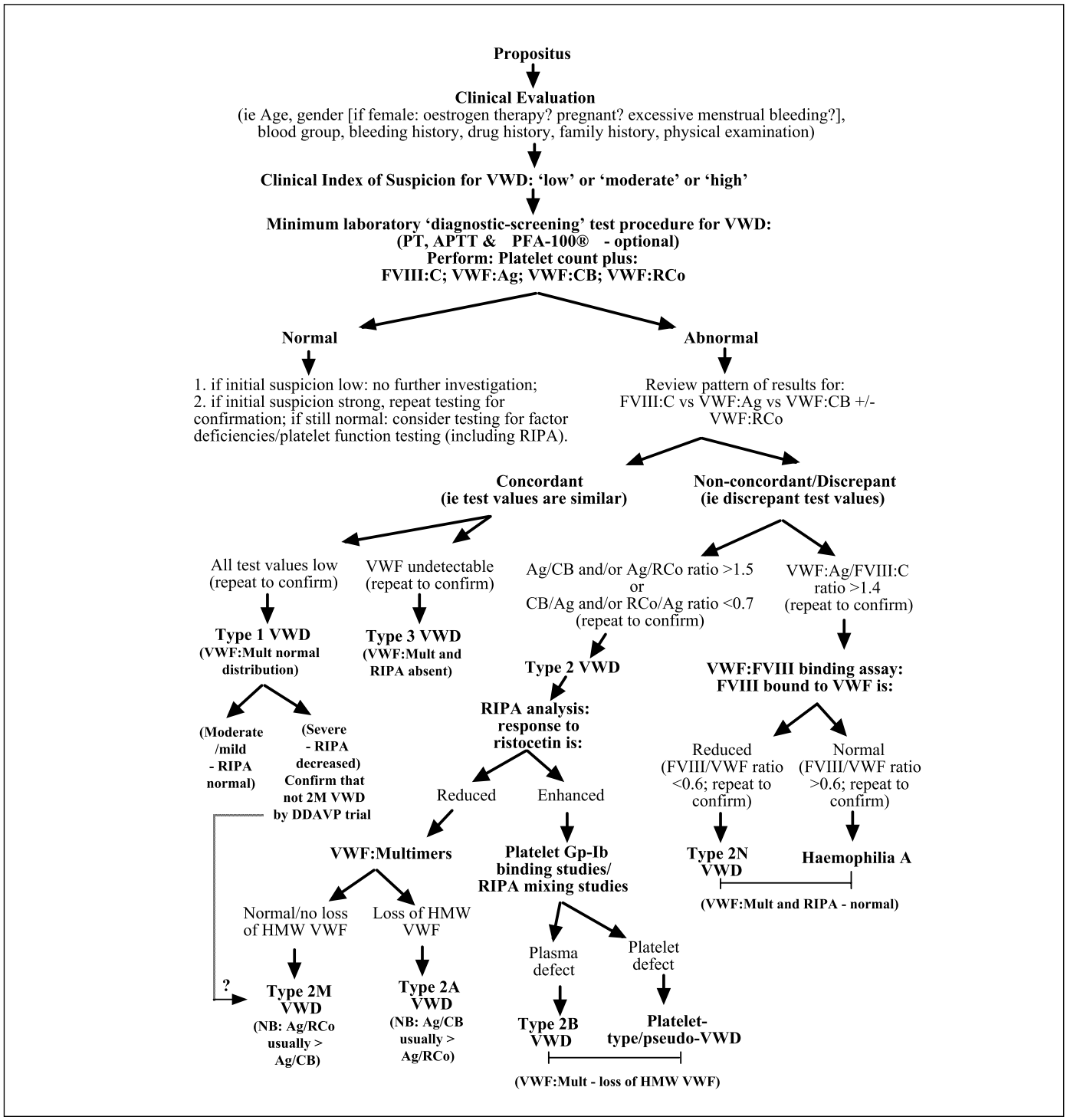

Figure 9: An algorithm containing one recommended approach to the current investigation of VWD. The important considerations are: perform a thorough clinical and family review; request the appropriate tests for investigation (at a minimum, for diagnostic-screening: platelet count/morphology, FVIII:C, VWF:Ag, VWF:RCo, VWF:CB; if clinical history is strong, RIPA as part of a platelet function study should also be considered); repeat tests for confirmation (when normal with strong history or when abnormal for all test cases); follow up abnormal test results further as required (e.g. VWF:Multimers [e.g. if type 2M suspected and management indicates differential therapy], RIPA [particularly to help identify/discriminate type 2B/platelet-type VWD, but also in some other cases of suspected VWD], VWF:FVIII binding assay [to help identify/discriminate type 2N VWD/haemophilia A]; perform a DDAVP challenge test if deemed appropriate [will help better identify some forms of VWD plus determine subsequent management]. This algorithm should be treated as a guide only; although it will help appropriately define the majority of cases, some isolated cases may not fit this scheme, and it is important that all patients be treated and managed as individuals. Finally, please seek expert local opinion and also refer to other expert recommendations (1–5), as different geographic localities may require a slightly different approach, and some experts may have a different working approach. Data using the VWF:Act assays described in the current report is too preliminary to include in this figure, which has been updated and modified from reference 5.

diagnostics is by no means clear, but results with the LIA based assays suggest that continued assessment is warranted.

We caution that our findings should not be construed as support for performing only the limited VWF test panel evaluated here; this panel simply reflects the current range of the RCPA QAP's proficiency-testing capacity and the participant testing set. In addition to clinical criteria, the correct diagnosis of VWD requires a comprehensive and appropriate panel of tests. At a minimum diagnostic screening level, this panel must include FVIII:C, VWF:Ag and at least one, but preferably more than one, functional VWF assay. Subsequent additional testing, including VWF:Multimer analysis, RIPA, and VWF:factor VIII binding will provide supplementary or confirmatory information and/or VWD-subclassification (1–5), and must be performed if it will help guide clinical decisions and subsequent therapeutic support. In particular, RIPA is essential in order to identify and differentiate type 2B VWD from other VWD subtypes (1, 6), and RIPA mixing studies are also recommended to help distinguish type 2B VWD from pseudo-, or platelet-type, VWD (6, 17). Although not recommended as a general investigative procedure, genetic studies should also be considered for difficult or interesting cases (6–9). Whether or not to take into consideration the patient's blood group when investigating patients or diagnosing VWD is contentious, but we would recommend that such testing be undertaken and considered in a broad sense when VWF levels are found to be around the reference range cut-off values, or if a conclusion of 'mild type 1 VWD' is being considered (18). A DDAVP-challenge test is also recommended as part of the patient work up wherever appropriate (2, 40). Finally, if laboratories can not provide a comprehensive testing process for investigations into VWD, then samples and/or patients should be referred to expert VWD testing centres for investigation. An algorithm of a general and currently recommended laboratory test approach is provided in Figure 9.

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Abbreviations

FVIII:C, factor VIII: coagulant (assay); MAB, monoclonal antibody; NRR, normal reference range; QAP, Quality Assurance Program; RIPA, ristocetin-induced platelet agglutination /aggregation; VWD, von Willebrand disorder/disease; VWF, von Willebrand factor; VWF:Ag, von Willebrand factor antigen (assay); VWF:CB, collagen binding activity/assay) for VWF; VWF:Rco, ristocetin cofactor assay for VWF.

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Regular Article

Evaluating errors in the laboratory identification of von Willebrand disease in the real world

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ABSTRACT

Introduction: von Willebrand disease (VWD), reportedly the most common bleeding disorder, arises from deficiency and/or defects of von Willebrand factor (VWF). Assessment requires a wide range of tests, including VWF activity and antigen. Appropriate diagnosis including differential identification of qualitative *vs* quantitative defects has important management implications, but remains problematic for many laboratories and clinicians.
Methods: Data using a large set (n = 29) of varied plasma samples comprising both 'quantitative' VWF deficiency ('Type 1 and 3' VWD) vs 'qualitative' defects ('Type 2 VWD') tested in a cross-laboratory setting has been evaluated to assess the ability of real world laboratories to differentially identify these sample types.
Results: Different VWF assays and activity/antigen ratios show different utility in VWD and type identification. VWD identification errors were often linked to high inter-laboratory test variation and result misinterpretation (i.e., laboratories failed to correctly interpret their own test panel findings). Thus, moderate quantitative VWF deficient samples were misinterpreted as qualitative defects on 30/334 occasions (9% error rate); 17% of these errors were due to laboratories misinterpreting their own data, which was instead consistent with quantitative deficiencies. Conversely, whilst qualitative VWF defects were misinterpreted as quantitative deficiencies at a similar error rate (~9%), this was more often due to laboratories misinterpreting their data (~50% of errors). For test-associated errors, ristocetin cofactor was associated with the highest variability and error rate, which was at least twice that using collagen binding.
Conclusion: These findings in part explain the high rate of errors associated with VWD diagnosis.

Introduction

von Willebrand disease (VWD) is reportedly the most common congenital bleeding disorder; nevertheless, its differential identification and clinical diagnosis remains problematic for many laboratories and clinicians [1,2]. VWD arises from deficiency and/or defects of von Willebrand factor (VWF), an adhesive plasma protein essential for effective primary haemostasis. VWF possesses many functional properties, including binding to platelets via several receptors, most notably glycoprotein Ib (GPIb), binding to sub-endothelial matrix components (most notably collagen), and binding and protecting factor VIII (FVIII) function [3]. The first two noted functions are essential for overall primary haemostasis and are respectively assessed in the laboratory by surrogate tests such as VWF ristocetin cofactor (VWF:RCo) and collagen binding (VWF:CB) [1,2,4,5], although alternative assays may also be employed.

The most recent classification scheme from the International Society on Thrombosis and Haemostasis (ISTH) recognizes six different types of VWD [5]. Type 1 represents a partial quantitative VWF deficiency, with VWF essentially functionally normal, but produced in lowered quantity. Type 3 VWD represents 'complete' deficiency of VWF. Type 2 VWD represents a heterogeneous group of qualitative VWF defects that comprise (i) 2A VWD (loss of high molecular weight (HMW) VWF), (ii) 2B VWD (enhanced functional binding of VWF that leads to loss of HMW VWF and typically mild thrombocytopenia), (iii) 2N VWD (loss of VWF-FVIII binding), and (iv) 2M VWF (VWF dysfunction not associated with loss of HMW VWF). The proper identification of VWD and differentiating its type is important for therapeutic management [6].

Abbreviations: AVWS, acquired von Willebrand syndrome; ELISA, enzyme linked immunosorbent assay; EQA, external quality assessment; FVIII:C, FVIII coagulant; GPIb, glycoprotein Ib; HMW, high molecular weight (VWF); LIA, latex-immuno-assay; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:Act, VWF activity (assays [generic]); VWF:Ag, VWF antigen; VWF:CB, collagen binding (assay for VWF); VWF:RCo, ristocetin cofactor (assay for VWF).

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In normal practice, VWD and VWD type can be determined by laboratory testing that encompasses a broad panel of different tests [1,2,4,5]. Virtually all laboratories perform VWF antigen (VWF:Ag) and FVIII coagulant (FVIII:C) [1], respectively measuring VWF protein and FVIII activity. VWF:Ag is most usually assessed using either ELISA (enzyme linked immunosorbent assay) or LIA (latex-immuno-assay) technologies (Table 1). The most common activity based test is VWF:RCo [1,2,4,5], usually performed as a platelet agglutination assay using aggregometry or automated methods with standard coagulation instruments. VWF:CB is assessed by a proportion of laboratories, most usually by ELISA [1,2,4,5]. Additional 'activity' assays include newly released commercial assays, many based on LIA, although representing distinct assays, using distinct reagents, and detecting VWF differently [7–10]. Two such assays do not use ristocetin [7,8], so cannot claim to be VWF:RCo assays, although many laboratories may use these instead of VWF:RCo. VWF:CB and/or VWF:RCo can also be detected by other methodologies including LIA, fluorescence and flow cytometry [9–12], and VWF can also be assessed by other methods including multimer analysis to determine loss of HMW VWF as well as structural abnormalities [13].

In brief, type 1 VWD is identified as a quantitative deficiency of VWF, with the level of deficiency correlating with the severity of the disorder, so that terms such as 'mild', 'moderate' or 'severe' type 1 VWD are sometimes employed. A new concept related to 'low VWF' as a risk factor for bleeding, without a formal diagnosis of VWD, has also recently emerged [14]. Importantly, for all these cases, low levels of VWF:Ag, VWF:RCo, VWF:CB and other VWF activity assays (generically abbreviated 'VWF:Act' in this paper) will be identified by laboratories, but since VWF is functionally normal, similar ('concordant') levels will be identified using all assays, so the ratio of any assay to another will be close to one. In practice, these cases are identified by a low level of VWF together with a ratio of VWF activity (VWF:RCo, VWF:CB and/or VWF:Act) to VWF:Ag above 0.7 [1,2,4,5,14].

In contrast, in qualitative defects (i.e., type 2 VWD), activity-based assays identify some VWF defect, with this defect helping to characterize VWD type. Thus, loss of HMW VWF (e.g., type 2A and 2B VWD) is identified by a relatively larger reduction in VWF:RCo, VWF:CB and VWF:Act compared to VWF:Ag; this VWF 'discordance' is expressed by a ratio of VWF activity to VWF:Ag typically below around 0.6 or 0.7 [1,2,4,5,14]. Type 2M reflects a variety of potential functional defects, with most common forms representing platelet GPIb binding defects; hence, VWF:RCo/Ag will be low, but VWF:CB/Ag may be normal.

Although we and others have previously reported on variability of cross-laboratory results for VWD, as well as associated errors in diagnostic interpretations from composite test panels [15–25], our last major report in this area was in 2007 [21].

We have therefore assessed the contemporary ability of real world laboratories to differentially identify VWF deficiency (potentially representative of type 3 VWD, type 1 VWD or low VWF) and qualitative deficiency (potentially representative of types 2A, 2B or 2M VWD) using a large composite set (n = 29) of plasma samples. The differential utility of different VWF assays and different VWF activity to antigen ratios, the variability of laboratory test data, identification errors related to VWD and VWD 'type', and their cause have all been evaluated.

Methods

Setting

This study derives from the Royal College of Pathologists of Australasia Quality Assurance Program (RCPAQAP) in Haematology (<http://www.rcpaqap.com.au>). Table 2 details the samples (n = 75) distributed to the external quality assessment (EQA) participants from the start of data collection (in 1998) to current (spanning 15 years in total). The principal author's laboratory acts as a 'host-laboratory', including sample preparation and homogeneity/stability testing prior participant dispatch. Participants to this VWF/VWD EQA and therefore of the current study (current n = 55) derive from Australia (n = 34), New Zealand (n = 5), Asia (n = 10), South Africa (n = 4), India (n = 1) and France (n = 1). The majority of these laboratories perform VWF testing for the investigation of VWD for clinical diagnosis and management and most would not be considered expert laboratories. Thus, the composite of laboratories would be generally representative of 'real-world' laboratories, defined here as the realm of practical or actual experience, as opposed to the abstract, theoretical, or idealized experience.

Samples

The current report details results using 29 samples from 2006 to 2013 inclusive (Tables 2 and 3), comprising a mixture of true patient and in-house prepared samples. Although patient samples are preferred in research evaluations, within an EQA setting it is important to balance this desire against ethical and logistical challenges around regular collections from VWD-affected patients. In-house prepared material also carries specific design advantages related to desired specifications. The 29 samples distributed comprised:

(i) Four pooled normal samples (two different batches; target VWF ~100U/dL);
(ii) One sample from a heterozygous type 3 VWD carrier ('2008-3het'); VWF ~60U/dL;
(iii) Seven samples mildly to moderately deficient in VWF (one patient sample and three different batches of in-house prepared material; targets VWF 25-40 U/dL);
(iv) Three samples severely deficient in VWF (two different batches of in-house prepared material; target VWF ~ 10 U/dL);

Table 1
Summary of VWF test methods employed in VWD diagnostic practice.

VWF assay	Description
VWF:Ag	Assessment of VWF protein level using an 'antigen' assay. Typically performed by either ELISA or LIA based methods. Alternative methods including flow cytometry utilised infrequently.
VWF:Rco	Assessment of VWF activity level utilising ristocetin and an 'agglutination' assay. Typically performed by either platelet agglutination or LIA based methods. For platelet agglutination, tests can be performed using an aggregometer (semi-automated method) or a coagulation based instrument (automated method). Alternate methods including flow cytometry utilised infrequently.
VWF:CB	Assessment of VWF activity level utilising collagen. Typically performed by ELISA. Alternative methods including flow cytometry utilised infrequently.
Werfen-IL 'activity' assay	Assessment of VWF activity level utilising a monoclonal antibody binding assay, where the antibody is directed against the platelet Glycoprotein Ib binding region of VWF. Performed by LIA based method. An ELISA based method from a different manufacturer (i.e., not Werfen-IL) using a similar antibody and concept was previously utilised.
Siemens Innovance 'activity' assay	Assessment of VWF activity level utilising a Glycoprotein Ib binding method. The system employs two gain of function Glycoprotein Ib mutations within a recombinant molecule that facilitates VWF binding. Performed by LIA based method. ELISA based methods employing a similar concept (but not from Siemens) have also been previously utilised.

Abrreviations: ELISA, enzyme linked immunosorbant assay; LIA, latex-particle immunoassay; VWF, von Willebrand factor; VWD, von Willebrand disease.

Table 2
Summary of surveys undertaken and samples distributed.

Year	No. samples	No. laboratories	Sample types									Data published in
			Normal	Equivocal	VWD or VWD-like samples						Other	Other Detail	
					1	**2A**	**2B**	**2M**	**2N**	**3**			
1998	10	25	3	2	1	1	2			1			[15]
2000	7	19	1	1	1		1	1	1	1			[16]
2002	8	44	4	2		1				1			[17]
2003	8	44	1	4	1	1				1			[18]
2004	6	45	2	1	1						2	[VWF]	[19]
2005	4	49	2	1	1								[20]
2006	4	52					3						[21]
			1										current report
2007	4	55			1		1			1	1	AVWS	current report
2008	4	56	1	1		1					1	AVWS	current report
2009	4	55			2		1			1			current report
2010	4	56	1		1					1	1	AVWS	current report
2011	4	58			2		1				1	AVWS	current report
2012	4	59			1			1		1	1	AVWS	current report
2013	4	57	1		2	1							current report
Totals:	***75***		***17***	***12***	***14***	***5***	***9***	***2***	***1***	***8***	***7***		
Current report	***29***		**4**	**1**	**9**	**2**	**3**	**1**	**0**	**4**	**5**		

Abbreviations: AVWS, acquired von Willebrand Syndrome; VWF, von Willebrand factor; VWD, von Willebrand disease [VWF], VWF concentrate.

(v) Three samples totally deficient in VWF (commercially obtained; VWF = 0 U/dL; purchased from Banksia Scientific (Bulimba, Australia) and prepared by Affinity Biologicals (Ontario, Canada));
(vi) Five samples selectively deficient in HMW VWF (two different batches of type 2B VWD patient plasma plus two different batches of type 2A VWD-like plasma prepared in house; one of the latter represented a small pool of cryosupernatant plasma, the other was prepared using a process similar to that previous identified [27]);
(vii) One type 2M VWD (patient) sample;
(viii) Five samples from two separate cases of acquired von Willebrand syndrome (AVWS); both showed substantial loss of VWF and some selective loss of HMW VWF.

In-house prepared VWF deficient and HMW deficient samples represents material with similar test matrix to true patient VWD samples as evidenced by similar reactivity profiles using standard VWF tests (Supplementary Fig. 1), and multimer analysis (Fig. 1).

Normal pool samples were primarily derived from fresh frozen blood bank plasma from the Australian Red Cross Blood Bank. Samples severely to moderately deficient in VWF were prepared as mixtures of

Table 3
Summary of samples utilised for current study.

Sample Type	Sample represents or mimics	Sample ID*	Sample Source	Details
Normal pool	Normal	**2006-N, 2008-N, 2010-N**	Prepared in house	target ~100U/dL VWF
Normal pool	Normal	**2013-N**	Prepared in house	target ~100U/dL VWF
Equivocal	Borderline normal VWF	**2008-3het**	Patient sample	Mother of Type 3 VWD daughter; obligate carrier type 3 (heterozygous); VWF:Ag ~60U/dL; VWF activity/Ag >0.7
Mildly VWF deficient	'Low VWF'	**2007-1 m**	Patient sample	VWF:Ag ~40U/dL; no mutation in exon 28; mild personal history of bleeding/bruising
Moderately VWF deficient	'mild/moderate VWD type 1'	**2010-1 m, 2011a-1 m**	Prepared in house	target ~30U/dL VWF; VWF activity/Ag >0.7
Moderately VWF deficient	'mild/moderate VWD type 1'	**2009-1 m, 2011b-1 m, 2012-1 m**	Prepared in house	target ~30U/dL VWF; VWF activity/Ag >0.7
Moderately VWF deficient	'mild/moderate VWD type 1'	**2013-1 m**	Prepared in house	target ~25U/dL VWF; VWF activity/Ag >0.7
Severely VWF deficient	'severe VWD type 1'	**2009-1 s, 2013-1 s**	Prepared in house	target ~10U/dL VWF; VWF activity/Ag >0.7
Severely VWF deficient	'severe VWD type 1'	**2012-1 s**	Prepared in house	target ~10U/dL VWF; VWF activity/Ag >0.7
Totally VWF deficient	'Type 3 VWD'	**2007-3, 2009-3, 2010-3**	Commercial VWF deficient Plasma	actual 0U/dL VWF
HMW deficient	'2A VWD'	**2008-2A**	Prepared in house	Type 2A Mimic (Cryosupernate); target ~20U/dL VWF:Ag; with RCo/Ag and CB/Ag < 0.5
HMW deficient	'2A VWD'	**2013-2A**	Prepared in house	Type 2A Mimic; target ~30U/dL VWF:Ag; with RCo/Ag and CB/Ag < 0.5
HMW deficient	2B VWD	**2007-2B**	Patient sample	Well characterised 2B VWD; VWF mutation (R1306W); VWF:Ag ~30U/dL; RCo/Ag and CB/Ag < 0.3
HMW deficient	2B VWD	**2009-2B, 2011-2B**	Patient sample	Well characterised 2B VWD; VWF mutation (R1306W) VWF:Ag ~40U/dL; RCo/Ag and CB/Ag < 0.3
Platelet binding defect VWD	2M VWD	**2012-2M**	Patient sample	Well characterised 2M VWD; VWF mutation (H1322P); VWF:Ag ~50U/dL; RCo/Ag < 0.3 and CB/Ag > 0.7
AVWS (2A-like)	AVWS (2A like)	**2007-AVWS, 2008-AVWS**	Patient sample	Well characterised AVWS case; no VWF mutation in Exon 28; no evidence of inhibitory antibodies.
AVWS (1/2A-like)	AVWS (1/2A-like)	**2010-AVWS, 2011-AVWS, 2012-AVWS**	Patient sample	Well characterised AVWS case secondary to multiple myeloma; increased clearance post VWF concentrate infusion; no evidence of inhibitory antibodies.

Abbreviations: Ag, Antigen; AVWS, acquired von Willebrand Syndrome; HMW, high molecular weight; VWD, von Willebrand disease; VWF, von willebrand factor; RCo, ristocetin cofactor; CB, collagen binding.
* samples listed in the same cell indicate multiple despatches of same plasma sample in different years.

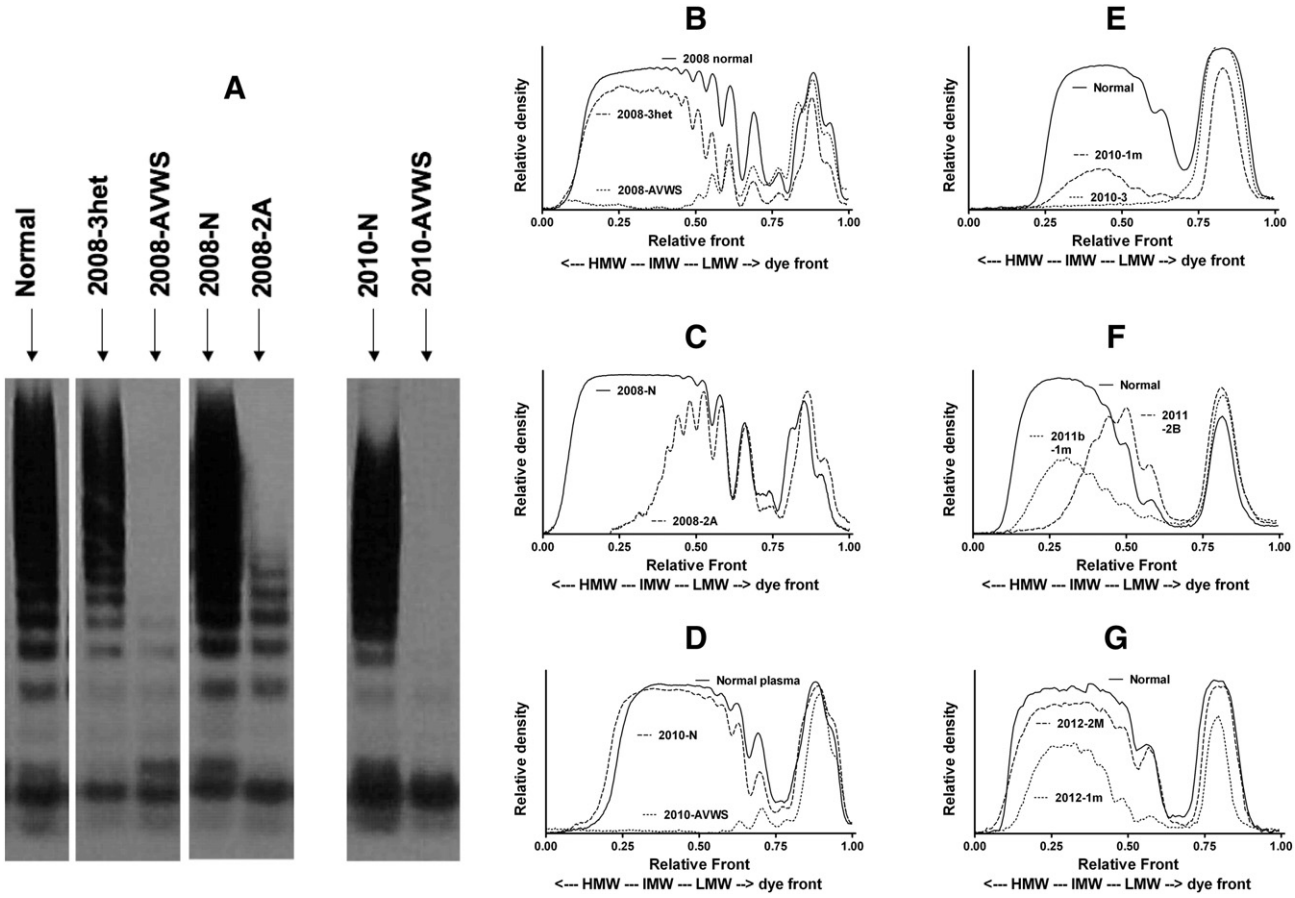


Fig. 1. Representative VWF multimer gels (***A***) and densitometry (***B to G***) results for representative samples investigated in current report, including normal, VWF deficient, high molecular weight (HMW) VWF deficient, and acquired von Willebrand syndrome (AVWS). For densitometry figures, relative density shown on y-axis, and relative front on x-axis, showing approximate regions of HMW, intermediate molecular weight (IMW) and low molecular weight (LMW) VWF, as well as the dye front. Normal samples are shown as reference samples; quantitative VWF deficient samples and the type 2M sample retain all molecular weight forms of VWF, but at a reduced level compared to normal plasma; in contrast, the HMW VWF deficient samples each show a reduction in HMW forms.

normal pool plasma with VWF deficient plasma to generate different samples with VWF ranging between 10 and 40 U/dL (Table 3).

All samples sent to participants were lyophilized plasma, typically 0.5 or 1.0 ml/vial. All samples passed stability and homogeneity testing to ensure sample integrity (data not shown).

Strategy of Sample Dispatches and Aims of the EQA Program

The overall aim of the program is to provide laboratories with a wide selection of test material that reflects a similar heterogeneity to that seen in VWD diagnostic practice. Samples (2x twice a year) are dispatched in semi-random rotation, with different samples tested in each year, and repeat samples tested in separate years (permits assessment of repeatability and provides additional evidence of sample stability). Although FVIII testing is also performed, this report focuses on VWF assays, given the large amount of data generated, and because of the type of VWF samples dispatched over the period of analysis (viz, no type 2N VWD samples).

Data Analysis

All laboratories report numerical values to the program for the tests performed, and these are analyzed using descriptive statistics (median, ranges, intra-method or inter-laboratory coefficient of variation [CV%], etc). Laboratories are also encouraged to interpret overall test findings for the likelihood or otherwise of 'VWD', as well as potential 'VWD type' if applicable. It is well recognized that diagnosis of VWD requires more than laboratory testing, and some samples used were not derived from VWD patients. Nevertheless, such interpretation exercises are valid within the context of EQA, and in-house prepared VWF-deficient and HMW-VWF-deficient samples cannot be otherwise distinguished from true VWD patient samples using the tests performed by these laboratories. Laboratory interpretations were assessed in the context of the provided sample, for both the variety of different interpretations as well as for 'errors' in interpretation. An error was defined to occur when the laboratory assigned (i) an 'incorrect VWD type' to the sample (e.g., qualitative defect assigned to a quantitative VWF deficiency, or visa versa), (ii) a VWD interpretation assigned to a clearly normal plasma sample, or (iii) a normal interpretation assigned to a moderate/severe deficient VWF sample or qualitative defect.

Results

Participant Numbers and Test Methodologies

Numbers of program participants have varied only slightly during the past seven years (n = 52-59; Table 2). All participants (i.e., 100%) have always performed FVIII:C testing, and >95% have always also performed VWF:Ag testing. VWF activity-based testing is performed by a large proportion of (but not all) laboratories, with the makeup changing appreciably over time (Fig. 2). Almost all laboratories (>80%) performed VWF:RCo testing in the past, but this has declined to just over 50% currently. VWF:CB testing has been performed by ~50% of laboratories throughout the past 15 year period, with this increasing slightly to ~60% currently. Other VWF activity ('VWF:Act') assays are performed by a smaller proportion (initially <10%, but currently nearly a third) of

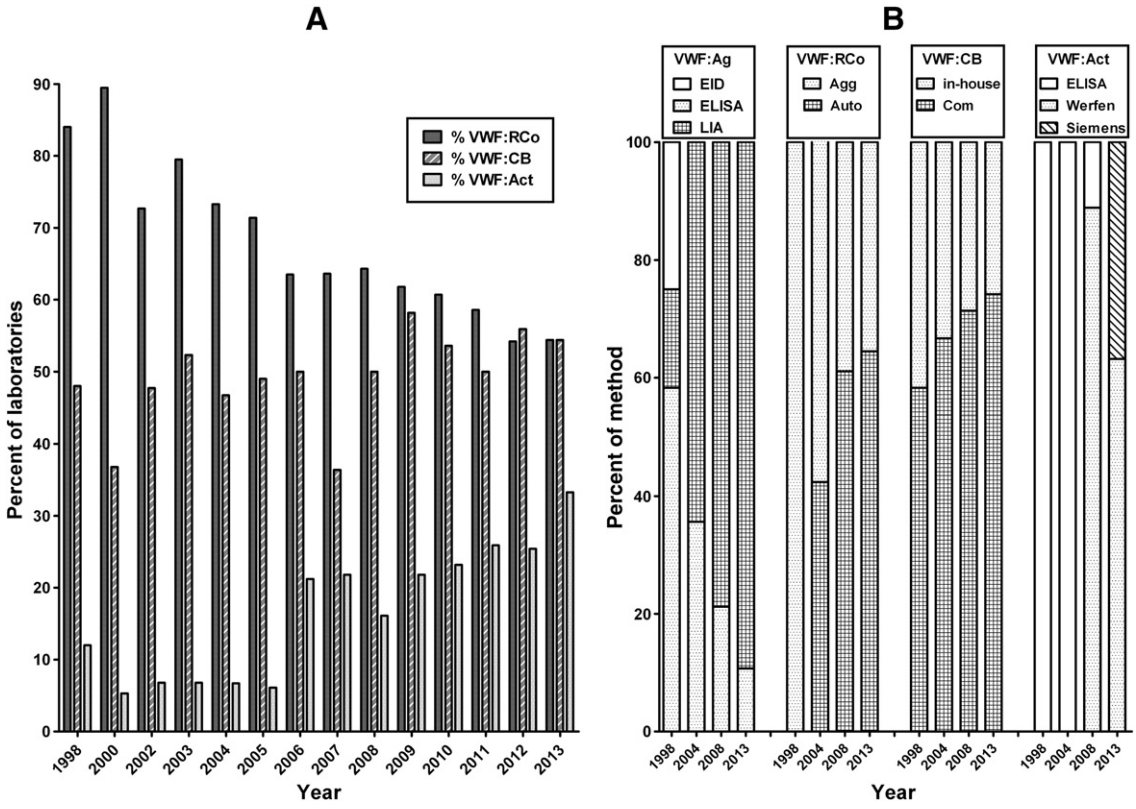


Fig. 2. ***A.*** Proportion of program participants performing various VWF activity assays over the past 15 years. VWF:RCo, ristocetin cofactor; VWF:CB, collagen binding; VWF:Act, other VWF activity assays. Note: >95% of laboratories have consistently performed VWF:Ag testing during this period, and all perform FVIII:C testing. ***B.*** Methodological detail for VWF:Ag and VWF activity assays as per ***A***, but shown as percentage of methods in approximate 4 year steps.

laboratories. Thus, the use of VWF:CB has been fairly stable over all years, but VWF:RCo usage has fallen, in many cases replaced by other VWF:Act assays.

VWF:Ag assays used to be primarily performed by electro-immuno diffusion (EID) and ELISA, but EID has completely disappeared, and ELISA methods have recently reduced in favor of LIA. VWF:RCo used to be performed exclusively by platelet aggregometry ('agg'), but this is reducing in favor of automated ('auto') methodologies. VWF:CB is performed exclusively by ELISA, with similar numbers of in-house and commercial ('com') methods originally, although the latter now dominates. VWF:Act assays originated as monoclonal antibody ELISA-based methods, with these giving way to the Werfen-IL LIA monoclonal antibody based method, and only recently in 2013 has the Siemens Innovance rGPIb-binding LIA method emerged (Table 1; Fig. 2).

Inter-laboratory and Intra-method Variability

Considerable inter-laboratory and intra-method variability was observed with all samples (Fig. 3), and CVs generally increase as VWF falls (Fig. 4A). Moreover, CVs are lowest for VWF:Ag, highest for VWF:RCo, and intermediate for VWF:CB and VWF:Act (Fig. 4B). Occasional identified outlier data (Fig. 3) reflect random error events, transcription errors, and occasional systematic error. Irrespective, these reflect real events that identify errors in laboratory reporting of numerical data that then impact on test result interpretation. Occasionally, laboratories reported outliers for several tests perhaps reflecting problems with a common assay calibrant standard.

Lower Limit of VWF Sensitivity

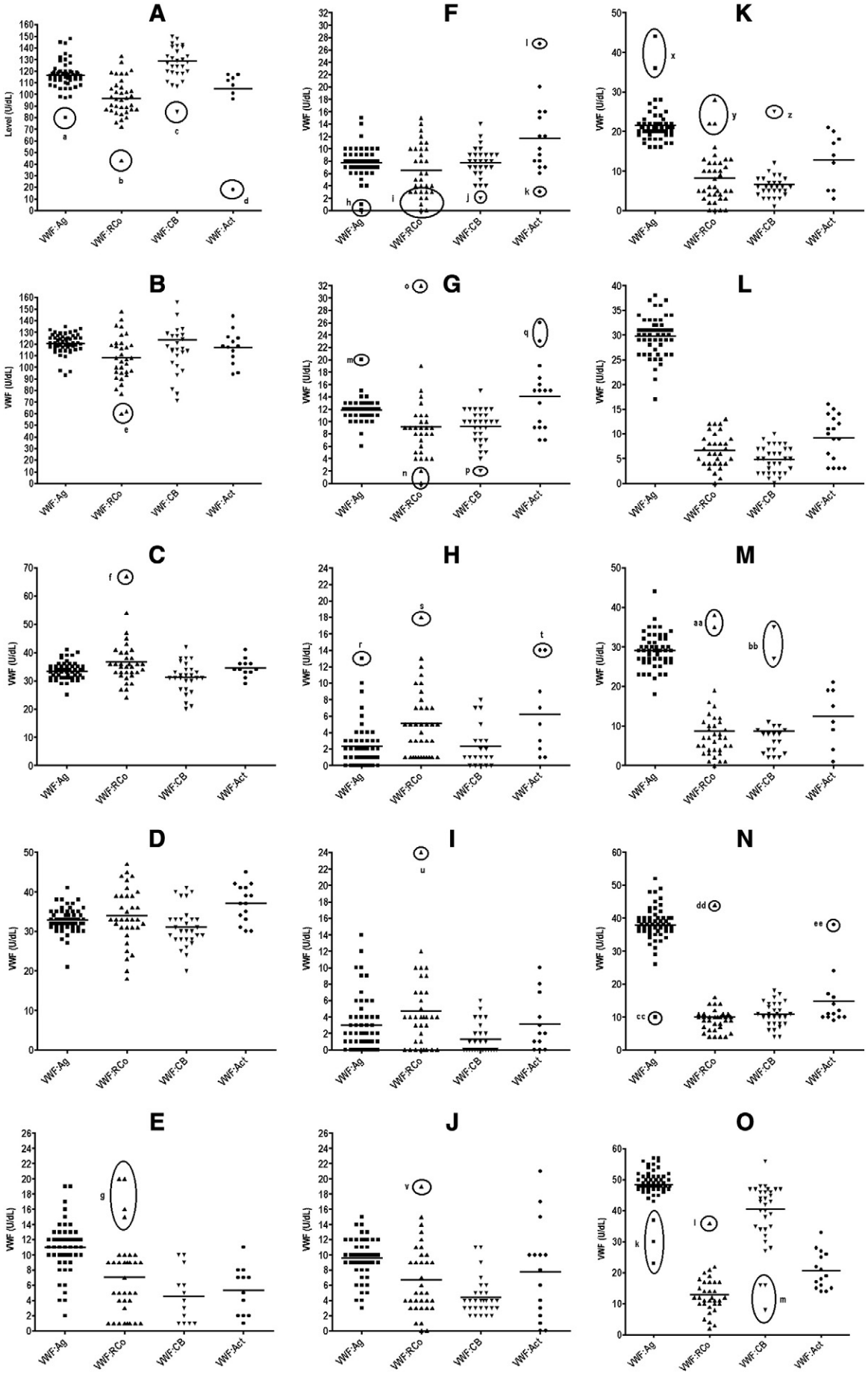
This was explored by assessment of samples totally (=0 U/dL VWF; equivalent to type 3 VWD) or severely deficient in VWF (~10 U/dL VWF) (Fig. 3). Even when testing samples that do not contain any VWF, most laboratories in fact report values above 0 U/dL for most VWF assays. This is comparatively worse for VWF:RCo than other assays, approaching 20U/dL in some laboratories; however, limits are close to 5-10U/dL for many laboratories for all VWF assays. Thus, many cannot distinguish total from severe VWF deficiencies. Sensitivity limits will also lead to problems in type 2 VWD identification.

Utility and Variability of VWF Activity/Ag Ratios

This was assessed by comparison of assay ratios derived using normal plasma and mild/moderate VWF deficient samples, *vs* those with qualitative defects or HMW VWF deficient (Fig. 5). In normal and VWF deficient (equivalent to low VWF or type 1 VWD) samples, there is expected concordance of VWF activity and VWF:Ag. In practice, different laboratories assign different cut-off values that in general range from 0.5-0.7. Conversely, samples that express qualitative defects and/or are specifically deficient in HMW VWF (i.e., type 2A, 2B and 2M VWD) would be expected to yield VWF activity/Ag ratios below these cut-off values. As identified in Fig. 5, best discrimination between normal/VWF deficient *vs* qualitative/HMW VWF deficient samples was obtained using VWF:CB/Ag ratios. In contrast, considerable overlap of data was observed with VWF:RCo/Ag ratios, and an intermediate overlap was observed with VWF:Act/Ag ratios. For the type 2M VWD case, functional discordance was observed using VWF:RCo/Ag but not with VWF:CB/Ag; VWF:Act/Ag ratios were again intermediate.

Variability in Test Result Interpretation

Considerable inter-laboratory variability in test result interpretation was also observed, which on occasion reflected subtle differences regarding the perception of 'severity' in VWF deficiency (mild, moderate, severe *vs* total), and on other occasions 'diagnostic errors' related to incorrect VWD type assignment. For example, for the five mildly/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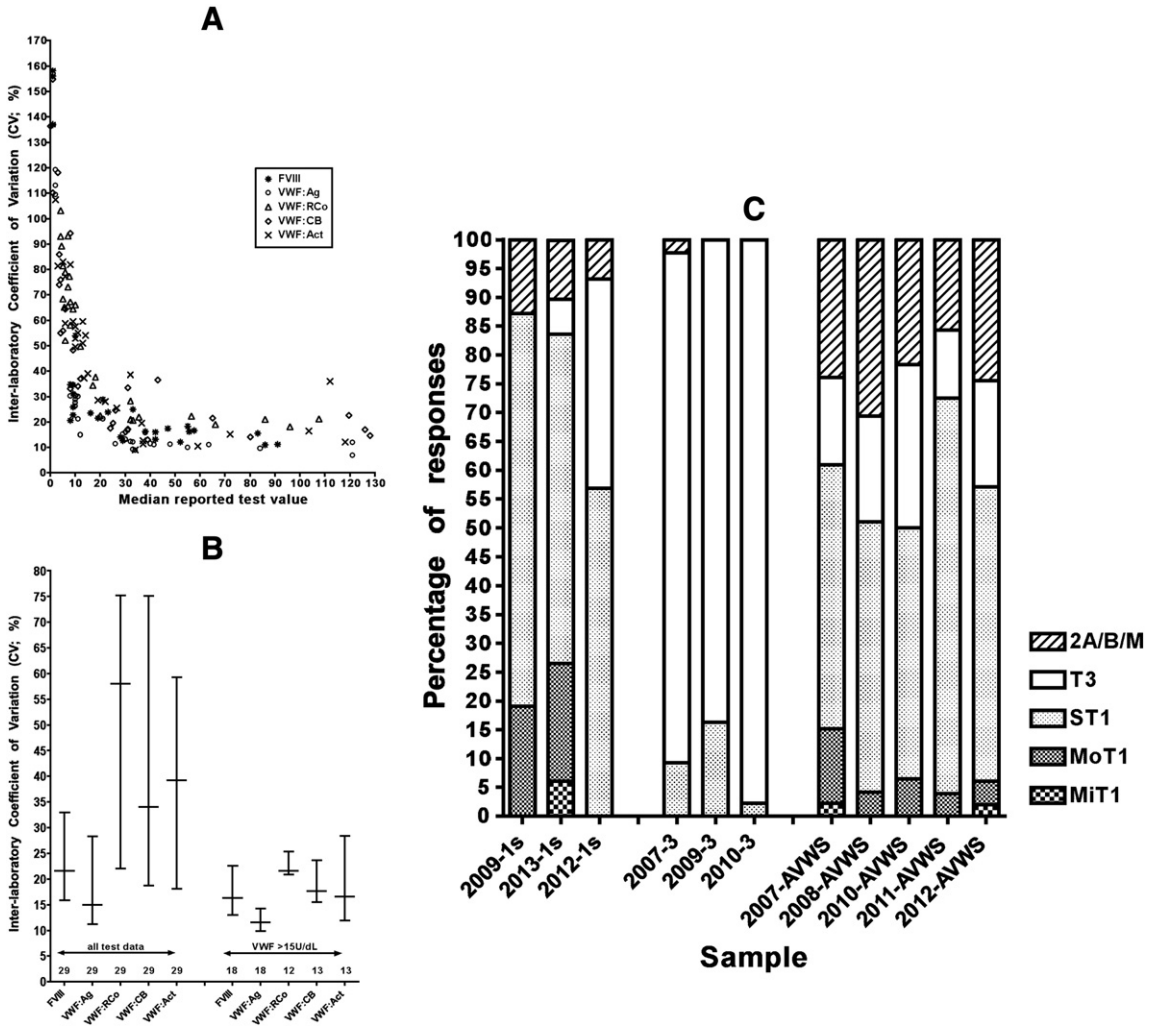

Fig. 4. ***A and B:*** Inter-laboratory variability for all performed tests expressed as coefficient of variation (CV%). FVIII:C data also shown here as comparator. ***A.*** CV% (y-axis) vs median reported assay values (x-axis). ***B.*** range of CV values (y-axis) expressed as 25th/median/75th percentiles for all test data (left portion of figure) and for select test data (median VWF levels > 15U/dL; right portion of figure). 15U/dL chosen since CVs for values below 15U/dL begin to increase exponentially (refer to Figure A). Median CVs for VWF:RCo are highest, and for VWF:Ag are lowest. Those for VWF:CB and VWF:Act are intermediate. Note the larger spread of CV data for VWF:Act where VWF > 15U/dL. Some of this variability is driven by data from acquired von Willebrand syndrome (AVWS) as partly shown in Fig. 3J. ***C:*** Variability in laboratory provided interpretations for severe VWF deficient (~10U/dL VWF) samples, totally VWF deficient samples (VWF = 0U/dL) and for AVWS samples. Data shown as percentage of responses (y-axis); samples identified on x-axis. MiT1, mild VWF deficiency/mild type 1 VWD; MoT1, moderate VWF deficiency/moderate type 1 VWD; ST1, severe VWF deficiency/severe type 1 VWD; T3, total VWF deficiency/type 3 VWD; 2A/B/M, high molecular weight (HMW) VWF deficient/'qualitative VWF defect'/type 2A/2B/2M VWD.

moderately deficient samples (VWF 25-40 U/mL), laboratories provided a variable mixture of interpretations of primarily moderate and mild type 1 VWD (data not shown). More interesting perhaps, were the comparative interpretations for severely deficient *vs* totally deficient VWF samples, which challenged many laboratories. While most identified these appropriately, several identified the former as 'type 3 VWD' and the latter as 'severely' VWF deficient (Fig. 4C). This partially relates to the lower limit of VWF sensitivity issue noted previously (Fig. 3). Also interesting was the wide range of interpretations provided for the AVWS samples. As these showed a specific loss of HMW VWF in addition to severe loss of VWF and VWF activity (Figs. 1 & 3), interpretations of type 2A/2B/2M VWD were expected; however, most laboratories interpreted these as quantitative deficiencies of VWF ranging from severe (most laboratories) to moderate to even mild (Fig. 4C).

Reproducibility of Test Results Using Repeat Test Samples

As identified in Table 3, several identical samples were dispatched in different years. In general, returned numerical data showed similar

Fig. 3. Numerical data returned by participants for representative samples from each sample category shown in Table 3; including two of four normal samples (***A*** [2008-N] and ***B*** [2010-N]), two of seven samples with mild/moderate VWF deficiency (***C*** [2010-1 m] and ***D*** [2011a-1 m]), two of five samples of acquired von Willebrand Syndrome (***E*** [2007-AVWS] and ***J*** [2011-AVWS]), two of three samples of severe VWF deficiency (***F*** [2012-1 s] and ***G*** [2013-1 s]), two of three samples with total VWF deficiency (***H*** [2007-3] and ***I*** [2010-3]), four of five samples with high molecular weight [HMW] VWF deficiency (***K*** [2008-2A], ***L*** [2013-2A]), ***M*** [2007-2B] and ***N*** [2011-2B]), and the type 2M VWD sample (***O*** [2012-2M]), for all VWF assays (VWF:Ag (antigen), VWF:RCo (ristocetin cofactor), VWF:CB (collagen binding), VWF:Act (other VWF activity assays)). VWF level shown on y-axis; assays shown on x-axis. Circled values indicate representative outlier values. For example, ***A*** 2008-N: a-d = four outlier points for each VWF assay as reported by four different labs; a. interpretation given as normal; b. interpretation given as normal despite VWF:RCo/Ag = 0.4; c. interpretation given as normal; d. Interpretation given as 2A/2B VWD with VWF:Act/Ag = 0.2. ***All figures:*** note high outlier points, especially for VWF:RCo, but also on occasion for other assays, as well as low outlier points. These were often associated with VWF/VWD identification errors, and yielded false VWF activity to antigen discordance in normal and quantitative VWF deficient samples, and false concordance in HMW VWF deficient samples. Note also the high scatter of points for VWF:Act for the AVWS sample shown in Figure J; apart from one or two participant points, these values reflect data from the Werfen-IL VWF activity assay. Similar high variability for this assay for the other AVWS samples was also evident.

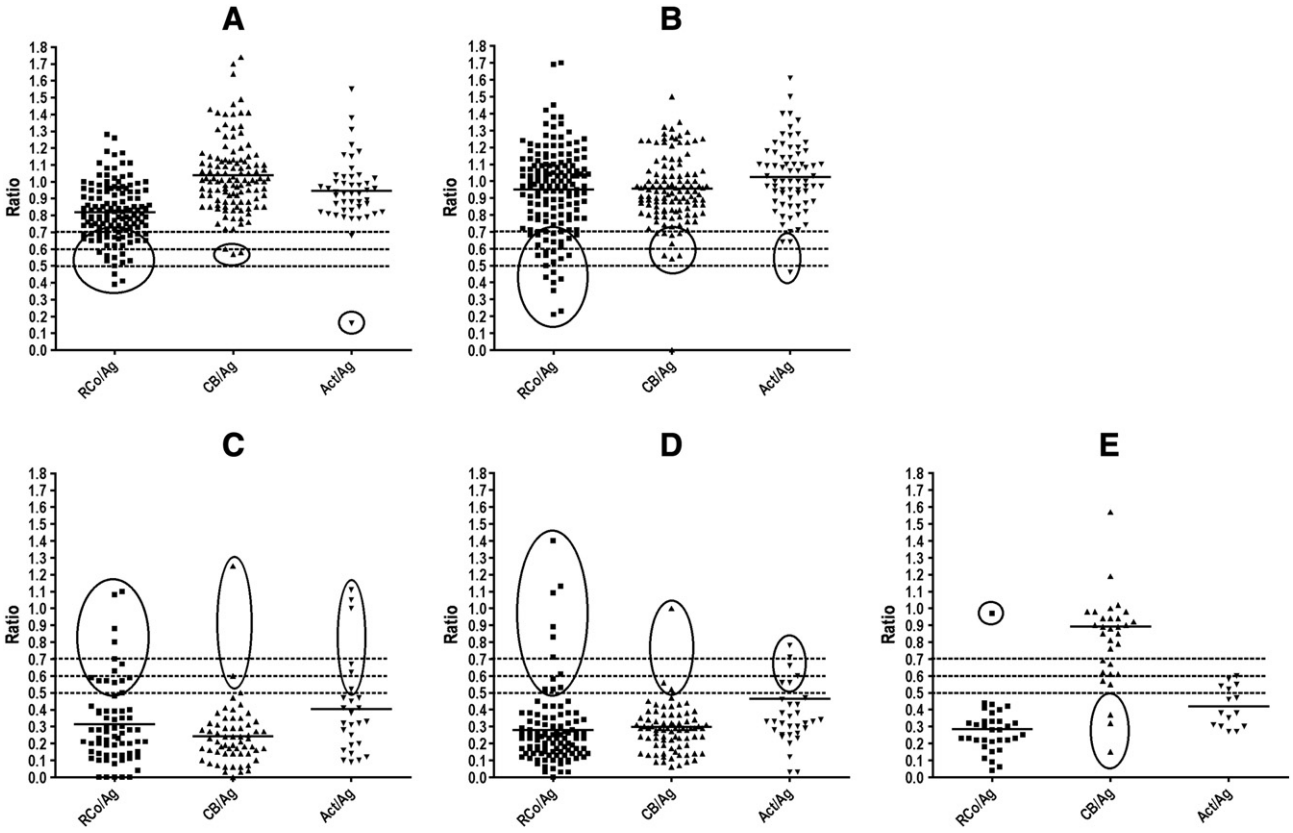

Fig. 5. VWF Activity/Ag ratios. ***A & B.*** Normal plasma (***A***) and mild/moderate VWF deficiency (***B***) samples using a representative subset of four plasma samples in each category. ***C & D***. high molecular weight (HMW) VWF deficient samples ('2A VWD like' [x2] and 2B VWD [x3] respectively); ***E***. The 2M VWD case as shown in Fig. 3. Circled values in each case reflect false (discordant) low ratios (<0.7) for normal and VWF deficient samples (***A*** and ***B***) and false (concordant) high ratios (>0.7) for HMW deficient samples, or areas of overlap, and therefore causing difficulties and errors in quantitative *vs* qualitative VWD type assignment. Apart from noted (circled) outliers, the type 2M case (***E***) showed VWF:RCo/Ag ratios <0.5 and VWF:CB/Ag ratios >0.5; the VWF:Act/Ag ratios were intermediate between those for VWF:RCo/Ag and VWF:CB/Ag.

median and ranges of VWF test data (examples shown in Fig. 3), and similar but not identical overall interpretations (examples in Fig. 4C).

Errors in Result Interpretation

Please also refer to highlighted outlier data points in Fig. 3.

Normal Samples

For the four normal samples (Table 3), acceptable interpretations were reported in all but four cases; one laboratory reported moderate type 1 VWD based on a normal VWF:Ag (86U/dL) but low VWF:RCo (39U/dL) for a VWF:RCo/Ag ratio of 0.5; three laboratories reported normal samples as 'type 2A/2B VWD' – two of these based on false discordance with VWF:RCo/Ag ratios of 0.4 and 0.5 and one on false discordance with VWF:Act/Ag (0.2).

Mild/moderate/severe Deficient VWF Samples

In total, there were 30 misinterpretations of qualitative defects (i.e., type 2A/2B/2M VWD) and false VWF Activity/Ag discordance reported, with errors reported for all seven samples (Table 4). In 5/30 (16.7%) cases these reflected laboratory misinterpretation of their own test data since VWF assay concordance (ratios > 0.7) was actually reflected in returned test data. In the majority of other cases (n = 24), false discordance was reported (i.e., VWF Activity/Ag ratios < 0.7). False low VWF:RCo/Ag ratios were reported in 19/24 (79.2%) of cases, false low VWF:CB/Ag ratios in 8/24 (33.3%), and false low VWF:Act/Ag ratios in 2/24 (8.3%). Adjusted error rates (taking into account numbers of laboratories performing testing with each type of activity assay) were 9.5%, 4.6% and 2.3% respectively for VWF:RCo, VWF:CB and VWF:Act.

Severe Deficient VWF Samples

Misinterpretations of qualitative defects (i.e., type 2A/2B/2M VWD) were reported with each of three samples for a total 14 occasions (Table 4). In 5/14 (35.7%) cases these reflected laboratory misinterpretation of test data since assay concordance was actually obtained. In the majority of other cases, 'false discordance' in various activity/Ag ratios was 'identified' by laboratories. Again, most errors related to use of VWF:RCo and VWF:RCo/Ag ratios.

HMW VWF Deficient Samples

Misinterpretations of type 1 VWD were reported with each the five HMW VWF deficient samples for a total of 26 occasions (Table 4). In most cases (13/26 = 50%) this reflected a misinterpretation of laboratory test data, which was instead consistent with the test sample (i.e., evidencing VWF activity/Ag ratios < 0.7). In many other occasions (5/26 = 19.2%) a limited test panel (i.e., lacking VWF:Ag or VWF activity assays) was identified. On other occasions, false VWF activity/Ag concordance was reported; this occurred more frequently for VWF:RCo/Ag ratios than VWF:CB/Ag or VWF:Act/Ag.

Misidentification of Type 2M VWD

One sample dispatched in this study was a type 2M VWD sample with a novel *VWF* mutation (Table 3). Consistent with expectations of a GPIb binding defect, multimer analysis showed retention of HMW VWF (Fig. 1), and this sample expressed a relative reduction in VWF:RCo/Ag but essentially normal VWF:CB/Ag ratio (Fig. 5). Even in the absence of multimer analysis, this pattern is highly suggestive of a platelet GPIb binding defect type 2M VWD. However, only 21/48 (43.8%) laboratories identified type 2M VWD and a higher proportion

Table 4
Interpretation Error Rates for select study samples.

Sample type	Sample sets	Total errors[a]	Total interps[b]	Error rate (%)[c]	Lab misinterp[d]	Insuf panel[e]	False VWF activity / Ag concordance or discordance[f]			Adjusted error rates[g]		
							All (n/N; %)	VWF:RCo (n/N; %)	VWF:CB (n/N; %)	VWF:Act (n/N; %)	VWF:RCo events (n/N; %)	VWF:CB events (n/N; %)	VWF:Act events (n/N; %)
Mild/ moderate VWF deficient	**2007-1 m, 2009-1 m, 2010-1 m, 2011a-1 m, 2011b-1 m, 2012-1 m, 2013-1 m**	30	334	9.0	5/30 (16.7%)	1/30 (3.3%)	24/30 (80.0%)	19/24 (79.2%)	8/24 (33.3%)	2/24 (8.3%)	19/200 (9.5%)	8/175 (4.6%)	2/86 (2.3%)
Severe VWF deficient	**2009-1 s, 2012-1 s, 2013-1 s**	14	146	9.6	5/14 (35.7%)	1/14 (7.1%)	8/14 (57.1%)	5/8 (62.5%)	2/8 (25.0%)	1/8 (12.5%)	5/97 (5.2%)	2/96 (2.1%)	1/46 (2.2%)
Qualitative defect/ HMW VWF deficient	**2008-2A, 2013-2A, 2007-2B, 2009-2B, 2011-2B**	26	233	11.2	13/26 (50.0%)	5/26 (19.2%)	8/26 (33.3%)	6/8 (75.0%)	1/8 (12.5%)	2/8 (25.0%)	6/136 (4.4%)	1/108 (0.9%)	2/55 (3.6%)
All samples above	**All samples above**	70	713	9.8	23/70 (32.9%)	7/70 (10.%)	40/70 (57.1%)	30/40 (75.0%)	11/40 (27.5%)	5/40 (12.5%)	30/433 (6.9%)	11/379 (2.9%)	5/187 (2.7%)

Notes:
a. Errors defined as per Materials and Methods; essentially, when a laboratory identified a qualitative defect/specific loss of HMW VWF (or types 2A/2B/2M VWD) in quantitative VWF deficient samples, or failed to identify qualitative defect/specific loss of HMW VWF in HMW deficient samples (e.g. false identification of type 1 or 3 VWD).
b. Total interps = total number of interpretations provided by participants for the combined sample set for that sample group.
c. Error rate = total errors/total interps as percentage.
d. Lab misinterp = misinterpretation of laboratory's own data set, which was actually consistent with the sample type presented.
e. Insuf panel - laboratory had an insufficient test panel to make an appropriate interpretation (generally, these labs ran (in addition to FVIII:C) only VWF:Ag or VWF:RCo.
f. False VWF activity / Ag concordance or discordance = false low ratios (i.e. <0.7) in mild/mod VWF deficient samples or false high ratios (i.e. >0.7) in HMW VWF deficient samples.
g. Adjusted error rates = False VWF activity / Ag concordance or discordance / total number of possible events for each test group (i.e., adjusted for numbers of laboratories reporting test results for these tests for each sample set included in data set).
Abbreviations: HMW, high molecular weight; VWD, von Willebrand disease; VWF, von willebrand factor; VWF:RCo, ristocetin cofactor assay for VWF; VWF:CB, collagen binding assay for VWF; VWF:Act, other 'activity' assays for VWF.

(23/48 = 47.9%) identified type 2A or 2B VWD. Three participants identified mild type 1 VWD, and one identified the sample as normal.

Discussion

In this study, a large number of test samples (n = 29) of diverse nature have been cross-laboratory tested by 'real world' laboratories. Despite the large assay variability and occasional outlier results (Fig. 3) most participants reassuringly provided both numerical and interpretation results consistent with the nature of the sample tested. However, some sample types challenged some laboratories, in particular the identification of HMW VWF deficient plasma samples. In our laboratory cohort, only 2/57 (3.5%) laboratories perform multimer analysis, and therefore loss of HMW VWF in these exercises, and likely also in diagnostic practice, is identified using surrogate laboratory markers, most notably the ratio of VWF activity/Ag. Thus, for loss of HMW VWF, all VWF activity assays as normally performed by laboratories (VWF:RCo, VWF:CB, VWF:Act) would be lower than VWF:Ag and yield VWF Activity/Ag ratios below 0.5 to 0.7. Consistent with previous reports from us [15–21,28–30], the VWF:RCo/Ag ratio was in general less able to discriminate HMW VWF deficient plasma samples (i.e., types 2A and 2B VWD) from normal or quantitative VWF deficient (i.e., type 1 VWD) plasma samples compared to the VWF:CB/Ag ratio. False VWF discordance using quantitative VWF deficient samples, and false VWF concordance using HMW VWF deficient samples, was previously associated with use of the VWF:RCo/Ag ratio at rates at least three times that of VWF:CB/Ag [19,20], and this continues currently. The association of higher relative error rates using VWF:RCo compared with VWF:CB was also most recently reported by NASCOLA [25], where overall diagnostic interpretation error rates ranged from 3% for normal samples, to 28% for type 1 VWD, to a staggering 60% for type 2 VWD. Notably, the type 2 VWD samples were identified correctly by all laboratories using VWF:CB/Ag ratios, but by only one-third using VWF:RCo/Ag or VWF:Act/Ag ratios. Much of the error rate associated with use of VWF:RCo seemed to be related to the higher assay variability of VWF:RCo testing compared to other VWF activity assays. Although some of the reported errors may be circumvented should laboratories undertake multimer analysis, multimer analysis *per se* may not protect laboratories from making errors. For example, in the NASCOLA study, 27% of laboratories performed multimer analysis, but VWD diagnostic and VWD typing error rates were higher overall than those reported in the current study. Reasons for this are not entirely clear, but may reflect poor performance in multimer assays or misinterpretation of overall test patterns even when multimers are performed.

Other recent comparative data using various VWF activity assays for identification of VWD is also available [7–10,31], but not in a cross-laboratory setting. Although these previous studies all individually 'validate' various VWF activity assays for assisting the diagnosis of VWD, comparative evaluation in the real world setting is best achieved by cross-laboratory studies. In another recent exercise using samples selectively and sequentially deficient in HMW VWF, the VWF:CB and VWF:RCo were better able to discriminate loss of HMW VWF than the Werfen-IL activity assay [27]. Unfortunately, the higher discriminating ability for VWF:RCo at the 'median' level is sometimes lost due to the high assay variability between laboratories, and therefore occasional failures occur. Cross laboratory data using the newest VWF activity assay, the Siemens Innovance assay [8], is currently lacking, as this assay is only just making inroads into diagnostic practice.

An important aspect of our report is the data using the type 2M VWD sample, which is a difficult form of VWD to identify. Most laboratories in most geographies misidentify type 2M VWD as either 2A or type 1 VWD [1,30–36]. Type 2M VWD is consequently a significantly under-diagnosed disorder. Identification requires evidence of VWF dysfunction not associated with loss of HMW VWF. However, most VWD testing laboratories do not perform multimer analysis [37], leaving the diagnosis to be achieved using surrogate markers.

Even when multimer analysis is performed, false concordance of VWF:RCo/Ag ratios may still lead to misidentification of 2M VWD as type 1 VWD (even in expert laboratories in upwards of 20% of type 1 VWD cases [35]). Most forms of type 2M VWD are platelet binding defect cases identified by relative loss of VWF:RCo (i.e., VWF:RCo/Ag ratio <0.7), but without a major effect on collagen binding (i.e., VWF:CB/Ag ratio >0.7). Despite this pattern being observed with our case (Figs. 3 and 5) most participants still failed to identify this as type 2M VWD. In most cases, this was simply a failure to recognize this pattern, and perhaps reflects a lack of general recognition of type 2M VWD. Thus, most laboratories instead identified the sample as type 2A VWD, of which they are more aware.

Low level sensitivity for VWF detection as reported here is consistent with an earlier report [26]. Although this largely reflects a difficulty in differentially identifying type 3 *vs* severe type 1 VWD, there are also adverse effects on identification of type 2A/2B/2M VWD. For example, when tested in some laboratories, a type 2A, 2B or 2M VWD sample could yield ~20U/dL for both VWF:Ag and VWF:RCo, and therefore be identified as a (moderate) type 1 VWD.

Conclusion

We report on assay and laboratory variability in numerical and interpretative reporting for VWF and VWD using a large number of samples of varying type. The data reflects on the challenges in laboratory identification of VWD and VWD type in real world laboratories, and this will subsequently reflect on the clinical diagnosis of VWD. In most cases, interpretive errors ('misdiagnosis' of VWD or VWD type) are due to performance of insufficient test panels or to misinterpretation of the laboratory's own test data, which may instead be consistent with the sample tested. In regards to a sufficient 'test panel', laboratories should employ a comprehensive panel that comprises FVIII, VWF:Ag, and preferably two independent activity assays. For the latter, the additional combination of VWF:RCo and VWF:CB will in general permit differential identification of types 2A/2B *vs* 2M *vs* 1. Performance of VWF multimers will also assist identification of type 2 VWD. Some workers believe that multimers may be replaced in a screening process by the combination of VWF:Ag, VWF:RCo and VWF:CB [38,39]. This is important as in fact, few laboratories worldwide perform multimer analysis [37], and only a small proportion of laboratories can generate good multimers results. In addition, performance of multimers *per se* will not protect laboratories from diagnostic errors [25,35]. It is hoped that this report highlights the challenges to accurate diagnosis of VWD, and that laboratories and clinicians adopt comprehensive test panels and best test practices, as well as repeat test all patients investigated using a fresh sample to confirm original test findings.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.thromres.2014.05.020.

Conflict of Interest Statement

The authors have no conflicts of interest in relation to this work.

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ORIGINAL ARTICLE *Von Willebrand disease*

Type 2M von Willebrand disease – more often misidentified than correctly identified

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Introduction: Appropriate diagnosis of von Willebrand disease (VWD), including differential identification of qualitative vs. quantitative von Willebrand factor (VWF) defects has important management implications, but remains problematic. **Aim:** The aim of the study was to assess whether 2M VWD, defining qualitative defects not associated with loss of high molecular weight (HMW) VWF, is often misidentified, given highly variable reported frequency ranging from 0 to ~60% of all type 2 VWD. **Methods:** A comparative evaluation of laboratory ability to appropriately identify 2M VWD ($n = 4$) vs. HMW VWF reduction ($n = 4$), as sent to participants of an international external quality assessment programme. **Results:** Laboratories had considerably greater difficulty identifying type 2M VWD, correctly identifying these on average only 29.4% of occasions, with the 70.6% error rate representing use of insufficient test panels (41.7%), misinterpretation of test results (10.0%) and analytical errors (13.3%). One type 2M case, giving a median of 49 U dL^{-1} VWF:Ag, was more often misidentified as type 2A/2B VWD (46.7%) than 2M (34.8%). Another 2M case, giving a median of 189 U dL^{-1} VWF:Ag, was instead often misidentified as being normal (non-VWD) (36.4%), with identifications of type 2A/2B VWD (13.6%) also represented. In comparison, errors in identification of HMW VWF reduced samples only averaged 11.5%, primarily driven by use of insufficient test panels (6.3%) or misinterpretation of results (4.2%) and infrequently analytical errors (1.0%). **Conclusion:** Type 2M VWD is more often misidentified (70.6%) than correctly identified as 2M VWD (29.4%), and potentially explaining the relative under-reported incidence of 2M VWD in the literature.

Keywords: 2M VWD, diagnosic, errors, interpretation, laboratory testing, von Willebrand disease, von Willebrand factor

Background

von Willebrand disease (VWD) is reportedly the most common congenital bleeding disorder; nevertheless, its differential identification and clinical diagnosis remains problematic for many laboratories and clinicians [1,2]. VWD arises from deficiency and/or defects of von Willebrand factor (VWF), an adhesive plasma protein essential for effective primary haemostasis. VWF possesses many functional properties, including binding to platelets via several receptors, most notable glycoprotein Ib, binding to sub-endothelial matrix components (most notably collagen) and binding and protecting factor VIII (FVIII) function [3]. The first two noted functions are essential for overall primary haemostasis and are, respectively, assessed in the laboratory by surrogate tests such as VWF ristocetin cofactor (VWF:RCo) and collagen binding (VWF:CB) [1,2,4,5], although alternative assays may also be employed [6].

The most recent classification scheme from the International Society on Thrombosis and Haemostasis (ISTH) recognizes six different types of VWD [5]. Type 1 represents a partial quantitative VWF deficiency, with VWF essentially functionally normal, but produced in lowered quantity. Type 3 VWD represents

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'complete' deficiency of VWF. Type 2 VWD represents a heterogeneous group of qualitative VWF defects that comprise (i) 2A VWD (loss of high molecular weight (HMW) VWF), (ii) 2B VWD (enhanced functional binding of VWF that leads to loss of HMW VWF and typically mild thrombocytopenia), (iii) 2N VWD (loss of VWF-FVIII binding) and (iv) 2M VWF (VWF dysfunction not associated with loss of HMW VWF). The proper identification of VWD and differentiating its type is important for therapeutic management [7].

Of particular relevance to the current report, type 2A VWD is often represented as being the most common type 2 VWD type, and 2M alternatively represented as being a relatively uncommon form of VWD [1]. Indeed, depending on the centre reporting data, type 2M VWD has been variously reported in the literature as representing between 0 and nearly 60% of all type 2 VWD cases [1]. Rather than this representing a geographically variable incidence, we believe this simply represents a relative difficulty in correctly identifying type 2M VWD in some centres, with this instead being often misidentified as either type 2A or type 1 VWD, depending in part on the test panel being used as well as the expertise of the reporting laboratory [8]. We have tested this hypothesis in this study by assessing the relative correctness of identification of test samples representing type 2M VWD ($n = 4$), vs. a comparator set of test samples showing HMW VWF reduction (2A or 2B VWD cases; $n = 4$), and as sent to participant laboratories of an international external quality assessment (EQA) programme, over the past 6 years.

Methods

Setting

This study derives from the Royal College of Pathologists of Australasia (RCPAQAP) Haematology EQA programme (<http://www.rcpaqap.com.au>). Participants to this VWF/VWD EQA (current $n = 60$) derive from Australia ($n = 33$), New Zealand ($n = 5$), Asia ($n = 13$; Malaysia=9, Hong Kong=4), South Africa ($n = 6$), India ($n = 1$), France ($n = 1$) and Spain ($n = 1$), and generally represent 'real-world' laboratories investigating VWD for clinical diagnosis and management; however, most would not be considered 'expert laboratories' (defined here as a laboratory regarded by others as having a particular expertise in the field of VWF/VWD testing; generally, these laboratories are associated with academic teaching hospitals or haemophilia centres).

Samples

Table 1 details the samples ($n = 8$) distributed to EQA participants for the current report. These samples comprised a set of four type 2M VWD samples derived from two separate well characterized patients with type 2M VWD (sent as duplicates in separate years), and a comparator set of four HMW VWF reduced samples. One of the type 2M VWD samples (a c.3965A>C *VWF* mutation, with p.His1322Pro) presented with VWF:Ag ~50 U dL^{-1} (plus VWF:RCo/Ag <0.3, but with no loss of HMW VWF), whereas the other (a c.3974C>T *VWF* mutation, with p.Ser1325Phe, homozygous), presented with a VWF:Ag level of ~190 U dL^{-1} (again plus VWF:RCo/Ag <0.3, and with no loss of HMW VWF). These two cases would therefore reflect different challenges to correct VWD subtype; we hypothesized that the first 2M case, reflecting a 'text-book' 2M VWD-case, would be often misidentified as a type 2A-VWD (or similar) sample. On the other hand, the latter 2M VWD case might instead be missed as a VWD case, given the high VWF:Ag level, and depending on the participant test panels performed, plus the resultant laboratory test panel interpretations.

The comparator set comprised four HMW VWF reduced samples, including one well characterized type 2B VWD case (*VWF* mutation (c.3916C>T; p.Arg1306Trp), and two samples that were purpose generated to express reduction in HMW VWF, and similar to those previously reported by this EQA [9,10]. These samples are produced by a propriety process of disulphide bond reduction with N-acetylcysteine, similar to that recently described by Chen

Table 1. Summary of samples utilized for current study.

Sample type	Sample represents	Sample ID*	Sample source	Details
HMW deficient	2B VWD	2009-2B, 2011-2B	Patient sample	Well-characterized 2B VWD; *VWF* mutation (c.3916C>T (p.Arg1306Trp)); VWF:Ag ~40 U dL^{-1}; with loss of HMW VWF and RCo/Ag and CB/Ag <0.3
	2A VWD'	2013-2A	Prepared in house	Type 2A mimic; target ~30 U dL^{-1} VWF:Ag; with loss of HMW VWF and RCo/Ag and CB/Ag <0.3
		2015-2A	Prepared in house	Type 2A mimic ('pregnancy'); target ~80 U dL^{-1} VWF:Ag; with loss of HMW VWF and RCo/Ag and CB/Ag <0.3
Platelet binding defect VWD	2M VWD	2012-2M, 2014-2M(a)	Patient sample	Well characterized 2M VWD; *VWF* mutation (c.3965A>C (p.His1322Pro)); with no loss of HMW VWF and VWF:Ag ~50 U dL^{-1}, RCo/Ag <0.3 and CB/Ag >0.7
		2014-2M(b), 2015-2M	Patient sample	Well characterized 2M VWD; *VWF* mutation (c.3974C>T (p.Ser1325Phe)); with no loss of HMW VWF and VWF:Ag ~190 U dL^{-1}, RCo/Ag <0.3 and CB/Ag >0.7

HMW, high molecular weight; VWD, von Willebrand disease; VWF, von Willebrand factor; RCo, ristocetin cofactor; CB, collagen binding.
*Samples listed in the same cell indicate repeated despatches of the same plasma sample in different years.

et al. [11]. Such samples generate VWF and FVIII test results and VWF multimer patterns that are very similar to true type 2A VWD samples. Although patient samples are preferred in research evaluations, within an EQA setting it is important to balance this desire against ethical and logistical challenges centred on regular collections of VWD-affected patients. In-house prepared material also carries specific design advantages related to preferred specifications. Thus, for this study, one such sample was prepared with a VWF:Ag level of ~30 U dL^{-1} and was meant to mimic a standard type 2A VWD-like sample, whereas another was prepared with a VWF:Ag level of ~80 U dL^{-1}, and was meant to mimic a type 2A VWD-like sample as might be derived from a pregnant woman [12]. In pregnancy, VWF:Ag levels can normalize in type 2A VWD, but HMW VWF and VWF activity by assays such as VWF:RCo, do not rise in unison [12]. As noted, the two other HMW VWF reduced samples derived from a single well-characterized type 2B VWD patient, sent as a duplicate sample in separate years. These four samples would also potentially reflect different challenges to correct VWD (2A/2B) subtype identification, given the different levels of VWF:Ag.

All samples sent to participants were buffered lyophilized plasma, and tested blind by laboratories. All samples passed local stability and homogeneity testing to ensure sample integrity (data not shown). Information on stability and homogeneity testing is available on request. VWF multimers and densitometry patterns for most of the samples are shown in Fig. 1, and were consistent with expectations (i.e. no HMW-VWF reduction in the four dispatched 2M VWD samples, but clear HMW-VWF reduction in the comparator four 2A/2B VWD cases).

Data analysis

All laboratories report numerical values to the RCPA-QAP Haematology for the tests performed, and these are analysed using descriptive statistics [median, ranges, intra-method or inter-laboratory coefficient of variation (CV%), etc.]. Although participants to this EQA also perform FVIII testing, this report focuses on VWF assays and test results, given the type of VWF samples assessed herein. In this study, we have not distinguished between different collagen binding or ristocetin cofactor methods, since there was no

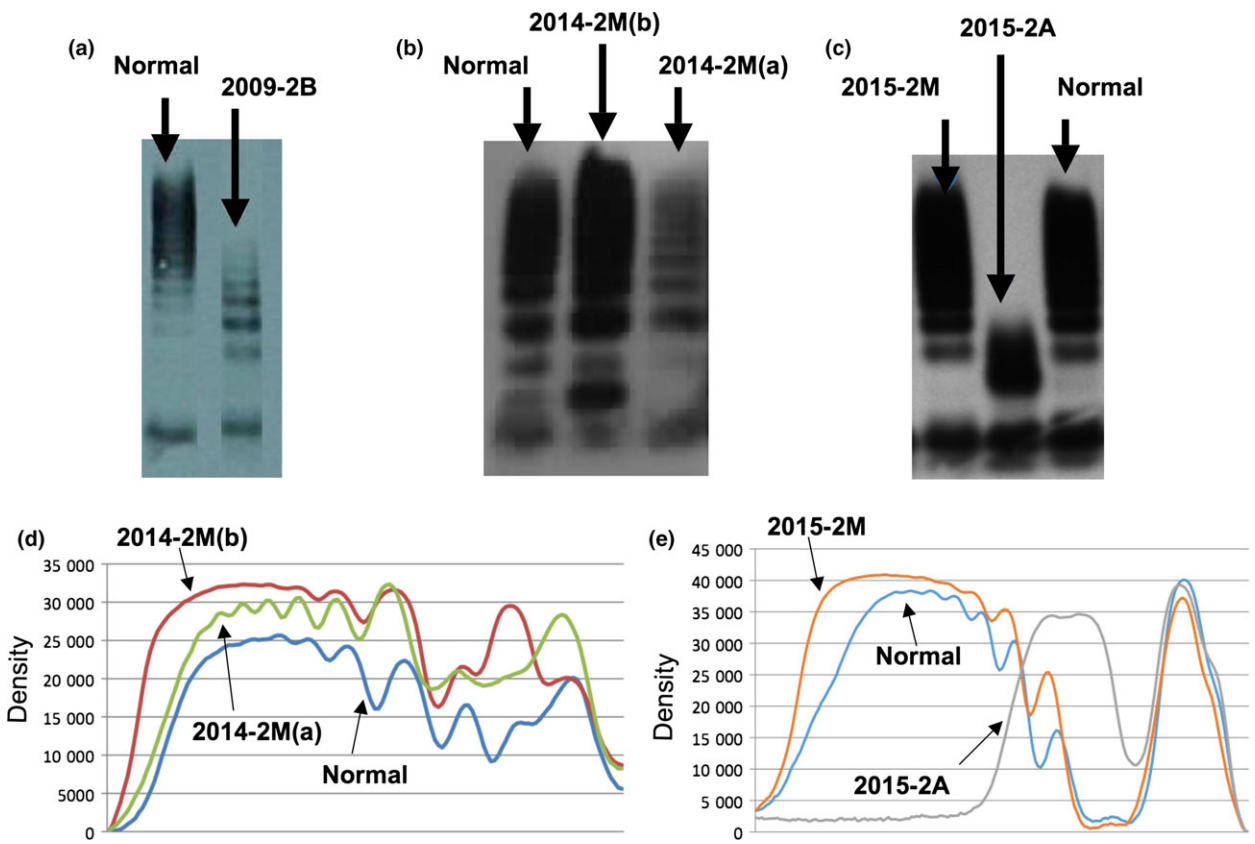


Fig. 1. Sample von Willebrand factor (VWF) multimer gels (a–c) and densitometry (d, e) results for representative samples reported in this study, including normal, high molecular weight (HMW) VWF reduced (2009-2B, 2015-2A; a, c, e), and type 2M VWD (2014-2M(b), 2015-2M; b, c, d, e). For densitometry figures, relative density is shown on the *y*-axis, and relative front on *x*-axis, including the dye front at the extreme right. Normal samples are shown as reference samples; the type 2M VWD samples retain all molecular weight forms of VWF; the HMW VWF reduced samples show a reduction in HMW forms.

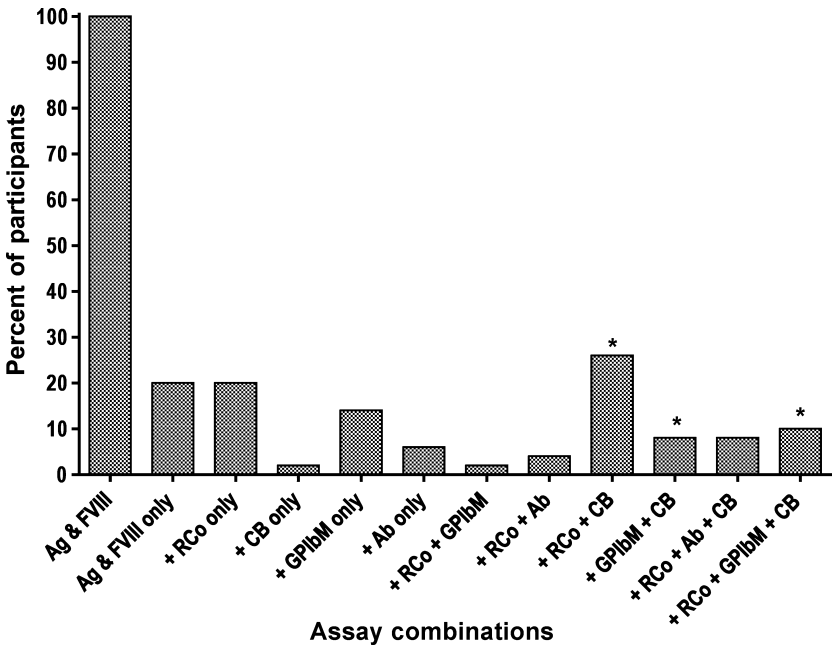


Fig. 2. Percentage of RCPAQAP Haematology participants currently performing various von Willebrand factor (VWF) assays and test panels. Note that all (i.e. 100% of) laboratories perform VWF:Ag (Ag) and factor VIII:C (FVIII) testing, but that performance of additional ('+') VWF activity assays and consequent test panels varies. RCo, ristocetin cofactor; CB, collagen binding; Ab, IL VWF activity assay; GPIbM, Siemens Innovance activity assay. Asterisks identify test panel combinations showing fewest errors in identification for the sample set reported in this study.

evidence of any differential patterns based on these different methods. We have, however, differentially assessed data reported for the Werfen-IL latex-immuno assay (LIA) monoclonal antibody based method ('VWF:Ab'), or (from 2013) the Siemens Innovance recombinant platelet glyoprotein I (rGPIb)-binding LIA method ('VWF:GPIbM') (using the recent nomenclature proposed by the International Society on Thrombosis and Haemostasis (ISTH) Scientific and Standardization Committee (SSC); [13]).

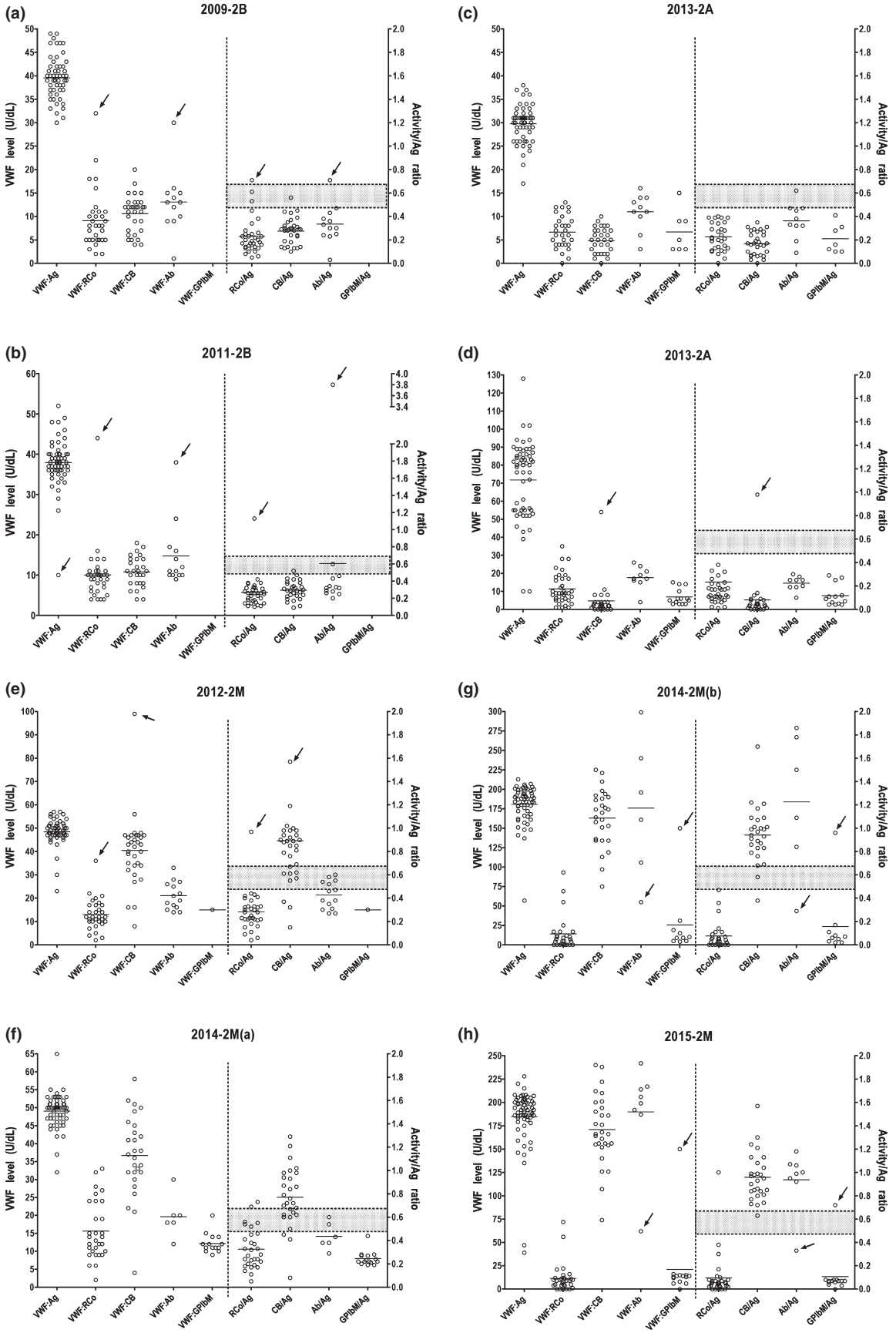
Laboratories are also encouraged to interpret overall test findings for the likelihood or otherwise of 'VWD', as well as potential 'VWD type' if applicable. It is well recognized that diagnosis of VWD requires more than laboratory testing, and some samples used are not derived from true VWD patients. Nevertheless, such interpretation exercises are valid within the context of EQA, and in-house prepared HMW-VWF reduced samples provide similar FVIII and VWF test results to true 2A VWD patient samples, and thus should be identified as representing HMW-VWF reduction (or '2A/2B VWD') using the tests performed by these laboratories. The selection choices provided to laboratories for interpretation comprise: normal/not VWD, type 1 VWD, type 2A or 2B VWD, type 2M VWD, type 2N VWD, type 3 VWD and other (specify). Laboratory interpretations reported to our EQA were assessed in the context of the sample provided, for both the variety of different interpretations as well as for 'errors' in interpretation. For the current report, an error in VWD type identification was defined to occur when the laboratory failed to identify a HMW-VWF reduced sample as either a type 2A or 2B VWD sample (from the choices available as above) or when a laboratory failed to identify the type 2M VWD cases as being 2M VWD. It is recognized that misidentification of type 2M VWD cases as 2A VWD may reflect a relatively non-serious misidentification in clinical practice, given that these cases are managed similarly [8,14]; however, we were interested in identifying how often laboratories correctly identified 2M VWD vs. how often this diagnosis was missed.

Results

Participant numbers and test methodologies

Numbers of RCPAQAP participants have varied only slightly during the period of analysis (n = 52–60). All participants (i.e. 100%) perform VWF:Ag and FVIII:C testing, but thereafter the test makeup differs substantially between laboratories (Fig. 2). A large proportion

Fig. 3. Numerical data returned by participants for all samples from current study, and as summarized in Tables 1 and 2. HMW-VWF reduced samples shown in (a–d), and type 2M VWD samples shown in (e–h). Figures show data for all VWF assays (VWF:Ag (antigen), VWF:RCo (ristocetin cofactor), VWF:CB (collagen binding), VWF:Ab (IL VWF activity assay), VWF:GPIbM (Siemens Innovance VWF activity assay), with VWF level shown on y-axis (U dL^{-1}); assays shown on x-axis and activity/Ag ratios on right y-axis. The shaded area defines the cut-off region for discrimination of type 2 (qualitative) defects, which may differ according to laboratory and test methodologies used, but which tends to be between 0.5 and 0.7. Arrowed values indicate representative outlier values, representing analytical error, potential transcription error, or incorrect assay type reported to EQA. For example, (a, b, d, e): high outlier VWF:RCo, VWF:CB and VWF:Ab values here likely reflect analytical error; in contrast, (g, h) arrowed outliers for VWF:Ab and VWF:GPIbM may reflect participants incorrectly identifying their VWF activity assays to our EQA.



of laboratories (~60%) still perform VWF:RCo testing, although many have now replaced this test with alternate VWF activity assays (in particular either the Werfen-IL LIA monoclonal antibody based method ('VWF:Ab'), or (from 2013) the Siemens Innovance rGPIb-binding LIA method ('VWF:GPIbM')). VWF:CB testing is also performed by ~60% of participant laboratories. However, as shown in Fig. 2, the actual composite test panels currently used vary widely.

Numerically reported values

This is shown in Fig. 3 and summarized in Table 2. Occasional identified outlier data may reflect random error events, transcription errors, and occasionally systematic error (e.g. potentially reflecting problems with a common assay calibrant standard if a laboratory reports outliers for several tests). Nevertheless, in general, the reported test findings were consistent with the sample type being presented to laboratories. Thus, the HMW-VWF reduced samples showed a general reduction in all VWF activity/VWF:Ag ratios (all generally <0.5), reflecting similar sensitivities of all VWF activity assays to HMW-VWF-reduction. However, the type 2M VWD cases showed a different test pattern, with low RCo/Ag ratios, but more variable test patterns with other VWF activity/Ag ratios. Thus, CB/Ag ratios were normal (i.e. >0.7) for one 2M VWD case, and in general higher and closer to normal values than RCo/Ag for the other 2M VWD case. The Siemens Innovance VWF:GPIbM/Ag ratios followed those for RCo/Ag, whereas the IL VWF:Ab/Ag ratios showed discrepancies between the two represented 2M VWD cases.

Test result interpretation and VWD type identification

This is highlighted in Figs 4 and 5. Most participants correctly identified the HMW-VWF reduced samples as being 2A/2B-VWD-like (viz, correctly identified on 88.5% of occasions; Table 2). Overall, then, errors in identification of these HMW VWF reduced samples averaged 11.5% (Table 2), and were primarily driven by laboratories utilizing insufficient test panels (6.3% contributory error rate) or misidentification of their own test panel results (which as reported were actually consistent with a HMW-VWF reduced sample; 4.2%), and infrequently due to analytical errors (1.0%). In contrast, laboratories had considerably greater difficulty in identifying type 2M VWD. In total, laboratories correctly identified type 2M VWD in only 29.4% of occasions, with the average 70.6% error rate represented by insufficient test panels (41.7% contributory error rate), misidentification of participant's own test panel results (10.0%) and analytical errors (13.3%). As expected, the two different 2M VWD cases lead to different interpretation challenges. The case giving a median of ~50 U dL^{-1} VWF:Ag was more often misidentified as a type 2A/2B VWD sample (46.7%) than 2M (34.8%), with type 1 VWD (14.1%) also represented. The other 2M VWD case, giving a median of ~190 U dL^{-1} VWF:Ag, was instead often misidentified as being normal (non-VWD) (36.4%), with identifications of type 2A/2B VWD (13.6%) less often represented.

Overall, fewer errors in interpretation were identified from laboratories using comprehensive test panels including GPIb and collagen binding compared to those using only one or other of GPIb and collagen binding. For the 2M VWD case with ~50 U dL^{-1} VWF:Ag, there were a total of 92 interpretative events, with near equal numbers of events for those performing both types of tests (n = 45) vs. those only performing one type of test (n = 47). Of these, 54.8% of those performing both types of tests correctly identified 2M VWD, compared to only 8.9% of those performing only one type of test. For the 2M VWD case with ~190 U dL^{-1} VWF:Ag, there were a total of 88 interpretative events, again with near equal numbers of events for those performing both types of tests (n = 45) vs. those only performing one type of test (n = 43). Of these, 66.8% of those performing both types of tests identified 2M VWD, compared to only 25.6% of those performing only one type of test. For this sample, participants performed better with the second sample dispatch, perhaps learning from the first dispatch and subsequent EQA report. Thus, for the first dispatch, 50.0% of those performing both types of tests correctly identified 2M VWD, compared to only 15.8% of those performing only one type of test, whereas for the second dispatch the respective values were both higher at 82.6% and 33.3%.

Reproducibility of test results using repeat test samples

As shown in Table 2 and Fig. 3, there was good reproducibility in test data for identical samples dispatched in different years, both for HMW-VWF reduced (type 2B VWD) samples and type 2M VWD samples. Thus, returned numerical data showed similar median and ranges of VWF test data, again highlighting some robustness in analytical test results. Interestingly, however, although the interpretations and VWD type identification were similar in different dispatches, they were not identical (Fig. 4). For one type 2M VWD case (VWF:Ag ~50 U dL^{-1}), the initial sample yielded a high level of misidentification as a HMW-VWF reduced (2A/2B VWD) sample, whereas the repeat testing showed a similar error rate but a higher level of misidentification of this samples as type 1 VWD. The second 2M VWD case (VWF:Ag ~190 U dL^{-1}) was initially misidentified primarily as a non-

Table 2. Median test values and summary error rates for study samples.

Sample type	Sample sets	Sample information	Median VWF: Ag	Median VWF: RCo	Median VWF: CB	Median VWF: Ab	Median VWF: GPIbM	Median RCo/Ag	Median CB/Ag	Median Ab/Ag	Median GPIbM/Ag	Total errors*	Total interps†	Error rate (%)‡	Analytical issue (*n*; %)§	Insufficient panel¶	Participant misinterpretation**
HMW VWF deficient samples	2009-2B	2B VWD; *VWF* mutation (c.3916C>T; p.Arg1306Trp)	**40**	**8**	**12**	**13**	**N/A**	**0.19**	**0.29**	**0.32**	**N/A**	2	46	4.4	0	1 (2.2)	1 (2.2)
	2011-2B	Repeat of above sample	**38**	**10**	**11**	**11**	**N/A**	**0.26**	**0.29**	**0.32**	**N/A**	3	49	6.1	2 (4.1)	0	1 (2.0)
	2013-2A	HMW reduced 2A-VWD like	**30**	**6**	**5**	**11.5**	**5**	**0.21**	**0.17**	**0.36**	**0.16**	4	48	8.4	0	3 (6.3)	1 (2.1)
	2015-2A	HMW reduced; 'pregnant' 2A-VWD like	83	**9**	**4**	**18**	**5.5**	**0.11**	**0.04**	**0.25**	**0.04**	13	49	26.5	0	8 (16.3)	5 (10.2)
	All HMW VWF reduced samples above											**22**	**192**	**11.5**	**2 (1.0)**	**12 (6.3)**	**8 (4.2)**
Type 2M VWD samples	2012-2M	2M VWD; *VWF* mutation (c.3965A>C; p.His1322Pro)	**48**	**12**	**43**	**19**	NA	**0.28**	0.89	**0.39**	NA	27	49	56.0	3 (6.1)	18 (36.7)	6 (12.2)
	2014-2M (a)	Repeat of above	**50**	**14**	**37**	**19**	**11**	**0.3**	0.74	**0.41**	**0.22**	35	43	81.4	7 (16.3)	21 (48.8)	7 (16.3)
	2M VWD (c.3965A>C; p.His1322Pro) samples above combined											***62***	***92***	***67.4***	***10 (10.9)***	***39 (42.4)***	***13 (14.1)***
	2014-2M (b)	2M VWD; *VWF* mutation (c.3974C>T; p.Ser1325Phe; homozygous)	187	**7**	167	178.5	**9.5**	**0.04**	0.94	1.29	**0.07**	29	41	70.7	7 (17.1)	18 (43.9)	4 (9.8)
	2015-2M	Repeat of above	192	**9**	166	202.5	**14**	**0.05**	0.97	1.00	**0.08**	26	47	55.3	7 (14.9)	18 (38.3)	1 (2.1)
	2M VWD (c.3974C>T; p.Ser1325Phe; homozygous) samples above combined											***65***	***88***	***73.9***	***14 (15.9)***	***36 (40.9)***	***5 (5.7)***
	All 2M-VWD samples above											**127**	**180**	**70.6**	**24 (13.3)**	**75 (41.7)**	**18 (10.0)**

HMW, high molecular weight; VWD, von Willebrand disease; VWF, von willebrand factor; Ag, antigen; RCo, ristocetin cofactor; CB, collagen binding; Ab, IL Activity assay; GPIbM, Siemens Innovance activity assay.

*Errors defined as per Methods; essentially, when a laboratory failed to identify qualitative VWF defects correctly; viz, failed to identify a 2M VWD as 2M VWD or failed to identify a HMW VWF reduced sample as either a 2A or 2B VWD-like sample.

†Total interps = total number of interpretations provided by participants for the (combined) sample set for that sample group.

‡Error rate = total errors/total interpretations as percentage.

§Analytical problem = when the test result for a given assay yielded a false normal result (when the median reported result was abnormal) or yielded a false abnormal result (when the median reported result was normal).

¶Laboratory performed an insufficient test panel to make an appropriate interpretation (generally, these labs only performed VWF:Ag and FVIII:C assays, or VWF:Ag and FVIII:C and VWF:RCo assays, or VWF:Ag and FVIII:C and VWF:CB assays).

**Participant misinterpretation of their own data set; i.e. the data set reported by the participant was actually consistent with the sample type provided, and not the interpretation they reported.

Bold text for median test values indicate abnormal test results. Bold text for summary errors indicates data for combined sample sets (either HMW VWF deficient samples or 2M VWD samples), with italic bold indicating data for combined sample set pairs (i.e., same sample sent in different surveys).

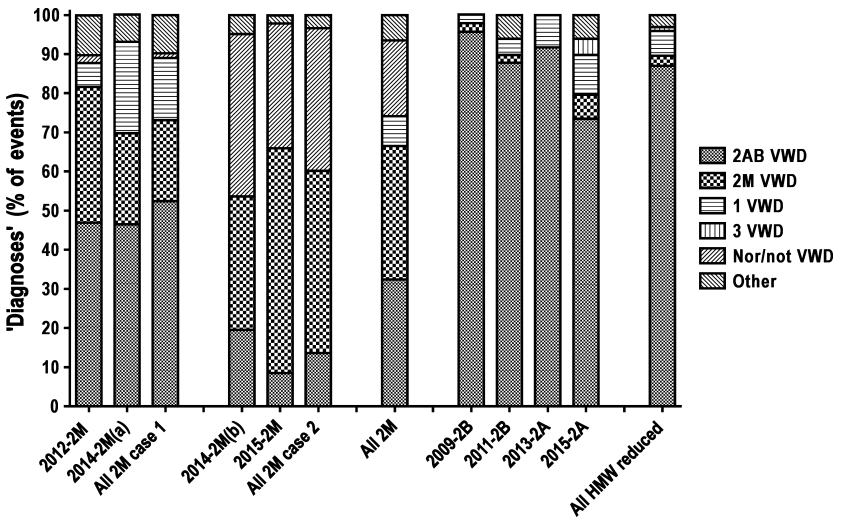


Fig. 4. Participant interpretations ('VWD diagnoses') for the sample set reported in this study. Other comprised occasional responses of 'equivocal', haemophilia, or type 2N VWD.

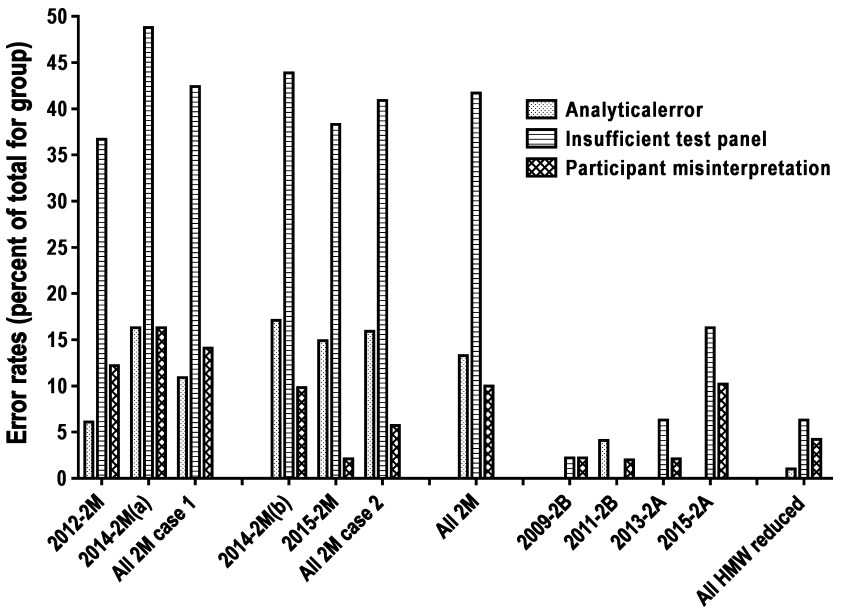


Fig. 5. Error rates for the sample set reported in this study, according to error type. Insufficient test panels and participant misinterpretations accounted for far more errors in VWD type identification than analytical errors.

VWD sample, and although the repeat testing identified fewer overall errors, these were still predominantly that of a failure to identify VWD.

Discussion

This study was focused on a comparative evaluation of HMW-VWF reduced samples ('type 2A or 2B-like VWD') vs. type 2M VWD to address the question of whether 2M VWD is a rare type of VWD, as largely reported in the literature [1], or alternatively (as we hypothesized) might just not be identified correctly by many laboratories [8]. Four plasma samples representing repeat dispatches of two different cases of 2M VWD identified a high error rate of VWD misidentification, as either being 2A/2B-like (i.e. 'HMW-VWF reduced') or not VWD. In our EQA only 2/60 (3.3%) laboratories currently perform multimer analysis; thus, HMW-VWF reduction (or identification of samples as 2A/2B-VWD like), is achieved using surrogate laboratory markers, most notably the ratio of VWF activity/Ag. This is likely to also be the case worldwide, as more laboratories do not perform multimer analysis than do perform multimer analysis [15]. Thus, data from other EQAs indicate VWF multimers are only performed by 1–20% of VWF test laboratories, depending on the reporting EQA [15].

In HMW-VWF reduced samples (i.e. 2A or 2B VWD), a low VWF activity/Ag ratio would be expected using all standard VWF activity assays (i.e. VWF:RCo, VWF:CB, VWF:Ab and VWF:GPIbM), and reflecting similar sensitivity of these assays for HMW-VWF. This

was in general observed in our study, although the RCo/Ag, CB/Ag and GPIbM/Ag ratios appeared to show better sensitivity to HMW-VWF reduction than the Ab/Ag ratio (Table 2 and Fig. 3). That the VWF:CB and VWF:RCo are better able to discriminate HMW-VWF reduction than the Werfen-IL VWF:Ab assay has also been previously reported by us [9,10].

For type 2M VWD, the relative sensitivity of different VWF activity assays (i.e. VWF:RCo, VWF:CB, VWF:Ab and VWF:GPIbM) would instead reflect their ability to identify structural changes in VWF as represented by the specific *VWF* mutation. For the type 2M VWD case representing a heterozygous c.3965A>C *VWF* mutation (p.His1322Pro), the VWF:CB (and thus the CB/Ag ratio) was least sensitive, and would thus identify this case as a mutation that primarily affects the VWF-platelet GPIb binding domain (and not VWF-collagen binding). Nevertheless, the IL VWF:Ab (and thus the Ab/Ag ratio) was also relatively less sensitive to this mutation than the VWF:RCo (and RCo/Ag) and Siemens VWF:GPIbM (and GPIbM/Ag ratio) (Fig. 3). For the type 2M VWD case representing a homozygous c.3974C>T mutation (p.Ser1325Phe) [16], the VWF:CB (and CB/Ag ratio) was also again insensitive, and thus also identifying this case as a mutation that primarily affects the VWF-platelet GPIb binding domain (and not the VWF-collagen binding domain). In this case, both VWF:RCo (and RCo/Ag) and VWF:GPIbM (and GPIbM/Ag) were similarly sensitive to the mutational effect (Fig. 3). However, the IL VWF:Ab assay failed to identify the mutational change, and yielded normal Act/Ag ratios with this sample. We believe that this is at least the third reported type 2M VWD case that this assay has failed to identify [17,18]. One of these previous cases was not specifically reported, but merely shown as a discrepancy in VWF:RCo (nearly 0 U dL^{-1}) vs. the IL VWF:Ab at ~70 U dL^{-1} [17]. The other 'case' was that of a family expressing a p.Gly1324Ala change in VWF, where the VWF:Ab was either similar to VWF:Ag or where this resulted a normal value [18,19]; here, the cases of 2M VWD might have been missed as VWD (where VWF levels were normal), or else identified as type 1 VWD (where VWF levels were low).

In conclusion, this study has identified that for the cases reported here, type 2M VWD is more often misidentified (70.6% of interpretations) than correctly identified as 2M VWD (29.4%), and this therefore potentially explains the relative under-reported incidence of type 2M VWD in the literature. In the Westmead-based laboratory, we have previously identified type 2M VWD as potentially being as common as type 2A VWD [20]. A few other laboratories, either in Australia or Italy, have reported a similar incidence of Type 2M VWD (at least 50% of type 2VWD cases) based on their own experience [21–23]. In contrast, most centres (including some expert centres) report type 2M VWD as being a relatively uncommon Type 2 VWD disorder [1], instead 'preferentially' identifying a high proportion (>80%) of type 2 cases as being type 2A. We believe that this simply reflects a 'reporting bias' to identification of qualitative (type 2) VWD cases reflecting low VWF activity/Ag ratios as being due to reduction in HMW-VWF, whether or not HMW VWF is actually reduced, and because most laboratories do not perform multimer analysis. As VWF:RCo is the most commonly employed VWF activity assay, and this will yield low RCo/Ag ratios with most 2A, 2B and 2M VWD cases, many laboratories may simply assume that if a low RCo/Ag ratio is obtained, that this reflects HMW-VWF reduction (rather than a VWF dysfunction per se). A similar finding would be expected if laboratories performed the Siemens Innovance activity assay (VWF:GPIbM), either instead of VWF:RCo, or in addition to VWF:RCo.

Although many might therefore argue that this then validates the need for greater performance of VWF:-multimers, we do not believe that this would provide a 'panacea' to this issue, given the high error rate otherwise associated with this test. Thus, in one recent study, NASCOLA (North American Specialized Coagulation Laboratory Association) reported an overall average error rate of 15% associated with VWF multimer performance, with 5% of laboratories reporting a loss of HMW VWF in normal samples, 18% reporting a loss of HMW VWF in type 1 VWD samples, and 18% reporting a normal multimer pattern in HMW reduced type 2 VWD [24]. More concerning was an ECAT (External quality Control of diagnostic Assays and Tests) report [25], which associated VWF multimers with a 23% error rate for normal samples and an up to 52% error rate in type 1 VWD.

The experience of the RCPA QAP, and of the Westmead laboratory, therefore reiterates the value of the VWF:CB in helping to identify and discriminate type 2 VWD. Essentially, a low RCo/Ag plus low CB/Ag ratio, reflective of combined discordant VWF activity and antigen levels, most likely reflects a loss of HMW VWF. In contrast, low RCo/Ag but normal CB/Ag ratio is incompatible with a loss of HMW VWF and must be a type 2M VWD. This strategy presumes both the VWF:RCo and VWF:CB assays in use to be sensitive to loss of HMW VWF, and laboratories should confirm this to utilize this strategy. The Westmead laboratory knows this to be the case for our methodology, based on extensive assay characterization [26–28]. In our extended experience, this differential is further accentuated in DDAVP testing [29]. Moreover, addition of the VWF:CB to the VWD diagnostic panel consistently reduces diagnostic errors according to previous data from the RCPA QAP [10,30,31]. The

Siemens Innovance VWF:GPIbM assay provides data that is broadly similar to VWF:RCo [32].

We also caution on the use of the IL monoclonal antibody based VWF activity assay (VWF:Ab), as it may fail to identify some type 2M *VWF* mutations, with the case reported here being at least the third such case reported in the literature. We do acknowledge, however, that this may be a relatively rare event, given that others reporting their experience with this assay did not report such discrepant behaviour [33–35]. On the other hand, the IL VWF activity assay (VWF:Ab) also appears to be relatively less sensitive to HMW-VWF reduction than the other commonly utilized VWF activity assays (VWF:CB, VWF:RCo, VWF:GPIbM) (current report and [9,10]).

We acknowledge some limitations with our study. In particular, although this study comprises a comparative cross laboratory evaluation of two distinct and well characterized type 2M VWD cases against HMW VWF reduced samples, including a well characterized type 2B case sent twice, the 'type 2A' comparators comprised artificially prepared samples. This permits us to circumvent ethical concerns regarding unnecessary collection of patient samples, recognizing the volume of plasma required for EQA use. In addition, generation of 2A VWD 'mimic' samples facilitates specific design characteristics, such as a 'pregnancy' like sample in the current report. Moreover, such samples can be shown to provide laboratory test data for VWF:Ag and activity, as well as FVIII and VWF multimers, that is similar to real 2A VWD samples. Nevertheless, these are not real cases and this can raise concerns over conclusions. On the other hand, the error rates in identifying these artificial samples as 2A-like were comparatively low in comparison with the error rates associated with identifying 2M VWD samples as 2M VWD (Table 2), and the 'text book' 2 VWD like sample (2013-2A) yielded similar error rates to the 2B VWD cases reported here, as well as reported historically [10,31].

Some may also question the ultimate significance of some of our findings; in particular, what is the clinical significance if 2M VWD is instead identified as either 2A (or even type 1 VWD for that matter)? In essence, all such VWD cases may be given a DDAVP trial and may or may not show sufficient responsiveness for a particular clinical purpose (e.g. minor surgery); should DDAVP responsiveness not be sufficient, then all subtypes will be given VWF factor concentrates, essentially at the same 'dose', to achieve the 'same' target levels of FVIII and VWF:RCo. Moreover, additional therapeutic interventions (e.g. tranexamic acid) will also be similarly applied to both types of VWD [14,36]. On the other hand, the current classification scheme [5] distinguishes 2M VWD as a distinct qualitative subtype, and there are good reasons for this. Most importantly, although both 2M and 2A VWD are currently treated similarly, current practice may not reflect the best possible clinical practice. There is some evidence, for example, that 2A and 2M VWD may reflect different severity disorders, just as haemophilia A and B are now reported to reflect [8]. Also, with future formulations of recombinant (r) VWF, it may be possible to treat patients according to the concept of personalized therapy, and this will only progress if laboratories and clinicians better recognize the differential that 2A and 2M VWD may represent. Thus, for 2A VWD, an enriched HMW rVWF may become available, whereas for 2M VWD, a different approach may eventuate [37]. Better differential diagnosis of 2A vs. 2M VWD will also enable development of evaluable patient cohorts that will in turn permit better identification of differential clinical symptoms, as well as justifying future advances in rVWF development, therapy and personalizing patient therapy. At the moment, the lack of distinction of 2M VWD by most laboratories will thwart such possible future therapy landscapes, and currently causes a 'melding' of VWD types that in itself obscures the clinical differentials.

Finally, the United Kingdom Haemophilia Centre Doctors Organization/British Committee for Standards in Haematology recommend that diagnosis of VWD be undertaken using both GPIb binding and collagen binding assays [38], useful for the discrimination of type 2M VWD, but also for minimizing errors in diagnosis of other VWD types, including 2A, 2B, 1 and 3 VWD [10,30,31]. Our findings are in full support of these recommendations.

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Author contributions

E. J. Favaloro prepared some samples for this study, performed sample testing, undertook data analysis and wrote the original draft of the paper. R. A. Bonar undertook some data analysis, and assisted with sample distribution logistics. M. Meiring performed sample testing including multimer analysis and densitometry. S. Mohammed performed sample testing and some data analysis. J. Sioufi and K. Marsden are involved in the logistical and supportive aspects of the RCPAQAP Haematology VWF/VWD EQA programme. A. Arbelaez, J. Niemann and R Freney were involved in the work up for, and provided, one of the 2M VWD cases utilized in this study. All authors were involved in manuscript preparation and approved the final version.

Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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Potential supplementary utility of combined PFA-100 and functional von Willebrand factor testing for the laboratory assessment of desmopressin and factor concentrate therapy in von Willebrand disease

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We performed a retrospective audit of cross-laboratory testing of desmopressin and factor concentrate therapy to assess the potential utility of supplementary testing using the PFA-100 with functional von Willebrand factor (VWF) activity testing. Data were evaluated for a large number of patients with von Willebrand disease of type 1, type 2A or type 2M, as well as a comparative subset of individuals with haemophilia or carriers of haemophilia. Laboratory testing comprised pre and postdesmopressin, or pre and postconcentrate, evaluation of factor VIII, VWF antigen (VWF:Ag) and VWF ristocetin cofactor activity as traditionally performed, supplemented with collagen-binding (VWF:CB) testing and PFA-100 closure times. In brief, both therapies tended to normalize VWF test parameters and closure times in individuals with type 1 von Willebrand disease, with the level of correction in closure times related to the level of normalization of VWF, particularly the VWF:CB. However, although occasional correction of closure times was observed in patients with type 2A or type 2M von Willebrand disease, these did not in general normalize PFA-100 closure times either with desmopressin or factor concentrate therapy. In these patients, improvement in closure times was more likely in those in whom VWF:CB values normalized or when VWF:CB/VWF:Ag ratios normalized. This study confirms that there is a strong relationship between the presenting levels of plasma VWF and PFA-100 closure times, and that the supplementary combination of PFA-100 and VWF:CB testing might provide added clinical utility to current broadly applied testing strategies limited primarily to VWF:Ag, VWF ristocetin cofactor and factor VIII:coagulant. Future prospective investigations are warranted to validate these relationships and to investigate their therapeutic implications. ***Blood Coagul Fibrinolysis*** **20:475–483 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Von Willebrand disease (VWD) is the most common inherited bleeding disorder and is characterized by low levels, or abnormal function, of the plasma protein von Willebrand factor (VWF). Six types of VWD have been defined, namely types 1, 2A, 2B, 2M, 2N and 3 [1–3]. A number of recent guidelines [4–7] have been published on the diagnosis and management of VWD. Management typically comprises either desmopressin (DDAVP; Aventis Pharmaceuticals, Inc., Bridgewater, New Jersey, USA) or VWF/factor VIII (FVIII) concentrate therapy depending on the VWD type and the individual patient response. In brief, DDAVP therapy is typically utilized for most cases of type 1 VWD and for a subset of patients with type 2A, 2M or 2N VWD. As it is difficult to predict individual DDAVP responsiveness, it is a standard practice to undertake a DDAVP trial. VWF/FVIII concentrate therapy is used for patients unresponsive to DDAVP, or in whom DDAVP is thought to be contraindicated, or in patients with prolonged therapeutic need. Irrespective of the type of therapy, current recommendations suggest monitoring of therapy primarily using the ristocetin cofactor (VWF:RCo) assay and/or the FVIII coagulant (FVIII:C) assay [3–7], sometimes supplemented by testing of VWF antigen (VWF:Ag).

Recent international attention has begun to focus on the collagen-binding assay (VWF:CB) as a supplementary test of VWF activity [2,3] and also on the PFA-100 (Dade-Behring, Sydney, Australia), a simple point of care instrument utilized to assess primary haemostasis and sensitive to both VWD and its associated therapy [8].

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Some of us have previously reported on the potential added utility of the VWF:CB and the PFA-100 for monitoring therapy in VWD using a small number of patients [9]. To our knowledge, this has only been effectively evaluated by one other group [10–13], which most recently reported a large study in VWD, with differential findings according to VWD type. In the current study, we have further and independently assessed the question of supplementary utility, with a similarly large case series of VWD patients in a cross-laboratory audit process.

Materials and methods

Study aims and setting

We report a cross-laboratory, retrospective audit of DDAVP and VWF/FVIII concentrate usage and effect. The main aim of this study was to assess whether supplementary laboratory testing using PFA-100 closure times together with VWF activity using the VWF:CB would provide any potential improvement in the therapeutic monitoring of VWD compared with the international laboratory standard test panel of VWF:Ag, VWF:RCo and FVIII:C. Although some expert laboratories perform the supplementary test of VWF multimers, this test is no longer routinely available within our geographic locality and indeed to most 'real-world' pathology laboratories [2,3]. Furthermore, VWF multimer analysis, when available and as applied to typing of VWD, is often poorly applied in clinical practice, with high-associated error rates [14]. Accordingly, we wished to observe whether we could identify differential patterns according to type of VWD that would be potentially useful for most testing laboratories without the requirement for additional more complex or availability-limited testing.

The original study population comprised over 300 individuals treated with either DDAVP or VWF/FVIII concentrates for treatment of various bleeding disorders, but the current report is limited to individuals with VWD (type 1, 2A or 2M) or with haemophilia or carriers of haemophilia for comparison. Laboratory testing comprised pre and post-DDAVP or VWF/FVIII concentrate evaluation of the standard recommended test panel of FVIII:C, VWF:Ag and VWF:RCo [4–7], supplemented by VWF:CB and PFA-100 closure times using collagen/epinephrine (C/Epi) and/or collagen/ADP (C/ADP) test cartridges. Data were derived from five diagnostic and treatment centres broadly distributed within our geographic region (i.e. New Zealand as well as differential east, west and north parts of Australia). Data collection was from local hospital databases and included the period since 1991 (Westmead Hospital), 2003 (Royal Perth Hospital), 2002 (Canterbury Health) or more recently (Royal Brisbane Hospital and Fremantle Hospital). Patients were initially diagnosed by the respective diagnostic/treatment centre, although the specific type of VWD was occasionally revised following a re-review of overall data.

Study population and patient von Willebrand disease typing

The current study is an extension of a previous study performed by us that looked at the potential combined benefit of performing VWF:CB testing with VWF:RCo testing, together with DDAVP responsiveness, to help better identify and characterize different VWD patients [15]. That study incorporated data from a total of 208 patients comprising severe type 1 VWD ('VWD-1s', $n=17$), mild/moderate type 1 VWD ('VWD-1m', $n=30$), 'possible mild' type 1 VWD ('VWD-1p', $n=113$), haemophilia A/carriers ('HA', $n=19$), type 2A VWD ('VWD-2A', $n=19$) and type 2M VWD ('VWD-2M', $n=10$). The current study draws from that pool and incorporates patients where PFA-100 closure times were also available. Patient categories were as per our previous report [15]. Thus, type 1 VWD was defined by consistently low but concordant test values for all VWF tests performed and evident on initial and repeat testing. In practice, concordance is an evidence of VWF 'activity'/VWF:Ag ratios [i.e. both VWF:RCo/VWF:Ag (RCo/Ag) and VWF:CB/VWF:Ag (CB/Ag)] above 0.7 [1–3,14]. Alternatively, type 2 VWD was defined by low levels of VWF and consistent discordance in VWF test results (i.e. VWF 'activity'/VWF:Ag ratios of ≤ 0.7), with the type of discordance helping to identify the probable type of VWD. In this and the previous [15] study, the VWF:CB was used by us as a surrogate marker of high-molecular weight (HMW) VWF, as previously validated and reported [2,3]. Thus, a consistently reduced CB/Ag as well as a consistently reduced RCo/Ag ratio would suggest either VWD type 2A or type 2B, whereas consistently reduced RCo/Ag but consistently normal CB/Ag would suggest a VWD-2M (i.e. platelet function discordant type [1–3,15]). These patterns and classifications are both consistent with the current classification scheme [1] and with previous observations regarding the ability of optimized VWF:CB assays to most appropriately identify HMW VWF [3] more reproducibly than interlaboratory VWF:RCo testing. Type 2B VWD patients identified by heightened responsiveness to ristocetin in a ristocetin-induced platelet agglutination assay were excluded from the current study. Accordingly, a consistent test pattern comprising reduced RCo/Ag but normal CB/Ag would by definition comprise platelet function discordant VWD-2M and exclude VWD-2A phenotype, and a consistent test pattern comprising reduced RCo/Ag and reduced CB/Ag would most likely reflect a specific loss of HMW VWF, and thus a VWD-2A phenotype. Type 1 VWD was further separated according to 'severity' as previously identified [15], with VWD-1s defined in those with VWF:Ag of 15 U/dl or less, VWD-1m in those with VWF:Ag between 16 and 35 U/dl and VWD-1p in those with VWF:Ag between 36 and 65 U/dl.

Laboratory test methods

Methodologies for phenotypic VWF and FVIII:C assays have been previously reported [15]. In brief, all laboratories tested for FVIII:C using a standard automated one-stage clot-based assay and for VWF:Ag using either a standard in-house ELISA or a commercial immunoturbidimetric ('latex-immuno-assay') procedure. All laboratories performed VWF:CB (by ELISA) using an in-house or commercial assay, with all methods based on a type I/III collagen mixture (inline with current recommendations [3]) and validated to be sensitive to HMW VWF [2,3,15]. VWF:RCo was performed by agglutination either using a platelet aggregometer or automated analyser [15]. The interlaboratory variation for these VWF tests is approximately 5–10% for FVIII:C, 10–15% for VWF:Ag, 15–20% for VWF:CB and 20–30% for VWF:RCo [2,3]. All laboratories are specialized testing referral centres affiliated with tertiary level teaching hospitals and undertake internal assessments and validity of assays prior to incorporation into diagnostic practice. All laboratories also participate in ongoing external quality assurance processes and are accredited by local regulatory authorities for suitability of testing processes. Cross-validation of test processes for laboratories within the current study was evaluated using retrospective data from the Royal College of Pathologists of Australasia (RCPA) haematology external quality assurance program (QAP). PFA-100 closure times were performed according to manufacturer (Dade-Behring) instructions.

Desmopressin and von Willebrand factor/factor VIII concentrate sampling data

This was a retrospective sampling of data from the hospital databases of several tertiary level institutions. To help standardize data analysis, we have standardized post-DDAVP and post-VWF/FVIII concentrate samplings to a single time-point, namely the first time-point after therapy (median 1 h after; range 30–90 min after). Current guidelines recommend the use of 0.3 μg/kg desmopressin infused intravenously over a period of 30 min [4–6]. The institutions to which the authors belong all follow current guidelines. The VWF/FVIII concentrate currently used in Australia is Biostate (CSL Limited, Melbourne, Australia) having replaced antihaemophilic factor (AHF) (high purity) recently; the composition and pharmacokinetic profiles of both of these have previously been reported [16]. It is a recommended practice to trial DDAVP in certain VWD patients [4–6] and to utilize VWF/FVIII concentrate for patients or clinical needs in whom DDAVP would be ineffective. As the institutions to which the authors belong all follow current guidelines, retrospective patient data for DDAVP vs. VWF/FVIII concentrate would comprise distinct sets in which either both or only one or other were utilized for any given patient.

Results

Cross-laboratory comparisons

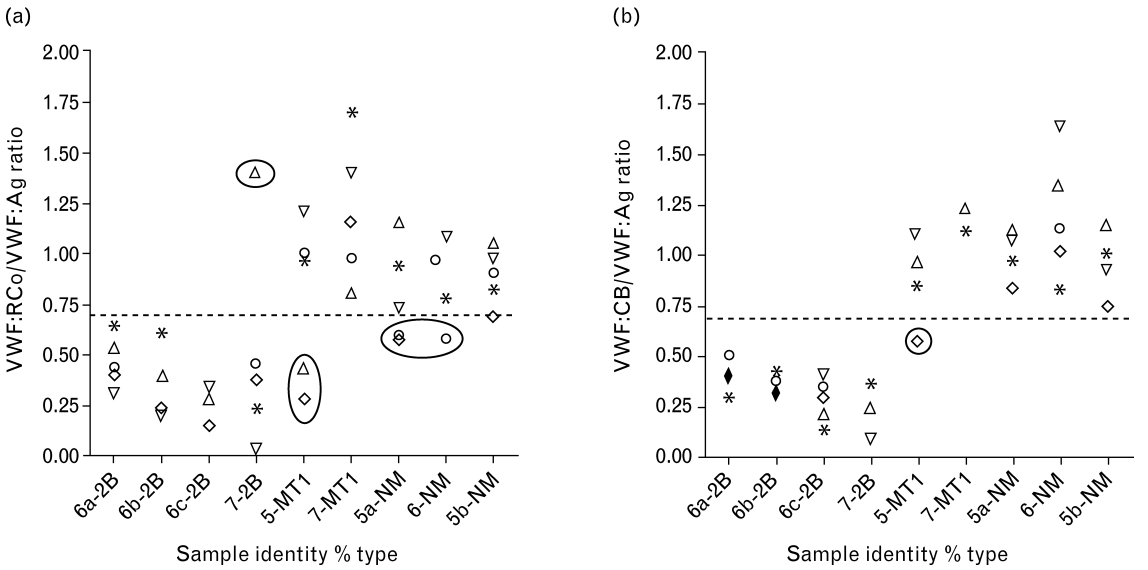
Given that we are reporting a cross-laboratory study in which laboratories are using different test processes, we also assessed the potential for cross-laboratory variance, as this might influence 'VWD diagnostic accuracy' on a laboratory-to-laboratory (or patient-by-patient) basis. Accordingly, cross-laboratory testing data, available through ongoing laboratory performance in an external QAP, were evaluated for comparability, as shown in Fig. 1. Multimer profiles for these cross-tested samples are shown in Fig. 2. Using a cut-off value of 0.7, all laboratories were consistently able to identify the loss of HMW VWF in a series of type 2B VWD samples using both CB/Ag and RCo/Ag ratios, whereas testing of normal samples or those from moderate/mild type 1 VWD patients (i.e. without loss of HMW VWF) typically yielded normal assay ratios. Consistent with previously published findings, the use of the CB/Ag ratio showed greater consistency in this discrimination.

Laboratory-identified responses to desmopressin

Data for VWF and FVIII test responses to DDAVP for differential patient groupings have previously been reported [15]. The salient points to report are: type 1 VWD patients: good rises in all VWF test parameters (e.g. VWF:Ag, VWF:RCo, and VWF:CB) and FVIII:C post-DDAVP for each VWD subgroup. Rises in VWF:CB tend to exceed those of VWF:RCo, and hence the increase in CB/Ag ratios tend to exceed those of RCo/Ag; Haemophilia A/carriers: similar to type 1 VWD, except that lower initial FVIII:C levels and moderate FVIII:C response. Type 2A VWD: good rises in VWF:Ag and FVIII:C but modest rises in VWF:CB and VWF:RCo, so that although CB/Ag and RCo/Ag both rise slightly, they both tend to remain below 0.7. Type 2M VWD: good rises in VWF:Ag, VWF:CB and FVIII:C but less rise in VWF:RCo, so that although CB/Ag rises, RCo/Ag tends to remain below 0.7. Summary of data for PFA-100 C/Epi closure times is shown in Fig. 3. Salient points to note are: Pre-DDAVP closure times are generally normal in haemophilia but prolonged in all VWD test groups, although in type 1 VWD, the extent of prolongation is related to the severity of the disorder. DDAVP results in normalization of closure times in type 1 VWD (with the extent of normalization related to the severity of VWD), but no normalization occurs in types 2A and 2M VWD (some minimal correction is observed in type 2M VWD). Data using the PFA-100 C/ADP test yielded similar findings (data not shown).

The relationship between VWF assays and FVIII:C vs. PFA closure times for C/Epi for type 1 VWD groups (combined data) is shown in Fig. 4 and for types 2A and 2M VWD in Fig. 5. Note the strong inverse relationship in type 1 VWD, whereas type 2 VWD patterns are different. DDAVP resulted in modest shortening of

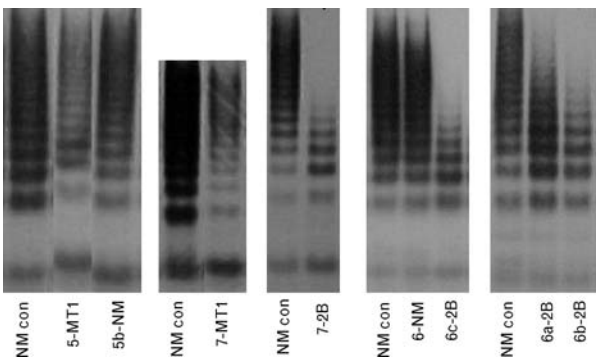
Fig. 1



Comparative retrospective cross-laboratory data for a series of samples cross-tested (via the RCPA Haematology QAP) by laboratories participating in the current study. A different symbol has been arbitrarily assigned to each laboratory. Data are shown for RCo/Ag (a) and CB/Ag (b) ratios using four type 2B VWD samples (6a-2B, 6b-2B, 6c-2B and 7-2B), two moderate/mild type 1 VWD samples (5-MT1 and 7-MT1) and three normal samples (5a-NM, 6-NM and 5b-NM). The dashed horizontal line indicates the cutoff value of 0.7. Ratio values below this value indicate functional VWF discordance, whereas ratio values above this indicate functional VWF concordance. All laboratories were able to identify the loss of HMW VWF in each type 2B VWD samples (i.e. ratios of RCo/Ag and CB/Ag below 0.7), although a single outlier data point (circled; above 0.7) was noted for RCo/Ag for sample 7-2B. Similarly, all laboratories obtained normal assay ratios for normal samples or those from moderate/mild type 1 VWD (i.e. ratios of RCo/Ag and CB/Ag above 0.7), although some outlier data points (circled; below 0.7) were occasionally obtained. Consistent with previously published findings, the use of the CB/Ag ratio (b) showed greater consistency in this discrimination compared with RCo/Ag (a). CB/Ag, VWF:CB/VWF:Ag; HMW, high molecular weight; RCo, ristocetin cofactor; RCo/Ag, VWF:RCo/VWF:Ag; VWD, von Willebrand disease; VWF, von Willebrand factor.

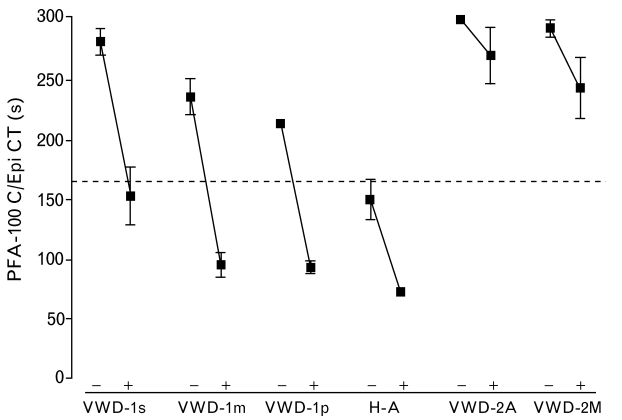
closure times in some patients only (primarily 2M); in most cases, closure times were virtually unchanged, despite normalization of VWF:Ag (Fig. 5a) and FVIII:C (Fig. 5d). Notably, VWF:CB and VWF:RCo did not tend to normalize in these patients (Fig. 5b and c). Although modest shortening of closure times was occasionally observed in type 2 VWD, this was often associated with normalization of VWF:CB and CB/Ag ratios (Fig. 5b and e) but not with normalization of VWF:RCo or RCo/Ag ratios (Fig. 5c and f).

Fig. 2



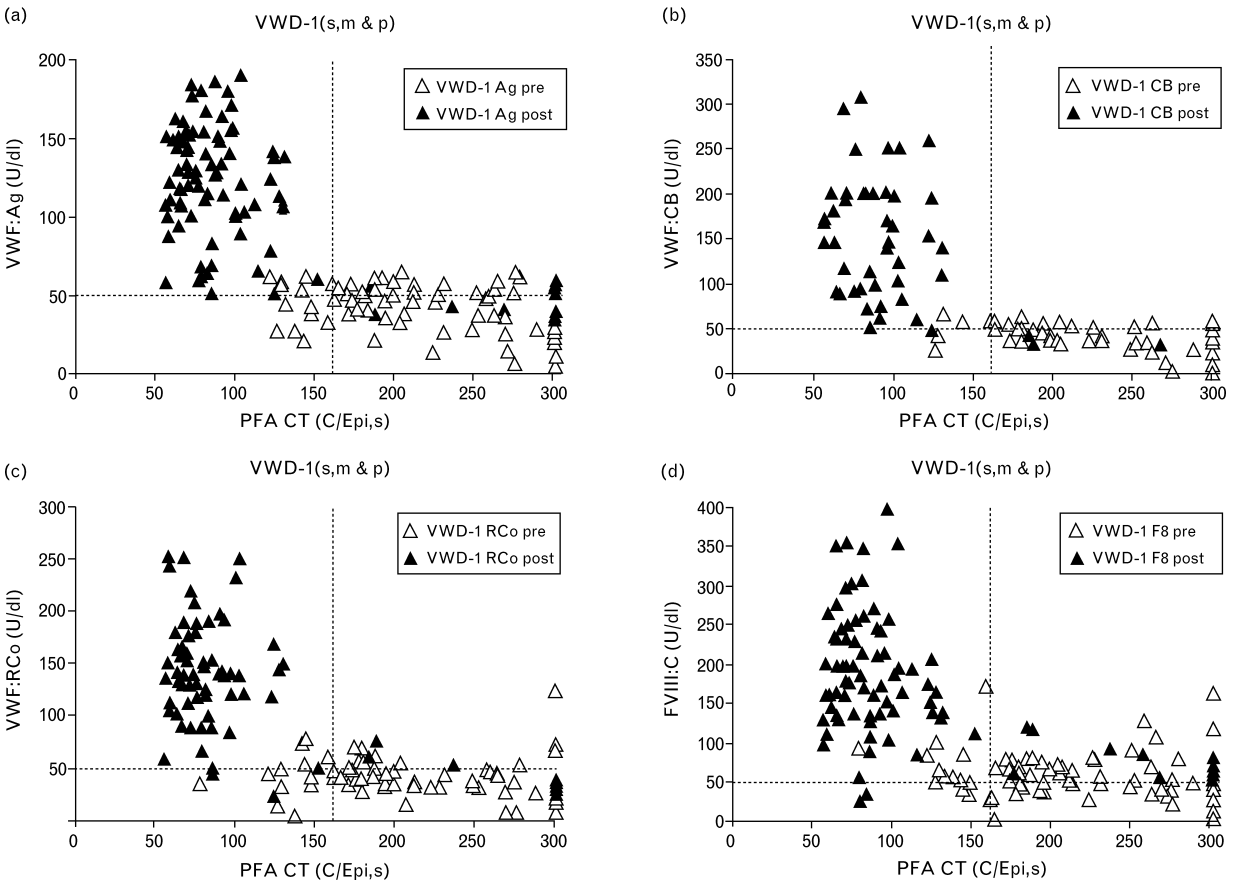
VWF multimer patterns for the samples cross-tested in Fig. 1, showing the loss of HMW VWF in the type 2B VWD samples. Test data are retrospective. Accordingly, test samples were run at different times, and a new normal control (NM con) sample was included with each test run. HMW, high molecular weight; VWD, von Willebrand disease; VWF, von Willebrand factor.

Fig. 3



PFA-100 C/Epi closure times (*y*-axis) before and after DDAVP (= ±) for patient groupings as identified in Materials and methods; notably, VWD-1s, $n = 7/7$; VWD-1m, $n = 18/17$; VWD-1p, $n = 69/66$; HA, $n = 19/17$; VWD-2A, $n = 10/10$; VWD-2M, $n = 6/6$. The dotted horizontal line indicates the upper limit of normal cutoff value. DDAVP, desmopressin; VWD, von Willebrand disease.

Fig. 4



Comparative data for PFA-100 C/Epi closure times (*x*-axis) versus plasma FVIII:C and various VWF test parameters [on *y*-axis, U/dl; graphs (a) VWF:Ag, (b) VWF:CB, (c) VWF:RCo, (d) FVIII:C], before and after DDAVP for combined VWD-1 patient group data set (i.e. VWD-1s and VWD-1m and VWD-1p). Dotted horizontal lines indicate the nominal VWF and FVIII:C cutoff value of 50 U/dl; values for VWF or FVIII:C below this would be considered low (or abnormal) and values above this would be considered normal. Dotted vertical lines indicate the nominal (manufacturer) PFA-100 closure time cutoff value of 165 s. Thus, all pre-DDAVP VWF:Ag values would be lower than 65 U/dl (depending on the derived patient set) with most data points falling below the 50 U/dl cutoff line, and most corresponding PFA-100 closure times would be abnormal or fall above the PFA-100 closure time 165 s cutoff value. Post-DDAVP, VWF and FVIII:C test values would generally normalize (i.e. rise above the horizontal 50 U/dl cutoff line) as would PFA-100 closure times (i.e. fall below the vertical 165 s cutoff line). Note the inverse relationship between closure times and VWF test parameters. DDAVP, desmopressin; FVIII, factor VIII; RCo, ristocetin cofactor; VWD, von Willebrand disease; VWF, von Willebrand factor.

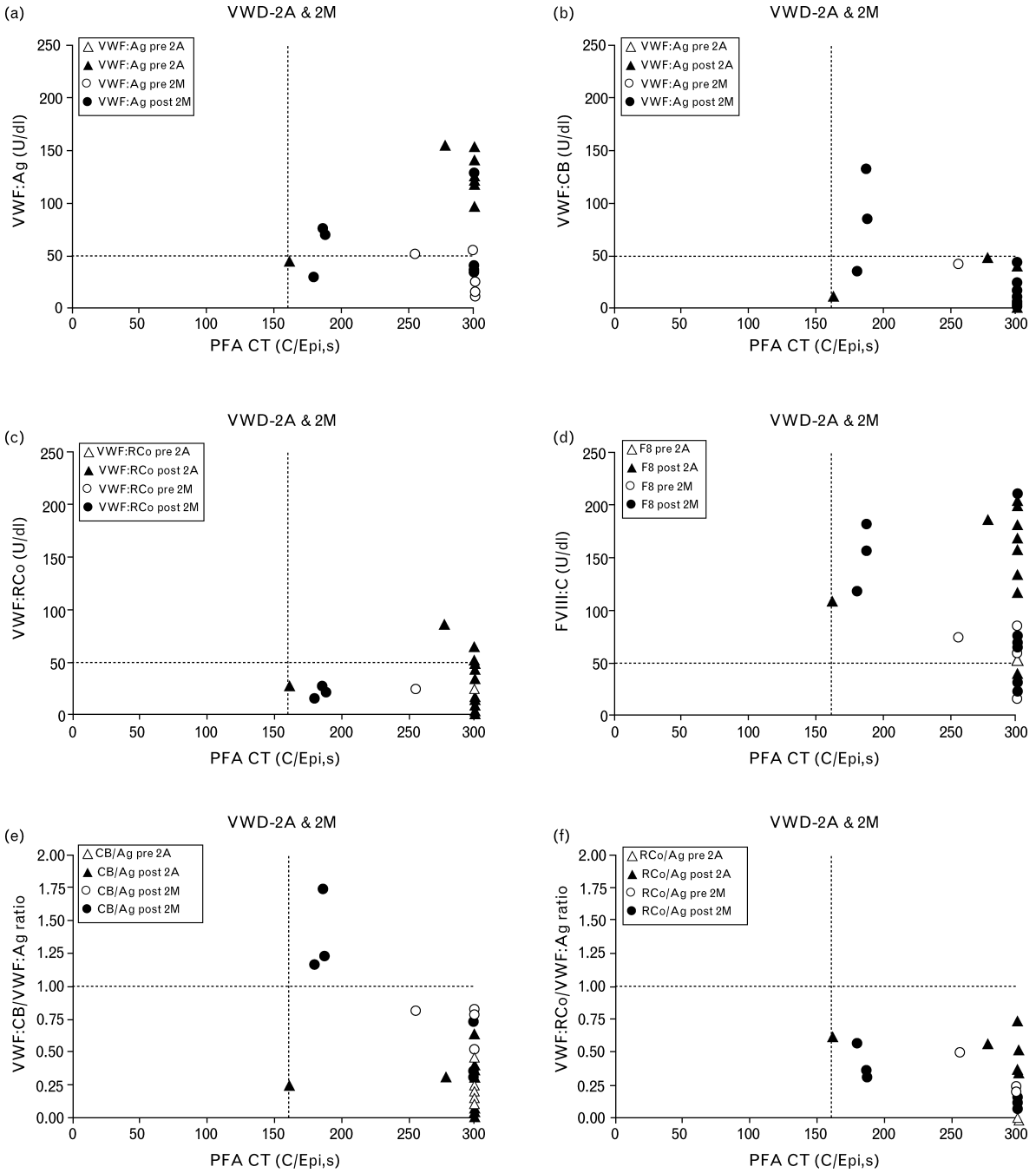
Laboratory-identified responses to von Willebrand factor/factor concentrate

The relationship between VWF and FVIII:C vs. PFA-100 C/Epi closure times for type 1 VWD groups (combined data) is shown in Fig. 6. Note once again the good inverse relationship observed. In contrast, closure times essentially failed to normalize or correct in type 2 VWD, despite occasional correction of VWF or FVIII (data not shown).

Discussion

Although a retrospective audit, this is the largest case series of VWD patients to our knowledge assessed with the panel of tests identified (e.g. FVIII:C, VWF:Ag, VWF:CB, VWF:RCo, and PFA-100 closure times), following DDAVP treatment or VWF/FVIII concentrate therapy. In particular, several differential test patterns could be identified, as also in part previously reported by us [15]. For type 1 VWD, there was also a noticeable inverse relationship between VWF test parameters and PFA-100 closure times (Fig. 4), with VWF test increases associated with normalization or correction of PFA closure times. VWF/FVIII concentrate therapy provided a similar conclusion regarding this relationship (Fig. 6). For types 2A and 2M VWD, differential test patterns could be identified compared with each other and with type 1 VWD, both in relation to functional VWF assays and assay ratios, and neither DDAVP nor VWF/FVIII concentrate therapy was able to substantially correct or normalize PFA-100 closure times. As data for C/ADP were not disparate to that using C/Epi, a case can be made for evaluating therapeutic responsiveness using only a single test (i.e. C/Epi) cartridge type.

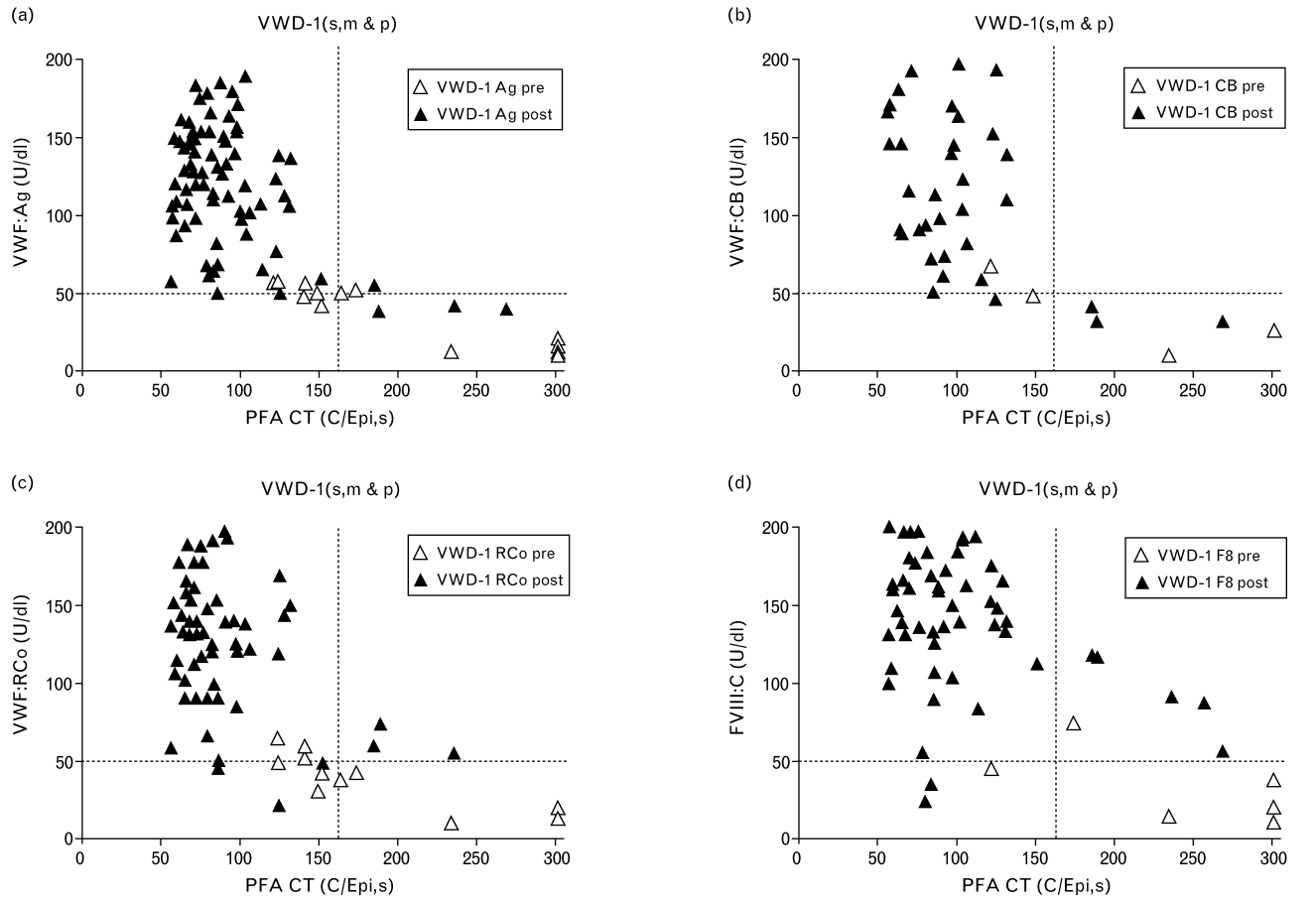
Fig. 5



Comparative data for PFA-100 C/Epi closure times (*x*-axis) versus plasma FVIII:C and various VWF test parameters [on *y*-axis, U/dl; graphs: (a) VWF:Ag, (b) VWF:CB, (c) VWF:RCo, (d) FVIII:C], before and after DDAVP for combined VWD-2A and 2M VWD patient group data set. Dotted horizontal and vertical lines show cutoff values as per Fig. 4. Graphs (e) and (f), respectively, show CB/Ag and RCo/Ag ratios vs. closure times. Dotted horizontal and vertical lines, respectively, identify ratios of 1.0 and PFA-100 cutoff values. DDAVP failed to normalize any closure time, and partially corrected closure times only occasionally. There was no obvious relationship between closure times and any test parameter, excepting that partial correction of closure times was more likely to occur if there was correction of VWF:CB or CB/Ag. DDAVP, desmopressin; FVIII, factor VIII; RCo, ristocetin cofactor; VWD, von Willebrand disease; VWF, von Willebrand factor.

Thus, to summarize for DDAVP, we could identify differential CB/Ag and RCo/Ag patterns among VWD groups, with elevation of both CB/Ag and RCo/Ag in type 1 VWD, selective elevation only in CB/Ag in type 2M VWD and no substantial elevation of either CB/Ag or RCo/Ag in type 2A VWD, in which ratios tended to remain below 0.7 [15]. Similar observations regarding comparative effects with FVIII:C, VWF:Ag, VWF:RCo

Fig. 6



Comparative data for PFA-100 C/Epi closure times (*x*-axis) versus plasma FVIII:C and various VWF test parameters [on *y*-axis, U/dl; graphs: (a) VWF:Ag, (b) VWF:CB, (c) VWF:RCo, (d) FVIII:C], before and after VWF/FVIII concentrate therapy for combined VWD-1 patient group data set (i.e. VWD-1s and VWD-1m and VWD-1p). Dotted horizontal and vertical lines show cutoff values as per Fig. 4. Note again the good inverse relationship between closure times and VWF test parameters, similar to the case for DDAVP therapy. DDAVP, desmopressin; FVIII, factor VIII; RCo, ristocetin cofactor; VWD, von Willebrand disease; VWF, von Willebrand factor.

and VWF:CB post-DDAVP were reported by some of us for type 1 VWD using small numbers of patient sample within a single institution over 10 years ago, with test correlations to PFA-100 closure times reported subsequently [9]. The working hypothesis at that time was that patterns showing higher relative VWF:CB increases reflected the initial release of (ultra) HMW VWF post-DDAVP, together with the higher comparative sensitivity of VWF:CB (e.g. compared with VWF:Ag and VWF:RCo) for detection of HMW VWF [3] and the similar effect of such HMW VWF on PFA-100 closure times. That this has been validated in a current larger cross-laboratory study ([15] and current report) is, we believe, a significant finding. The patterns with types 2A and 2M VWD are distinct, both in terms of relative increases in VWF post-DDAVP and in PFA-100 closure times.

VWF/FVIII concentrate therapy provided similar findings to that of DDAVP therapy for type 1 VWD, but for type 2 VWD, correction in PFA-100 closure times was an even rarer event than that observed with DDAVP, and there was only a minor relationship between closure time correction and correction in VWF:CB or CB/Ag. This latter finding was again similar to that previously reported for a small single institution pilot study [9]. Notably, those type 2M patients, in whom some shortening of closure times was observed, were those in whom DDAVP managed to increase CB/Ag ratios above 1.0. VWF/FVIII concentrate therapy generally failed to shorten closure times, and in no patient did CB/Ag ratios rise above 1.0.

There have been a large number of studies that have assessed the potential added utility of the PFA-100 in monitoring therapy in VWD and a smaller number of studies assessing the potential added utility of the VWF:CB in this setting, as recently reviewed and reported [9]. There are, however, very few studies that have assessed both the PFA-100 and the VWF:CB in this

setting, and data from only one other group provide good comparative data [10–13]. Thus, although Giannini *et al.* [17] and Hanebutt *et al.* [18] have recently reported on DDAVP therapy in VWD and included both VWF:CB and PFA-100 closure time testing in their respective studies, neither group provided detailed data on comparative responses within their patient groups.

Overall, our data for type 1 VWD are similar to that most recently reported by van Vliet *et al.* [13], but data are somewhat disparate for type 2 VWD, as these workers saw better correction of closure times for both DDAVP and VWF/FVIII concentrate therapy (i.e. a greater number of their type 2 VWD patients showed closure time corrections). Further, they identified that patients with type 2 VWD showed correction only if the CB/Ag ratio also normalized. We observed occasional cases in which correction of VWF:CB or CB/Ag ratios were associated with improved closure times, but overall data indicated that this was a rare event and essentially restricted to cases in which CB/Ag ratios exceeded 1.0. There are two plausible explanations for the discrepancy between these two studies for types 2A and 2M VWD, and namely that either the therapy was more effective in their study than in ours and/or that they utilized a milder type 2 VWD patient group. van Vliet *et al.* [13] utilized a different VWF/factor concentrate (i.e. Haemate P) to those that we used, but published data [16] would suggest that this concentrate is in fact of a similar composition to one of the concentrates assessed within the current study. Accordingly, our conclusion remains that van Vliet *et al.* [13] utilized a 'milder' set of types 2A and 2M VWD patients compared with our patient set, and that in general, PFA-100 closure times are unlikely to correct in type 2A or 2M VWD post-DDAVP or post-VWF/FVIII concentrate.

We believe that the current report supports our belief regarding the potential for additional clinical utility of supplementary PFA-100 and VWF:CB testing within context of DDAVP and VWF/factor concentrate in VWD. Thus, in type 1 VWD, and following either DDAVP or VWF/factor concentrate therapy, one should generally see correction of VWF and FVIII:C as well as PFA-100 closure times, except in very severe cases of type 1 VWD. Moreover, there is a strong inverse relationship between closure times and VWF test parameters, with the advantage that the PFA-100 can provide data in real time, taking only minutes to perform, whereas results using a comprehensive panel of VWF tests would typically not be available for some time. The situation with types 2A and 2M VWD is somewhat different. In our experience, PFA-100 closure times do not correct in these VWD types either using DDAVP or VWF/factor concentrate therapy. Nevertheless, testing of PFA-100 closure times after therapy might be useful in patients in whom VWD types are unclear and require definition (i.e. correction, likely type 1). In any case, the benefits of additional post-therapy testing with the PFA-100 are generally restricted to patient test cases in whom baseline VWF:Ag exceeds around 10%.

Conclusion

We report findings of a retrospective cross-laboratory evaluation of DDAVP and VWF/FVIII concentrate therapy in patients with VWD and haemophilia and incorporating testing with the standard panel of FVIII:C, VWF:Ag and VWF:RCo, supplemented with PFA-100 closure times and the VWF:CB to derive CB/Ag and RCo/Ag ratios. We recognize several limitations to our study, including its retrospective nature, the lack of genetic testing to explore genetic–phenotypic correlations and the lack of multimer analysis to confirm specific VWD types based on phenotypic testing. Nevertheless, we observed differential plasma VWF test result patterns in different patient groups (i.e. haemophilia/carriers and types 1, 2A and 2M VWD) and using different laboratory assays and in particular using comparative data with activity-based VWF assays (i.e. VWF:CB and VWF:RCo). We also noted inverse relationships between all plasma VWF test results and PFA-100 closure times following either DDAVP or VWF/factor concentrate therapy in type 1 VWD but not in types 2A or 2M VWD, with correction of closure times also requiring correction of VWF (and notably VWF:CB). Our study findings warrant appropriate validation with prospective studies to definitively address the role of these tests in monitoring therapy and to investigate genetic associations and therapeutic implications.

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Testosterone and acute stress are associated with fibrinogen and von Willebrand factor in African men: The SABPA study

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ABSTRACT

Background: Low testosterone, acute and chronic stress and hypercoagulation are all associated with hypertension and hypertension-related diseases. The interaction between these factors and future risk for coronary artery disease in Africans has not been fully elucidated. In this study, associations of testosterone, acute cardiovascular and coagulation stress responses with fibrinogen and von Willebrand factor in African and Caucasian men in a South African cohort were investigated.
Methods: Cardiovascular variables were studied by means of beat-to-beat and ambulatory blood pressure monitoring. Fasting serum-, salivary testosterone and citrate coagulation markers were obtained from venous blood samples. Acute mental stress responses were evoked with the Stroop test.
Results: The African group demonstrated a higher cardiovascular risk compared to Caucasian men with elevated blood pressure, low-grade inflammation, chronic hyperglycemia (HbA1c), lower testosterone levels, and elevated von Willebrand factor (VWF) and fibrinogen levels. Blunted testosterone acute mental stress responses were demonstrated in African males. In multiple regression analyses, higher circulating levels of fibrinogen and VWF in Africans were associated with a low T environment (R^2 0.24–0.28; $p \leq 0.01$), but only circulating fibrinogen in Caucasians. Regarding endothelial function, a low testosterone environment and a profile of augmented α-adrenergic acute mental stress responses (diastolic BP, D-dimer and testosterone) were associated with circulating VWF levels in Africans (Adj R^2 0.24; $p < 0.05$).
Conclusions: An interdependence between acute mental stress, salivary testosterone, D-dimer and vascular responses existed in African males in their association with circulating VWF but no interdependence of the independent variables occurred with fibrinogen levels.

1. Introduction

Black South Africans are facing an epidemic of hypertension and vascular disease but there is still inadequate information on the physiological factors contributing to this process [1–3]. Low testosterone (T) levels are associated with endothelial dysfunction, increased arterial stiffness and hypertension [4,5], atherosclerosis [6], and target organ damage like renal impairment [7]. Endothelial dysfunction and damage are involved in a number of these conditions suggesting that a low T environment may be involved in the aetiology of these conditions. Testosterone deficiency is also associated with an increase in haemostatic factors like fibrinogen and plasminogen activator inhibitor type 1 which, together with endothelial dysfunction, may increase the risk for thromboembolic events [6] and the development of hypertension related target organ damage [8].

Chronic and acute stress which are major health problems of our modern world, are also associated with low T [9] and increases in various coagulation factors (fibrinogen, von Willebrand factor (VWF) and D-dimer) [10,11]. Von Känel et al. [12] demonstrated that changes in VWF and D-dimer during acute mental stress are positively correlated as markers of coagulation activation VWF is almost exclusively produced by endothelial cells and is an important factor in the coagulation process as it mediates platelet adhesion and stabilization of clotting factor VIII. Alpha-adrenergic activity (rather than β-adrenergic activity)

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results in an increase in D-dimer [13], a covalently cross-linked product of fibrin degradation, indicating increased coagulation activation [14]. Together with D-dimer, VWF is regarded as a useful marker of endothelial damage and the risk of coronary artery disease (CAD) [15]. In addition, Spiel et al. proposed that VWF is pathogenically and directly involved as a causative agent in the development of CAD and acute myocardial infarction [16]. Both fibrinogen and D-dimer are independently associated with increased risk for future myocardial infarction, stroke and peripheral vascular disease [17,18]. The occurrence of target organ damage is, therefore, under the direct influence of the coagulation system.

The aim of this study is to determine whether a low testosterone environment and acute stress responses are associated with hypercoagulation markers in a cohort of African and Caucasian men.

2. Methods

2.1. Design and participants

Participants were recruited as part of the Sympathetic Activity and Ambulatory Blood Pressure in Africans (SABPA) comparative cohort population study conducted between February and May of 2008 and 2009. The study sample comprised of urban African and Caucasian teachers working in the Kenneth Kaunda Education district in the North West Province of South Africa. The motivation for this selection was to obtain a homogenous sample from a similar working environment and socio-economic status although cultural differences could not be excluded.

We invited all eligible participants between the ages of 25 and 65 years to participate. Exclusion criteria were an ear temperature above 37.5 °C, chronic use of α- and/or β-adrenergic blockers, psychotropic substance dependence or abuse, blood donors and individuals vaccinated in the past 3 months. A total of 200 men were initially included in the study. For the current sub-study participants who were infected with HIV (N = 16), clinically diagnosed with diabetes (N = 8), antidepressant users (N = 2) and/or diagnosed with renal dysfunction (N = 1) were excluded. A total of 75 Africans and 98 Caucasians were thus included in the study. Participants were fully informed about the objectives and procedures of the study prior to their recruitment. All participants provided written, informed consent. The study conformed to the Helsinki Declaration and was approved by The Ethics Review Board of the North-West University, Potchefstroom Campus: Approval number 0003607S6.

The protocol and methodology used in this study have been described extensively elsewhere [2].

Ambulatory blood pressure monitors (ABPM), electrocardiograms (ECG), as well as physical activity apparatus were fitted to the participants before 07 h00 min after which they commenced with their normal daily activities. Participants were transported at 16 h30 min to the Metabolic Unit Research Facility of the North-West University and were encouraged to go to bed at around 22 h00 min. They were woken at 05 h45 min the following morning to undergo a battery of clinical assessments.

2.2. Assessment of health behaviours

Physical activity was assessed by means of Actical® accelerometers (Mini Mitter, Bend OR, Montréal, Québec). The device was initialised using 15-s epochs and converted to 1-min epochs for data analysis [19]. Smoking status was assessed using serum cotinine [20,21]. Serum gamma-glutamyltransferase (γ-GT) was measured and considered as a marker of alcohol abuse [22].

2.3. Anthropometric measurements

All anthropometric measurements were performed in triplicate by registered level II anthropometrists according to standardised procedures [23]. Body surface area (BSA) was calculated based on the Mosteller formula [24].

2.4. Ambulatory blood pressure measurements

On the morning of the first clinical assessment day, ABPM and 2-lead electrocardiograph were attached to participants on the non-dominant arm at their workplace (Meditech CE120 CardioTens®; Meditech, Budapest, Hungary). This apparatus was validated and approved by the British Hypertension Society. The ABPM was programmed to measure BP at 30-min intervals during the day (07 h00 min–22 h00 min) and every hour during night time (22 h00 min–06 h00 min) [25]. The successful inflation rate over this period was 75.2% (±9.8) in Africans and 84.7% (±9.1) in Caucasians. Hypertensive status was classified from 24 h ABPM as SBP > 125 mm Hg and/or DBP > 80 mm Hg [26]. Apparatus was removed after the last BP measurement at 06 h00 min.

2.5. Acute mental stress testing

Participants were to avoid exercising, smoking and consuming alcohol and caffeine 8 h prior to saliva sampling commencement. Furthermore, they were requested to take care with dental hygiene, to prevent bleeding gums, and not to brush their teeth before saliva sampling [27]. Application of the Stroop colour-word-conflict (CWC) test was given.

At 06 h45 min the next morning participants were in semi-recumbent position for 10 min followed by beat-to-beat BP, saliva and blood sampling. A small amount of anti-clotting solution (0.5 ml of a Heparin Sodium-Fresenius 5000 IU/ml in 50 ml normal saline solution) to prevent blood clotting was left in situ. A 10 minute resting period followed to ensure resting BP values before the Stroop colour-word-conflict (CWC) test was applied. Successive series of five colour words written in incongruent colours in a random order on a cardboard were shown to the participant. The participants were instructed to recognize and verbally confirm colours [28]. The task lasted for 1 min with added time pressure and a monetary incentive on completion of the task. This was followed by 10 min post stress blood sampling where the infusion set was thoroughly flushed with 2–3 ml saline. A 2 ml blood sampling was discarded before post stress sampling was obtained. Saliva sampling followed 30 min after CWC application.

Beat-to-beat blood pressure was continuously assessed throughout CWC testing using the Finometer device validated for relative changes (Finapres Medical Systems®, Amsterdam) [29]. The last 15 s of the CWC test, Finometer measurements resembling maximum BP plateau values were used for analyses. Data was processed with the Beatscope 1.1 software (Finapres Medical Systems, Amsterdam, The Netherlands). The BeatScope® version 1.1a software further calculates an integrated age dependent aortic flow curve from the surface area beneath the pressure/volume curve determining β-adrenergic central cardiac responses including SBP, stroke volume (SV) and cardiac output (CO) while DBP and TPR were used to represent α-adrenergic vascular responses [2,30].

2.6. Biochemical analyses

Serum samples were analysed for total testosterone (T) using an electro chemiluminescence immunoassay on the Elecsys 2010 apparatus (Roche, Basel, Switzerland). Both the intra- and inter-assay coefficients of variation (CV) for all the assays were less than 10%. Saliva samples were obtained through chewing a Salivette (Sarstedt Inc., Leicester, UK) cotton swab for 1–2 min, which was snap-frozen immediately in liquid nitrogen and stored at −80 °C until analyses. Saliva samples were analysed with an immunoassay kit with chemiluminescence detection (CLIA; IBL Hamburg, Germany). Intra-assay and inter-assay coefficients of variation were less than 10%. High sensitivity C-reactive protein (CRP), cotinine and γ-GT were analysed in serum (Unicel DXC 800, Beckman and Coulter, USA; Modular ROCHE Automized, Switzerland and Konelab™ 20I Sequential Multiple Analyser Computer, ThermoScientific, Vantaa, Finland respectively). Citrate coagulation markers, fibrinogen and D-dimer were analysed with a viscosity-based Immunoturbimetric clotting method. Instrument: STA compact; Manufacturer: TAGO diagnostic, France). The citrate VWF antigen levels were measured with a "sandwich" ELISA assay. A polyclonal rabbit anti-VWF antibody and a rabbit anti-VWF-HRP antibody (DAKO, South Africa) were used to form the assay. The 6th International Standard for VWF/FVIII was used to set the standard curve against which the samples were measured [31].

2.7. Statistical methods

Data was analysed using Statistica® software version 11.0 (Statsoft Inc., Tulsa, USA, 2012). Skewness of data was tested and only γ-GT values were logarithmically transformed. Independent T-tests determined participant characteristic differences. Confounders which are implicated in higher sympathetic activity and cardiovascular risk were identified [age, body surface area (BSA), physical activity and log γ-GT] [2,26]. Chi-square (χ^2) statistics calculated and compared proportions. General linear model analyses, independent of covariates, were computed to test interactions within race and coagulation markers. ANCOVA's determined significant differences by comparing ethnic male groups from least square means analyses, while adjusting for confounders and resting values. Cardiovascular and coagulation responses (indicated by Δ) during the mental stressors were determined, as the ratio of the variable reaction and the resting value, by using the formula (stressor value/resting value).

Multiple linear regression analyses were computed. Forward stepwise multiple regression analyses were performed for various models from two perspectives. Both perspectives used circulating fibrinogen and VWF as the dependent variables. The first perspective included covariates: resting serum testosterone, Δ cardiovascular and Δ coagulation responses during the CWC. The second perspective included covariates: Δ salivary testosterone, Δ cardiovascular and Δ coagulation responses during the CWC. Covariates included in all the models were age, BSA, physical activity, log γ-GT and anticoagulants (aspirin and warfarin). Sensitivity analyses were computed for multiple linear regression analyses excluding one African and 9 Caucasians using oral anticoagulants. Significance was noted as $p \leq 0.05$ and tendencies were noted as $p < 0.1$.

3. Results

Table 1 shows baseline characteristics of African and Caucasian men. The African men displayed ($p \leq 0.05$) lower physical activity and BSA than the Caucasian men (Table 1). After adjusting for age, BSA, physical activity and log γ-GT, the African men, despite a smaller stature (height), displayed a higher body mass and waist circumference than the Caucasians. Furthermore, Africans demonstrated higher resting levels of CRP, VWF, fibrinogen and D-dimer than Caucasians. Sensitivity

Table 1
Comparing adjusted baseline characteristics between African and Caucasian men.

	Africans (N = 75)	Caucasians (N = 98)	P
Unadjusted values			
Age (years)	42.7 ± 8.39	44.7 ± 11.2	0.18
Body surface area (BSA) (m^2)	1.94 ± 0.23	2.18 ± 0.21	<0.0001
Physical activity (kcal/24 h)	2719.2 ± 827.7	3675.8 ± 2088.4	<0.001
γ-GT, U/L (geometrical mean)	58.9 (23.4, 182.0)	26.9 (10.0, 89.1)	<0.0001
Adjusted values: *Lifestyle and biochemical variables*			
Body mass (kg)	89.8 (89.0, 90.7)	86.0 (85.2, 86.8)	<0.0001
Height (cm)	172.7 (171.2, 174.1)	179.1 (177.8, 180.4)	<0.0001
Waist circumference (cm)	99.6 (97.7, 101.5)	95.1 (93.4, 96.9)	0.002
BMI (weight kg/length m^2)	30.0 (29.3, 30.7)	26.6 (26.0, 27.3)	<0.0001
Cotinine (μg/L)	19.0 (−1.78, 39.9)	37.3 (17.4, 57.3)	0.27
C-reactive protein (mg/L)	5.45 (4.04, 6.86)	2.01 (1.16, 2.86)	0.003
Resting blood glucose (mmol/L)	6.11 (5.71, 6.51)	5.89 (5.52, 6.25)	0.47
HbA1C (%)	6.30 (6.06, 6.54)	5.59 (5.36, 5.81)	<0.001
Endocrine variables			
Serum testosterone (nmol/L)	15.3 (13.9, 16.7)	18.6 (17.3, 19.9)	<0.01
Salivary testosterone (nmol/L)	0.47 (0.42, 0.53)	0.64 (0.59, 0.69)	0.0001
Cardiovascular and coagulation variables			
24 h SBP (mm Hg)	139 (136, 142)	127 (124, 129)	<0.0001
24 h DBP (mm Hg)	88 (86, 90)	79 (77, 81)	<0.0001
24 h heart rate (beats/min)	77 (75, 80)	73 (71, 76)	0.05
D-dimer rest (mg/L)	0.67 (0.38, 0.96)	0.25 (−0.02, 0.52)	0.06
von Willebrand factor rest (%)	85.8 (80.5, 91.1)	63.7 (58.6, 68.7)	<0.0001
Fibrinogen rest (g/L)	3.23 (3.09, 3,38)	2.90 (2.77, 3.03)	0.003
TPR resting (mm Hg/ml/sec)	1.09 (0.94, 1.23)	1.05 (0.92, 1.11)	0.77
Resting SBP (Finometer) (mm Hg)	147 (143, 152)	133 (129, 137)	<0.0001
Resting DBP (Finometer) (mm Hg)	87 (85, 90)	79 (77, 81)	<0.0001
Stroke volume (ml/min)	103.0 (97.2, 108.9)	101.1 (95.6, 106.7)	0.68
Cardiac output (L/min)	6.79 (6.36, 7.22)	6.48 (6.08, 6.89)	0.38
Hypertensive, n (%)	59 (78.7)	67 (68.4)	0.12
Coagulation medication, n (%)	1 (1.33)	9 (9.18)	

Variables adjusted for age, body surface area, physical activity, γ-GT and anticoagulant users. Mean (95% confidence interval); p values ≤ 0.05 regarded as statistically significant. Where: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; TPR, total peripheral resistance; HT, hypertension; γ-GT, γ-glutamyltransferase; HbA1C, glycated hemoglobin.

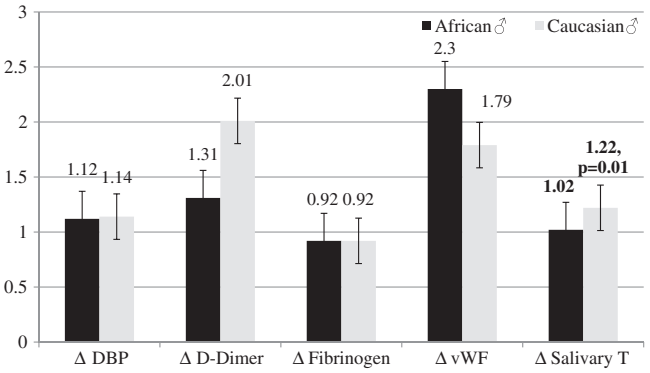

Fig. 1. Cardiovascular and coagulation acute responses (Δ) in African vs. Caucasian men where Δ values were calculated as Stroop value/Resting value. Data were adjusted for age, body surface area, physical activity, log γ glutamyl transferase, and use of anticoagulants.

analyses after excluding one African and 9 Caucasians using oral anticoagulants demonstrated no difference in the outcome (data not shown).

Significant interactions, independent of covariates, demonstrated an ethnic difference for resting coagulation markers, VWF (%) [F (1, 180), 17.70; p = 0000.4] and fibrinogen [F (1, 180), 8.16; p = 0.005]. Group differences maintained significance after controlling for covariates age, BSA, physical activity and log γ-GT. Higher ambulatory blood pressure (BP) as well as heart rate (HR) values were evident in African men compared to the Whites. Hypertension, according to the ESH guidelines [26] was prevalent in 78.7% of Africans and 69.0% of the Whites in this study.

In terms of stress responses (Fig. 1), no significant differences between groups, except for blunted Δ salivary testosterone responses were evident in African males compared to their Caucasian counterparts in general linear models (p = 0.01).

Forward stepwise linear regression analyses demonstrated associations between resting dependent variables fibrinogen and VWF including covariates (resting testosterone, Δ cardiovascular and Δ coagulation markers and confounders) following our first perspective (Table 2: models 1–4). D-dimer acute mental stress responses (ΔD-dimer) were positively associated with circulating fibrinogen (β = 0.33, p < 0.02) and VWF (β = 0.24, p = 0.03) in Africans only. Also only in Africans, Δ-DBP was positively associated with circulating VWF (β = 0.23, p = 0.04). In Caucasians, Δ-DBP showed a negative association with circulating fibrinogen (β = −0.36, p = 0.0001). Resting T was negatively associated with circulating fibrinogen in Africans (β = −0.49, p = 0.0001) with only a tendency in Caucasians (β = −0.17, p = 0.09), as well as with circulating VWF in Africans (β = −0.41, p < 0.01).

To pursue our second perspective of assessing acute mental stress responses we repeated linear stepwise forward regression analyses. We therefore aimed to demonstrate associations between circulating dependent variables fibrinogen and VWF including covariates (Δ salivary testosterone, Δ cardiovascular, Δ coagulation markers and confounders) following our second perspective (Table 3: models 5–8). ΔD-dimer remained positively associated with both circulating fibrinogen and VWF in the Africans and ΔDBP with VWF in the Africans only. In Caucasians, Δ-DBP showed a negative association with circulating fibrinogen (beta = −0.32, p = 0.001). ΔT was positively associated with VWF in the Africans (beta = 0.27, p = 0.01) but not with fibrinogen.

When either of T or the acute mental stress response of D-dimer was left out of the model, the association of the remaining variable with fibrinogen did not change indicating independent associations. However, when any of the acute stress responses (ΔD-dimer, Δ salivary T or ΔDBP) was left out of the model with VWF (dependent variable), the other variables remained in the model (p < 0.05) with unchanged β-values but with greatly reduced R^2-values. This indicates that ΔD-dimer, Δ salivary T and ΔDBP are interdependent in their association with VWF. Therefore, in models 3 and 7, interdependent associations between the independent variables were evident for VWF % in the African only. This may indicate endothelial risk. Augmented Δ-DBP, ΔD-Dimer and Δ salivary T acute stress responses and a low resting T environment were associated with elevated VWF % (p < 0.05).

4. Discussion

The aim of this study was to investigate the possible association between testosterone, acute stress and hypercoagulation in a biethnic male cohort. Main findings revealed a negative association between serum T and circulating VWF as well as a positive association between salivary T stress responses and circulating VWF. We also found interdependence between Δ salivary testosterone, ΔD-dimer and Δ vascular responses in their association with VWF, a marker of endothelial dysfunction and an increased risk for stroke events and cardiovascular disease in Africans.

4.1. Cardiovascular risk in Africans

Although the African and Caucasian groups differ in cultural environment, they were similar in socio-economic status (similar income, educational level and employment) and age. The African group's cardiometabolic health was in a poorer condition than that of the Caucasians with mean hypertensive blood pressure [2], low grade

Table 2
Forward stepwise regression analyses predicting relationships between coagulation, testosterone and acute cardiovascular STROOP responses.

Resting serum fibrinogen		
	Model 1: Africans	Model 2: Caucasians
Adjusted R^2	0.28	0.35
	β (±95% CI)	β (±95% CI)
ΔD-dimer	0.33 (0.13, 0.54), p < 0.02	–
ΔDBP	–	−0.36 (−0.53, −0.36), p < 0.0001
Testosterone (nmol/L)	−0.49 (−0.70, −0.28), p < 0.0001	−0.17 (−0.38, 0.03), p = 0.09
Resting von Willebrand factor		
	Model 3: Africans	Model 4: Caucasians
Adjusted R^2	0.24	0.15
	β (±95% CI)	β (±95% CI)
ΔD-dimer	0.24 (0.03, 0.46), p = 0.03	–
ΔDBP (mm Hg)	0.23 (0.01, 0.45), p = 0.04	–
Testosterone (nmol/L)	−0.41 (−0.66, −0.15) p < 0.01	–

β denotes standardised regression coefficient. Acute mental stress changes (delta, Δ) were calculated as STROOP values/Resting values. Covariates considered for models 1–4: Age, body surface area, physical activity, log γ-GT, and anticoagulants.

inflammation and pre-diabetic (HbA1c > 5.7%) status values. It is known that BP as well as coagulation factors like fibrinogen [11] and VWF [32] increase with age. However, after adjusting for age these values remained unchanged (data not shown). In accordance with Pieper et al., we found that the levels of the coagulation factors, VWF, fibrinogen and D-dimer were race related with higher values in Blacks than in Caucasians [33]. It is also known that increased BP as well as coagulation factors are risk factors for coronary artery disease (CAD) and other target organ damage (TOD) like renal impairment [8]. Sechi et al. also found a strong and independent association between fibrinogen as well as D-dimer and the presence and severity of hypertension-related TOD [8]. The fact that these variables were higher in the African group may indicate a higher risk in this group for the development of CAD and TOD.

Table 3
Forward stepwise regression analyses predicting relationships between coagulation and acute testosterone and cardiovascular STROOP responses.

Resting serum fibrinogen		
	Model 5: Africans	Model 6: Caucasians
Adjusted R^2	0.17	0.25
	β (±95% CI)	β (±95% CI)
ΔD-dimer	0.30 (0.08, 0.52), p = 0.03	–
ΔDBP	–	−0.32 (−0.51, −0.14), p = 0.001
Δsaliva testosterone	–	–
Resting von Willebrand factor		
	Model 7: Africans	Model 8: Caucasians
Adjusted R^2	0.29	0.14
	β (±95% CI)	β (±95% CI)
ΔD-dimer	0.24 (0.03, 0.44), p = 0.03	–
ΔDBP	0.37 (0.16, 0.57), p = 0.001	0.23 (0.03, 0.44), p = 0.03
Δsaliva testosterone	0.27 (0.07, 0.47), p = 0.01	–

β denotes standardised regression coefficient. Acute mental stress changes (delta, Δ) were calculated as STROOP values/Resting values. Covariates considered for models 1–4: Age, body surface area, physical activity, log γ-GT, and anticoagulants.

4.2. Low testosterone environment and cardiovascular risk

Serum T decreases with age and a low T environment is associated with increased BP and TOD in Caucasians as well as in Africans [6,7]. Furthermore, a low T environment will induce higher levels of coagulation factors like fibrinogen [6]. The serum levels of coagulation factors like fibrinogen and VWF will also increase with age [32]. Here, we showed that higher VWF is directly associated with a low T environment. The age adjusted T values were lower in Africans than in Caucasians and that may have contributed to their higher BP and coagulation factor values. The potential role of a low T environment on the BP of the Africans is supported by another SABPA sub-study where T was negatively associated with nocturnal SBP as well as daytime and nocturnal DBP [5].

4.3. Cardiovascular and coagulation acute mental stress responses

Acute and chronic mental or psychosocial stress triggers sympathetic nervous system hyperactivity and have been shown to be associated with increased α-adrenergic vascular responsiveness, pressure overload, hypertension and myocardial ischemic risk in Africans [2,34]. Previously, we have demonstrated that 24 h SBP predicted structural vascular changes and remodelling in low T Africans [5]. Interdependence between associated VWF, augmented ΔT, ΔD-dimer, Δ vascular DBP responses and a low T environment underscores the impact of α-adrenergic vasoconstrictive responses on the endothelium.

For Caucasians, there was only a negative association between coagulation markers and DBP stress responses suggesting less vasoconstriction [2] improving endothelial function [35].

Conversely, the increased DBP in the Africans associated with low T [5] strengthens the notion that a low T environment will enhance VWF levels and, therefore, also blood coagulability. The interdependent association between ΔD-dimer and ΔDBP in the Africans is an indication that acute stress may exert joint influences on coagulation factors and blood pressure. These results are in line with the findings of Wakabayashi and Masuda who found significant correlations of pulse pressure with blood fibrinolysis markers such as D-dimer [36]. It is well known that acute and chronic stress is positively associated with coagulation factors [10]. However in our study, acute stress responses (ΔD-dimer, ΔDBP and ΔT) revealed associations with VWF and fibrinogen only in the Africans and not in the Caucasians. Our previously reported result of an increased vulnerability of the Africans to a low T environment was again confirmed [5,7]. As the CWC test provokes an acute stress reaction which may simulate everyday stress situations there is a possibility that Africans are also more susceptible to the stressful situation in every-day life such as teachers in a very demanding environment [Jackson et al., 2006]. Job stress is one of the most important types of chronic stress associated with increased plasma fibrinogen and, therefore, a hypercoagulable state [37]. It may seem that the Africans find their working environment more stressful than their Caucasian counterparts [38]. It might translate into a less favourable cardiovascular health profile in general and increased VWF in particular.

There exists an independent association between fibrinogen, ΔT and ΔD-dimer responses but that is not the case with VWF. This indicates that ΔD-dimer, ΔT and ΔDBP are interdependent in their association with VWF and ultimately there may be a synergistic action on VWF when two or all three of these variables are present. To our knowledge, this is novel data where a low T environment will directly affect the endothelium as shown by VWF although it is well known that an association do exist between low T and fibrinogen [6].

Several limitations should be noted. The sex hormones were sampled only once, thus circadian patterns could not be considered. The cross sectional design of the current study prevents us from being inferring causality. Larger sample sizes are needed and should include interactions between sex hormone profiles and sympathetic drive. In addition, further data on autonomic and endothelial function are needed to delineate

possible physiological mechanisms at play. Direct immunoassays used in this study could have resulted in higher values in both the ethnic groups. Nevertheless, the assays used in the present study had intra- and inter-assay CVs below 10%.

We conclude that in Caucasians a low T environment is associated with elevated fibrinogen levels as has been reported previously, while no association between low T, ΔT and ΔD-dimer with circulating VWF could be observed. Blood coagulability and, therefore, the risk of cardiovascular thrombotic events and stroke in black Africans, may increase by the effect of a low T environment. If the individual is challenged during acute stress episodes the risk seems to increase as augmented ΔDBP, ΔD-dimer and ΔT responses predict increased levels of VWF, a marker of endothelial function. Indications were found that ΔT, ΔDBP and ΔD-dimer were interdependently associated with VWF but not with fibrinogen. These associations may set the stage for endothelial dysfunction, coronary artery disease and stroke risk in the African population.

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Procoagulant reactivity to laboratory acute mental stress in Africans and Caucasians, and its relation to depressive symptoms: The SABPA Study

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Summary

The risk of cardiovascular disease is dramatically increasing in Africans (black). The prothrombotic stress response contributes to atherothrombotic disease and is modulated by depressive symptoms. We examined coagulation reactivity to acute mental stress and its relation to psychological well-being in Africans relative to Caucasians (white). A total of 102 African and 165 Caucasian school teachers underwent the Stroop Color-Word Conflict test. Circulating levels of von Willebrand factor (VWF) antigen, fibrinogen, and D-dimer were measured before and after the Stroop. Cardiovascular reactivity measures were also obtained. All participants completed the Patient Health Questionnaire-9 and the General Health Questionnaire-28 for the assessment of depressive symptoms and total psychological distress, respectively. After controlling for covariates, resting levels of VWF, fibrinogen, and D-dimer were higher in Africans than in Caucasians (all p-values ≤0.006). Depressive symptoms and psychological distress were not significantly associated with resting coagulation measures. Stress reactivity in VWF ($p<0.001$) and fibrinogen ($p=0.016$), but not in D-dimer ($p=0.27$), were decreased in Africans relative to Caucasians with Africans showing greater reactivity of total peripheral resistance ($p=0.017$). Depressive symptoms, but not general psychological distress, were associated with greater VWF increase ($p=0.029$) and greater fibrinogen decrease ($p=0.030$) in Africans relative to Caucasians. In conclusion, Africans showed greater hypercoagulability at rest but diminished procoagulant reactivity to acute mental stress when compared with Caucasians. Ethnic differences in the vascular adrenergic stress response might partially explain this finding. Depressive symptoms were associated with exaggerated VWF reactivity in Africans relative to Caucasians. The clinical implications of these findings for Africans need further study.

Keywords

Cardiovascular disease, coagulation, depression, ethnicity, psychological stress

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Introduction

Enhanced coagulation, impaired fibrinolysis, endothelial activation, and hyperactive platelets play an important role in the development of atherogenesis, atherothrombosis, and acute coronary syndromes (ACS) (1). During acute mental stress, healthy individuals show a prothrombotic state that is viewed as an adaptive fight-flight response protecting the organism from excessive bleeding should injury occur (for review see [2, 3]). Fibrinogen, von Willebrand factor antigen (VWF:Ag) and D-dimer, the latter indicating fibrin turnover, are particularly responsive to acute mental stress (4, 5). Excessive stress procoagulant changes might potentially increase the risk of incident cardiovascular disease (CVD) and recurrent CVD events (3). For instance, stress-induced fibrinogen increase was associated with carotid artery stiffness (6) and predicted an increase in ambulatory systolic blood pressure (SBP) over three years in healthy individuals (7). Owing to impaired endothelial anticoagulant function, patients with atherothrombotic diseases showed greater platelet activation (8) and D-dimer increase (9) than controls free of CVD. Moreover, patients with emotional triggers like depressive feelings in the 2 hours before ACS onset, showed greater platelet aggregation during laboratory mental stress than patients reporting non-emotional triggers (10). Depressive symptoms have also been associated with stress-induced elevation in platelet reactivity (11) and D-dimer (12) in elderly subjects, thereby supporting the notion that hypercoagulability is one mechanism linking depression with an increased CVD risk (13).

Potential ethnical differences in the acute procoagulant response to stress and their relation to depressive symptoms have

not previously been explored. Such bio-behavioural research seems important for urbanised Africans in whom a concerning increase in CVD can be observed, much of it being a consequence of their transition from a traditional African to a modernised "Western" lifestyle (14). For instance, in Africans, the CVD risk predicted by fibrinogen becomes stronger with an increasing degree of urbanisation (15). Moreover, subclinical atherosclerosis, as measured by increased carotid intima media thickness (CIMT), was greater in Africans than in Caucasians (16) as well as in those with more severe depressive symptoms of both these ethnicities (17).

The autonomic nervous system and hypothalamic pituitary adrenal axis are the two major physiologic stress response systems in humans. As cortisol peaks between 15 and 30 minutes (min) after stress onset, prothrombotic changes within a few minutes of acute stress are largely governed by the sympathetic nervous system through release of catecholamines and adrenergic receptor stimulation (18, 19). Stress-hormone mediated stimulation of vascular beta2-adrenergic receptors releases VWF from endothelial cells (19-21). Stress-induced increases in D-dimer and in alpha-receptor activating norepinephrine are correlated with each other (5), but beta-adrenergic stimulation did not result in D-dimer increase (20). Compared to Caucasians, Africans show a greater alpha-adrenergic vascular response, which is reflected by a greater total peripheral resistance (TPR) (22). One specific hypothesis following from the above literature is that Africans would show greater D-dimer reactivity but lower VWF reactivity to acute stress than Caucasians. More severe depressive symptoms would hypothetically relate to greater procoagulant activity in both Africans and Caucasians.

The primary aim of this study was to investigate procoagulant reactivity between Africans and Caucasians through measurements of changes in circulating levels of VWF, fibrinogen and D-dimer to acute standardised laboratory stress. A secondary aim was to explore the association of depressive symptoms both continuously and categorically (i.e. based on a clinical cut-off level, with stress-induced coagulation changes. We further examined whether procoagulant reactivity would specifically relate to depressive mood or would rather be associated with elevated levels of general psychological distress.

Materials and methods

Study participants and design

The participants of this blood coagulation reactivity study were recruited as part of the Sympathetic Activity and Ambulatory Blood Pressure in Africans (SABPA) study conducted between February 2008 and May 2009. The study protocol was conducted in accordance with the Declaration of Helsinki (23) and was approved by the Ethics Review Board of the North-West University, Potchefstroom Campus (0003607S6). All participants provided written informed consent before participation. The SABPA study has a target population comparative design and recruited 409 teachers, aged 25–65 years, working in the Dr Kenneth Kaunda Education district in the North West Province, South Africa. This selection assured a homogenous sample from a similar socio-economic class. Exclusion criteria included pregnancy, lactation, and vaccination or blood donation within three months prior to participation.

For purposes of the present study, we excluded participants with a history of atherothrombotic disease (myocardial infarction, n=4; stroke, n=1), atrial fibrillation (n=16), and HIV-positive status (n=18), all of which are associated with hypercoagulability at rest and/or in response to acute stress (10, 24, 25), as well as participants with an ear temperature ≥37.5°C (n=3) because of a probable acute phase response that might increase coagulation factors like fibrinogen. Users of oral anticoagulants (n=2), aspirin (n=12), cortisone (n=3), oral contraceptives (n=21), antidepressants (n=3), anxiolytics (n=1), beta blockers (n=4), and calcium antagonists (n=12) were also excluded, as all of these might affect resting coagulation activity and/or coagulation reactivity with acute mental stress and/or mood (21, 26-29). To allow full multivariate statistical analyses, we also excluded subjects who missed data for coagulation measures (n=22), haemodynamic reactivity (n=10), psychological questionnaires (n=1), haemoglobin A1c (HbA1c) (n=1), creatinine (n=2), alcohol consumption (n=1), and physical activity (n=1). Finally, we excluded four participants because of excessively high D-dimer levels after stress (≥10,000 ng/ml), yielding a final study sample of 267 subjects. Excluded participants (n=142) were significantly more often Africans, women, and antihypertensive drug users, and they also had significantly higher screening systolic blood pressure (SBP), HbA1c, and gamma glutamyl transferase (γ-GT) levels than those included.

The participants were admitted at 4:30 PM to the multi-bedroomed Metabolic Unit Research Facility of the North-West University on Monday through Thursday. All received a standardised dinner at 06:00 PM after which they completed psychological questionnaires. They had their last beverages (tea/coffee) and biscuits at 8:30 PM followed by recreational activities such as reading, watching television, or interacting socially. We also obtained information on demographic data, general health, including medication, and health behaviours. All participants went to bed at around 10:00 PM and were woken at 05:45 AM. After the completion of anthropometric and manual BP measurements, a registered nurse obtained fasting blood samples which were handled according to standardised procedures. A sterile winged infusion set was left in situ with a heparin block (0.5 ml of a Heparin Sodium-Fresenius 5,000 IU/ml in 50 ml normal saline solution; Fresenius Kabi, Port Elizabeth, South Africa) to prevent clotting. Thereafter, all participants underwent the laboratory stress.

Acute laboratory stress testing

Mental stress test

All participants underwent the Stroop Color-Word Conflict test for 1 min. The Stroop is a standardised laboratory stressor showing reproducibility on cardiovascular reactivity (30). The Stroop requires identification of the ink colour of the word rather than the

name of the colour spelled by the word, under time pressure. The participants received a monetary motivation reward in line with performance. Perceived stress triggered by the Stroop was rated on a Likert scale (1="not at all stressful", 7="very stressful").

Cardiovascular data collection

With participants in a semi-recumbent position a registered nurse took a 5-min continuous measurement of resting cardiovascular parameters with the validated Finometer® device (Finapres Medical Systems, Amsterdam, the Netherlands) (31). The Finometer provides beat-by-beat BP and thus detects the full contour of the cardiovascular responses. The Finometer recorded the SBP, diastolic blood pressure (DBP) and heart rate (HR) and computed an integrated age dependent aortic flow curve from the surface area beneath the pressure/volume curve determining cardiac output (CO), stroke volume (SV), and total peripheral resistance (TPR) online (32); data were stored in the results files. Calculations of SV, CO, and TPR yield reliable measures (33, 34). We stabilised BP to resting state before participants underwent the Stroop. We obtained beat-to-beat BP responses throughout mental stress and the 5 min recovery phase. For analyses, we used the average of the last 2 min of the resting recordings and the averages of the last 15 seconds (s) of the stressor recordings as well as of the recovery recordings at 1, 3, and 5 min after the Stroop had ended. Cardiovascular reactivity was calculated as the area under the curve (AUC) with respect to increase from rest (35) across these five time points for SBP, DBP, HR, CO, SV, and TPR.

Blood collection

Citrated blood samples were taken before the Stroop to determine resting levels of coagulation measures. At 10 min post-stress, the infusion set was thoroughly flushed with 2-3 ml of saline, and the first 2 ml of blood were discarded before sampling was done for coagulation measures. Coagulation reactivity was calculated for each participant as the percentage change from the resting value.

Cardiometabolic risk factors

Body mass index

With participants in their underwear, we measured height and weight to the nearest 0.1 cm and 0.1 kg to calculate the body mass index (BMI, kg/m^2).

Screening blood pressure

After 5 min of rest, using a stethoscope and a mercury sphygmomanometer (auscultatory method), duplicate BP readings were taken 5 min apart and measurements averaged to obtain screening SBP and DBP.

Blood lipids

Total cholesterol (T-C) and high-density lipoprotein cholesterol (HDL-C) levels were measured in serum with the Konelab 20i (Thermo Fisher Scientific, Vantaa, Finland). For statistical analysis, we computed the T-C/HDL-C ratio.

Haemoglobin A1c

HbA1c levels were determined by a turbidometric inhibition immunoassay method from EDTA plasma (Integra 400, Roche, Basel, Switzerland).

Renal function

We used the Modification of Diet in Renal Disease (MDRD) Study equation to estimate glomerular filtration rate (eGFR) from creatinine levels, age, sex, and ethnicity (36). Creatinine was measured in serum using an enzymatic colorimetric test (Cobas Integra 400 plus, Roche, Basel, Switzerland).

Health behaviours

Smoking

Participants who indicated that they currently smoked and/or had smoked at least one cigarette per day during one year in the past were categorised as ever smokers.

Alcohol consumption

Serum levels of γ-GT activity were used as a marker of alcohol abuse (37) and measured with an enzymatic colorimetric assay (Cobas Integra 400 plus).

Physical activity

We used the Actical® accelerometers (Montréal, Québec) to quantify and index physical activity with 3 (vigorous intensity), 2 (moderate intensity) or 1 (light intensity) (38).

Coagulation measures

Blood processing

Citrated blood samples were centrifuged at 1,500 rpm (2,000 g) for 15 min at room temperature. Citrated plasma samples were aliquoted and frozen at -80°C until analysis. Fibrinogen and D-dimer were determined by an accredited laboratory.

Von Willebrand factor

Plasma VWF:Ag (%) were measured with a "sandwich" ELISA assay. A polyclonal rabbit anti-VWF antibody and a rabbit anti -VWF-HRP antibody (DAKO, Cape Town, South Africa) were

used to form the assay. The 6th International Standard for VWF/FVIII was used to set the standard curve against which the samples were measured (39).

Fibrinogen

Plasma fibrinogen levels (g/l) were determined using a viscosity-based method (STA Compact, STAGO Diagnostic, Roche, Asnieres, France).

D-dimer

Plasma D-dimer levels (ng/ml) were determined with an immuno-based method (STA Compact, STAGO). There were 47.6% and 39.7%, respectively, of D-dimer values at rest and after stress below the limit of detection (<220 ng/ml). Undetectable values were substituted using the maximum likelihood method that generates values from the lognormal distribution of uncensored data under the assumption that censored values follow a lognormal distribution.

Psychological questionnaires

Depressive symptoms

We used the 9-item Patient Health Questionnaire (PHQ-9) to measure the frequency of depressive symptoms during the prior two weeks corresponding to criteria in the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (40). Each item is rated on a 4-point scale (0="not at all", 1="several days", 2= "more than half the days", 3="nearly every day") giving a possible global score of 0-27. PHQ-9 scores ≥10 have 88% sensitivity and specificity for major depression (40). In our sample, Cronbach's α was 0.82 for Africans and 0.84 for Caucasians for the total scale.

Psychological distress

With the 28-item General Health Questionnaire (GHQ-28), we quantified perceived psychological distress in general related to symptoms of depression, anxiety, somatic complaints and social withdrawal over the past few weeks applying the binary scoring method. The global score provides a severity measure of total psychological distress. If exceeding the threshold of 4, subjects are classified as achieving "psychiatric caseness" (41). In our sample, Cronbach's α for the total scale was 0.91 for Africans and 0.90 for Caucasians.

Statistical analysis

We used SPSS version 21.0 for Windows to analyse the data with significance level at $p<0.05$ (two-tailed). In case of a non-normal distribution, dependent variables were log transformed; for clarity, all data are given in original units. Chi-square test and Student's t-test with Levene's correction in the case of unequal variances were applied for data comparison between groups. We used multivariate analysis of variance (MANOVA) and of covariance (MANCOVA) to test for a significant association of ethnicity, PHQ-9 measures, GHQ-28 measures, and cardiovascular reactivity measures with coagulation measures. We only deemed results of individual coagulation measures to be significant if the MAN(C)OVA test statistic was significant, as this procedure protects against inflated Type I errors due to multiple tests of (likely) correlated dependent variables. Covariates were selected a priori based on the literature about demographic, cardiometabolic and life style factors affecting circulating levels of coagulation measures (42-44). Effect sizes are expressed as partial eta squared (ηp^2) or partial correlation coefficients.

Results

Participant characteristics

►Table 1 shows the demographic and health characteristics of the 267 study participants per ethnic group. The percentage of men was higher in Africans than in Caucasians. In terms of cardiometabolic risk factors, Africans showed higher BMI, HbA1c, and screening BP – despite using more antihypertensives – than Caucasians, while Caucasians had a higher T-C/HDL-C ratio than Africans. Of the 20 subjects who took antihypertensive medications, nine had thiazide diuretics, six had angiotensin-converting enzyme (ACE) inhibitors, two had angiotensin receptor blockers (ARB), and three had combination therapy (i.e. thiazide diuretic plus ACE inhibitor or ARB). Regarding health behaviours, Africans were physically less active and had higher γ-GT indicating more alcohol abuse than Caucasians. Africans were significantly more depressed and they also perceived more distress than Caucasians.

Coagulation measures at rest

Ethnicity

Both MANOVA ($F_{3,263}=43.28$, $\eta p^2=0.330$, $p<0.001$) and MANCOVA ($F_{3,252}=36.31$, $\eta p^2=0.302$, $p<0.001$) showed a significant association between ethnicity and resting levels of the three coagulation measures. ►Table 2 shows that compared with Caucasians, Africans had significantly higher resting levels of VWF, fibrinogen, and D-dimer in the unadjusted as well as in the adjusted analyses.

Psychological questionnaires

To test for an association of PHQ-9 and GHQ-28 measures with resting levels of coagulation measures, we reran the above MANCOVA with continuous and categorical PHQ-9 and GHQ-28 measures as additional covariates (four separate models). Ethnicity remained associated with coagulation measures in all of these models (all p-values <0.001). However, coagulation measures showed no significant association with continuous PHQ-9 scores (p=0.46), categorical PHQ-9 scores (p=0.071), continuous GHQ-28 scores (p=0.59), and GHQ-28 caseness (p=0.47).

Interaction between ethnicity and psychological questionnaires

Taking into account main effects of ethnicity and PHQ-9 and GHQ-28 scores, the interaction terms between ethnicity and the continuous as well as categorical PHQ-9 and GHQ-28 scores were not significant (all p-values >0.10).

Stress-induced changes across all participants

The mean level of perceived stress from the Stroop was 3.70 ± 1.64 (range 0-7). Mental stress provoked significant responses in coagulation and haemodynamic measures. While VWF levels increased from 74.9 ± 26.1% to 99.9 ± 40.1% (p<0.001), fibrinogen levels decreased from 3.18 ± 0.69 g/l to 2.88 ± 0.68 g/l (p<0.001). D-dimer levels showed no significant change (320 ± 339 ng/ml vs 376 ± 503 ng/ml, p=0.44). Moreover, SBP (134.4 ± 16.2 mmHg vs 154.2 ± 20.8 mmHg, p<0.001), DBP (78.6 ± 9.3 mmHg vs 88.6 ± 11.4 mmHg, p<0.001), HR (66.8 ± 10.7 bpm vs 87.4 ± 16.6 bpm, p<0.001), and CO (6.59 ± 1.81 l/min vs 8.12 ± 2.26 l/min, p<0.001) all increased and SV (99.6 ± 23.9 ml vs 94.2 ± 23.1 ml, p<0.001) and TPR (1.02 ± 0.46 mmHg/ml/s vs 0.99 ± 0.51 mmHg/ml/s, p=0.013) both decreased.

Association between haemodynamic and coagulation reactivity

Reactivity measures of VWF:Ag, fibrinogen, and D-dimer were not significantly correlated with each other (all p-values >0.25). Separate MANOVA models for AUC measures of haemodynamic variables showed that reactivity in DBP ($F_{3,263}$=3.83, ηp^2=0.042, p=0.010), SV ($F_{3,263}$=3.35, ηp^2=0.037, p=0.020), and TPR ($F_{3,263}$=2.74, ηp^2=0.030, p=0.044) were significantly associated with coagulation reactivity, whereas reactivity in SBP (p=0.44), HR (p=0.83), and CO (p=0.52) were not. Correlation analysis on individual measures revealed that VWF reactivity increased with greater SV reactivity (r=0.18, p=0.003), but decreased with greater reactivity in DBP (r=-0.18, p=0.003) and TPR (r=-0.14, p=0.022). Fibrinogen and D-dimer reactivity were not significantly associated with DBP, SV, and TPR reactivity (all p-values >0.12).

Stress-induced changes and ethnicity

Perceived stress

Africans (3.69 ± 1.87) and Caucasians (3.70 ± 1.49) perceived the Stroop protocol as similarly stressful (p=0.94).

Coagulation reactivity

Using MANOVA (p=0.19) and MANCOVA (p=0.62) tests, there were no significant associations between ethnicity and post-stress levels of coagulation measures (see ►Table 2 for unadjusted and adjusted post-stress levels of VWF, fibrinogen, and D-dimer). However, there were significant association between ethnicity and coagulation reactivity in MANOVA ($F_{3,263}$=13.73, ηp^2=0.135, p<0.001) and MANCOVA ($F_{3,251}$=16.86, ηp^2=0.168, p<0.001) tests. ►Table 1 shows that relative to Caucasians, Africans experienced lower procoagulant reactivity with less of an increase in VWF reactivity and more of a decrease in fibrinogen reactivity; D-dimer reactivity showed no significant difference between Africans and Caucasians.

Haemodynamic reactivity

MANCOVA showed a significant association between ethnicity and AUC measures of haemodynamic variables ($F_{6,248}$=3.18, ηp^2=0.072, p=0.005). Relative to Caucasians, Africans had lower SBP AUC (29.3 ± 5.8 vs 45.1 ± 4.3, ηp^2=0.016, p=0.043), and lower SV AUC (–32.3 ± 8.1 vs 5.4 ± 6.1, ηp^2=0.049, p<0.001), but greater TPR AUC (0.40 ± 0.27 vs –0.30 ± 0.20, ηp^2=0.029, p=0.007). Ethnicity was not related to AUC measures of DBP (p=0.46), HR (p=0.12), and CO (p>0.09). Ethnicity did not significantly interact with AUC measures of any cardiovascular parameter to determine coagulation reactivity (all p-values >0.18).

Table 1: Characteristics of 267 study participants.

Variable	Africans (n=102)	Caucasians (n=165)	P-value
Gender (% men)	62.7	49.7	0.037
Age (years)	43.4 ± 8.2	44.1 ± 11.0	0.580
Body mass index (kg/m^2)	29.9 ± 7.8	27.6 ± 5.6	0.008
Total cholesterol (mmol/l)	4.76 ± 1.17	5.50 ± 1.22	<0.001
HDL-cholesterol (mmol/l)	1.15 ± 0.36	1.20 ± 0.41	0.339
Total cholesterol/HDL-cholesterol ratio	4.51 ± 1.86	4.98 ± 1.60	0.028
Systolic blood pressure (mmHg)	134.4 ± 19.6	126.7 ± 13.5	<0.001
Diastolic blood pressure (mmHg)	88.4 ± 12.6	83.1 ± 9.6	<0.001
Hemoglobin A1c (%)	6.05 ± 1.12	5.48 ± 0.41	<0.001
Creatinine (µmol/l)	77.6 ± 13.5	74.2 ± 15.3	0.069
Estimated GFR (ml/min/1.73 m^2)	114.7 ± 29.3	112.0 ± 27.2	0.453
Ever smoker (%)	25.5	21.2	0.419
Gamma glutamyl transferase (U/l)	64.6 ± 75.8	26.9 ± 36.2	<0.001
Physical activity index	1.33 ± 0.57	1.62 ± 0.70	0.001
Antihypertensive drugs (%)	12.7	4.2	0.010
Patient Health Questionnaire-9 (scores)			
Total depressive symptoms	9.40 ± 5.60	5.70 ± 4.73	<0.001
Moderate depression (cut-off ≥10) (%)	46.1	17.6	<0.001
General Health Questionnaire-28 (scores)			
Total psychological distress	8.50 ± 6.71	3.79 ± 4.80	<0.001
Cases (cut-off >4) (%)	64.7	33.3	<0.001

Data are given as means ± SD or percentage values. P-value refers to group differences. GFR, glomerular filtration rate; HDL, high-density lipoprotein.

Psychological questionnaires

After controlling for ethnicity MANCOVA tests showed no significant associations of continuous PHQ-9 scores (p=0.71), categorical PHQ-9 scores (p=0.94), continuous GHQ-28 scores (p=0.18), and GHQ-28 caseness (p=0.91) with coagulation reactivity. However, there were significant interactions of ethnicity with continuous PHQ-9 scores ($F_{3,249}$=3.03, ηp^2=0.035, p=0.030) and categorical PHQ-9 scores ($F_{3,249}$=3.80, ηp^2=0.044, p=0.011), but not with continuous GHQ-28 scores (p=0.24) and GHQ-28 caseness (p=0.62).

Between-subject analysis revealed significant associations between ethnicity and continuous PHQ-9 scores for reactivity of VWF (ηp^2=0.016, p=0.044) and fibrinogen (ηp^2=0.020, p=0.026), but not for D-dimer reactivity (p=0.54). Partial correlation coefficients differed significantly between ethnic groups for VWF reactivity (p=0.029) and for fibrinogen reactivity (p=0.030). With greater continuously scaled PHQ-9 scores, indicating greater severity of depressive symptoms, VWF increased more in Africans (r=0.202) than in Caucasians (r=-0.074), whereas fibrinogen decreased more in Africans (r=-0.215) than in Caucasians (r=0.058).

Similarly, ethnicity interacted with categorical PHQ-9 scores in determining VWF reactivity (ηp^2=0.023, p=0.015) and fibrinogen reactivity (ηp^2=0.018, p=0.035), but not D-dimer reactivity (p=0.28). ▶Figure 1 illustrates these interactions. Partial correlation coefficients significantly differed between groups for VWF reactivity (p=0.035) and for fibrinogen reactivity (p=0.047). PHQ-9 scores ≥ 10 were directly correlated with VWF reactivity in Africans (r=0.149), but inversely so in Caucasians (r=-0.119). Moreover, PHQ-9 scores ≥ 10 were inversely correlated with fibrinogen reactivity in Africans (r=-0.187), but directly so in Caucasians (r=0.064).

We performed six complementary univariate analyses of covariance to test whether sympathetic activity as indicated by haemodynamic reactivity might relate to the direct association between continuous PHQ-9 scores and VWF reactivity in Africans vs Caucasians. However, all six three-way-interactions between ethnicity, continuous PHQ-9 scores and AUC measures of any haemodynamic parameter were non-significant (all p-values ≥0.12).

Discussion

The main finding from our study is that Africans showed lower procoagulant reactivity in response to standardised acute laboratory mental stress when compared with Caucasians, controlling for a range of potentially confounding variables. Relative to Caucasians, VWF levels had increased less and fibrinogen levels had decreased more in Africans after stress relative to resting levels. One explanation for the diminished increase in VWF in Africans could be a "ceiling effect" as Africans started out with higher resting levels of VWF than Caucasians. For instance, previous studies in apparently healthy individuals reported stress-induced increases of VWF of 6%, 9%, and 30% from resting levels which were 91%, 98%, and 95%, respectively, in these studies (4, 45, 46). This amount of an increase in VWF is clearly lower than in our Caucasian subjects who showed an increase in VWF of 60% starting out from average resting values of 64%, but similar to our African subjects who showed an increase in VWF of 18% from average

Table 2: Coagulation measurements per ethnic group at rest and after mental stress.

Condition	Adj.	von Willebrand factor				Fibrinogen				D-dimer			
		Africans	Caucasians	ES	P	Africans	Caucasians	ES	P	Africans	Caucasians	ES	P
Rest	–	93.2 ± 28.4%	63.6 ± 16.5%	0.330	<0.001	3.35 ± 0.82 g/l	3.07 ± 0.57 g/l	0.030	0.004	354 ± 340 ng/ml	298 ± 338 ng/ml	0.015	0.044
	+	94.2 ± 2.4%	62.9 ± 1.8%	0.291	<0.001	3.33 ± 0.06 g/l	3.09 ± 0.05 g/l	0.029	0.006	379 ± 37 ng/ml	283 ± 28 ng/ml	0.030	0.005
Post-stress	–	101.8 ± 34.1%	98.7 ± 43.4%	0.010	0.101	2.95 ± 0.71 g/l	2.84 ± 0.65 g/l	0.006	0.208	398 ± 515 ng/ml	363 ± 496 ng/ml	0.004	0.302
	+	97.5 ± 4.4%	101.4 ± 3.3%	<0.001	0.817	2.91 ± 0.07 g/l	2.86 ± 0.05 g/l	0.001	0.593	426 ± 56 ng/ml	345 ± 42 ng/ml	0.006	0.217
Reactivity	–	18.1 ± 53.6%	60.1 ± 70.2%	0.110	<0.001	–11.2 ± 11.6%	–7.4 ± 12.6%	0.022	0.016	64.5 ± 241.9%	74.5 ± 319.9%	0.002	0.499
	+	10.2 ± 7.0%	65.0 ± 5.2%	0.140	<0.001	–11.7 ± 1.4%	–7.2 ± 1.0%	0.023	0.016	48.1 ± 32.6%	84.7 ± 24.6%	0.005	0.270

Data are given as means ± SD for unadjusted values (–) and as means ± SEM for adjusted values (+) with p-values for group comparisons. Adjustment was made for gender, age, body mass index, total cholesterol/high-density cholesterol ratio, screening systolic blood pressure, haemoglobin A1c, estimated glomerular filtration rate, smoking, gamma glutamyl transferase, physical activity, and antihypertensive drugs for resting values and additionally for perceived stress with the Stroop test for post-stress and reactivity values. Reactivity measures are expressed as percentage change of post-stress values from resting values. Adj., adjustment; ES, effect sizes (partial eta-squared).

resting levels of 93%. In contrast, a "floor effect" in Caucasians who started out on relatively lower resting fibrinogen levels might account for the greater decrease in post-stress fibrinogen levels relative to resting levels in Africans. Another explanation for the blunted VWF reactivity in Africans might be their alpha-adrenergic vascular stress response, as was suggested by their increased TPR reactivity (22). Across all participants, lower VWF reactivity correlated with both greater TPR reactivity and lower SV reactivity, the latter likely reflecting increased afterload due to elevated TPR (22). Moreover, in Caucasians, the systemic vasodilation response during the Stroop test is largely mediated by the beta2-adrenergic receptor (47) whose stimulation through stress hormones will result in endothelial release of VWF into the circulation (19-21).

A decrease in fibrinogen levels between rest and 10 min after the Stroop was seen across all study participants, and to an even greater extent in Africans than Caucasians. This is contrary to expectations, as fibrinogen levels were shown to increase in response to acute mental stress in several previous studies (2-4). The Stroop protocol provoked an average level of psychological distress that compares to similar protocols (10). However, previous studies suggest that the Stroop alone might be less effective in provoking a significant fibrinogen response (48) than combinations of the Stroop with other stressors (e.g. mental arithmetic) (45) or speech stressors (4). As there was no correlation of VWF reactivity with both fibrinogen and D-dimer reactivity in the present study, but in a previous one (49), it is also possible that the 1-min stressor was too short to evoke a significant fibrinogen increase with fibrin formation and degradation further downstream. Nevertheless, previous studies showed coagulation and fibrinolysis activation within 2-5 min of acute mental stress (50, 51) and infusion with the stress hormone epinephrine (52). D-dimer did not increase in a previous study that combined the Stroop with mental arithmetic (53), but in studies that applied speech stressors to inflict social evaluative threat (5, 9). The sensitivity of the various D-dimer assays used in previous stress studies might also explain heterogeneous results (54). Saying that, previous stress reactivity studies also used different methods to measure fibrinogen (e.g. the functional Clauss method) (4) and VWF (e.g. enzyme-linked immunosorbent assays) (46). To our knowledge, a direct comparison of the sensitivity between different assays to detect reactivity in prothrombotic measures has not been performed, but would seem important to reconcile heterogeneous study findings.

Coagulation reactivity was specifically associated with depressive symptoms (i.e. PHQ-9 scores) as opposed to more general psychological distress (i.e. GHQ-28 scores). More severe depressive symptoms were associated with greater VWF reactivity in Africans compared with Caucasians. This concurs with the notion that depressive mood along a continuum of severity is associated with an increased risk of incident CVD and recurrent cardiac events (55). However, post-stress levels of VWF and fibrinogen were similar in Africans and Caucasians, fibrinogen decreased more in depressed Africans than depressed Caucasians, and VWF reactivity was, on the whole, lower in Africans than in Caucasians; therefore, a depression-associated increase in CVD risk in Africans through a

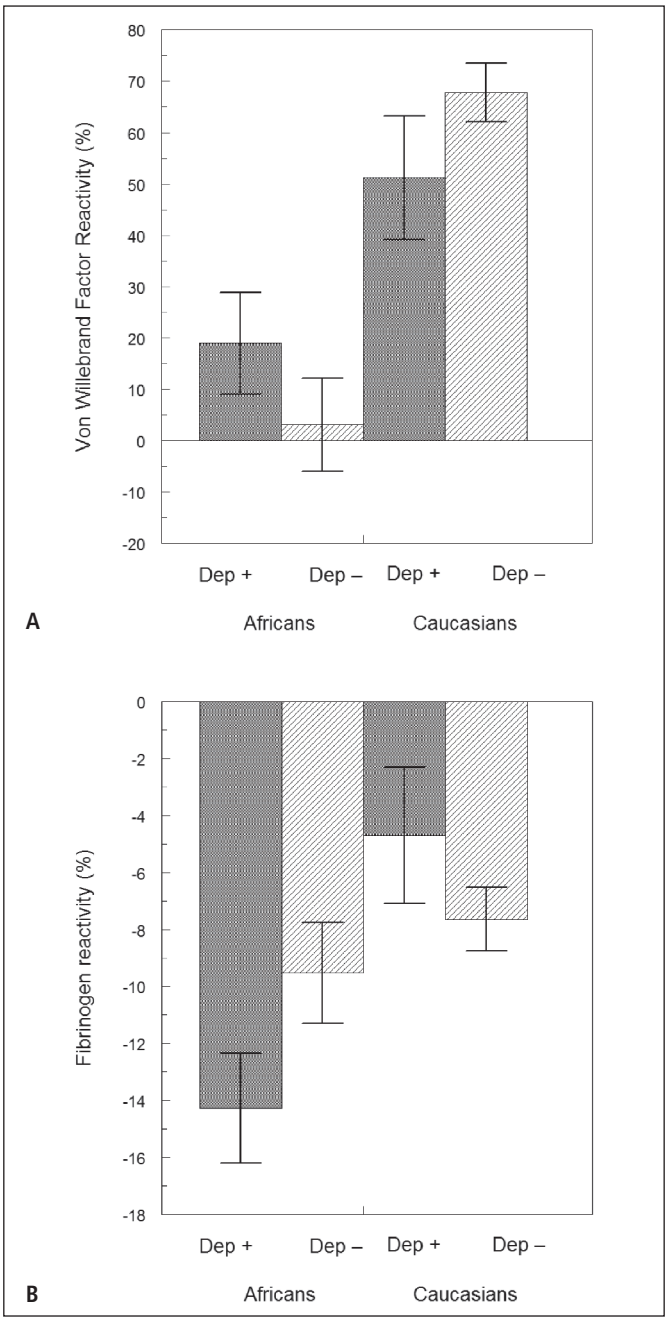

Figure 1: Coagulation reactivity related to ethnicity and depression. Depicted are multivariate-adjusted interaction effects between ethnicity and categorical depression (i.e. score ≥10 on the PSQ-9 total depressive symptom scale) on von Willebrand factor (VWF) and fibrinogen reactivity (i.e. relative change in levels of coagulation measures in % from resting levels to 10 min after the Stroop test). Relative to non-depressed individuals (Dep –) of the respective ethnicity, VWF reactivity was significantly greater in Africans vs Caucasians with depression (Dep +). Fibrinogen reactivity showed a significantly greater decrease in depressed Africans than in depressed Caucasians (see text for detailed statistics). Africans/Dep + (n=47), Africans/Dep – (n=55), Caucasians/Dep + (n=29), Caucasians/Dep – (n=136).

pathway of stress-induced VWF increase would need to be shown in prospective studies.

Ethnical differences in coagulation are still poorly understood with studies showing enhanced clotting but also increased bleeding tendency in various clinical settings in Africans compared with Caucasians (56). We found resting levels of VWF:Ag, fibrinogen, and D-dimer to be higher in Africans than Caucasians, which is in agreement with some previous studies (57-59). Theoretically, this potential predisposition to hypercoagulability might contribute to the CVD risk in Africans. In contrast, lower increase in VWF levels and greater decrease in fibrinogen levels both with acute mental stress could be viewed a hypoactive fight-flight response (60). As opposed to constitutively released VWF, the VWF released from the endothelium after stimulation consists of large and haemostatically highly active multimers (61). Moreover, although not investigated in our study, acute stress also activates fibrinolysis (2, 45, 50). Therefore, the inability to mount physiologic hypercoagulability might subject Africans to an increased risk of bleeding in fight-flight situations either upon injury or even spontaneously. Therefore, the almost absent VWF response in the non-depressed Africans might be as harmful as the increased VWF reactivity in the depressed Africans for the maintenance of the haemostatic balance between thrombosis and haemorrhage during acute mental stress.

Rigorous selection of participants on medical characteristics to minimise confounding of coagulation measures was a strength of our study, but also might reduce generalisability of study findings to the larger African population and those with established CVD. We selected three coagulation measures that were previously shown to be stress-responsive. However, they do not cover the entire dynamics of the coagulation and fibrinolysis pathways during acute mental stress. A speech stressor inflicting social evaluative threat and measuring coagulation measures longer into the recovery period from stress might have provided additional informative data. The Stroop test provoked substantial increases in BP and HR, but there is an ongoing debate as to whether cardiovascular reactivity data gained from standardised lab stressor are generalisable to real-life situations (62). We controlled our analysis for antihypertensive medications as a group but due to insufficient statistical power could not take into account potential class effects of the different antihypertensives on outcomes. We did not include a non-stress control group. Therefore we were unable to account for circadian changes in coagulation measures, particularly a decrease in plasma fibrinogen levels during the morning hours. We are unable to account for the possibility that stress-related changes in clearance impacted plasma concentrations of coagulation measures. For instance, glycosylation of VWF influences its clearance by the liver, which, moreover, occurs more rapidly in individuals with blood group O than in non-O individuals (63); unfortunately, information on ABO blood group was not available in our study. The difference in liver function between Africans and Caucasians seems not an apparent explanation for the ethnic difference in VWF reactivity because the relation between ethnicity and procoagulant reactivity persisted after adjustment for γ-GT activity.

Taken together, Africans seem to have lower procoagulant reactivity with acute mental stress than Caucasians. This observation might partially be explained by ethnic differences in the vascular stress response. Depressive symptoms may modulate coagulation reactivity against an ethnic background. The potential clinical implications of our findings for CVD risk and bleeding disorders in Africans need further studies.

What is known about this topic?

- Acute mental stress induces a hypercoagulable state that may contribute to atherothrombotic diseases.
- Depressive symptoms are associated with the procoagulant stress response.
- There are ethnic differences in coagulation between Africans and Caucasians.

What does this paper add?

- Africans show lower procoagulant reactivity in response to acute laboratory mental stress than Caucasians.
- Explanation might be higher resting levels of coagulation factors in Africans than Caucasians and ethnic differences in the vascular stress response.
- Depressive symptoms are associated with greater stress reactivity of von Willebrand factor in Africans relative to Caucasians.

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Conflicts of interest

None declared.

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S-33

HIV-associated Thrombotic Thrombocytopenic Purpura – What We Know So Far

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Abstract

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening disease characterised by microvascular platelet deposition and thrombus formation in selected organs, resulting in microangiopathic haemolytic anaemia, thrombocytopenia, neurological symptoms and renal failure. Typically a very rare disorder, TTP is being seen with increased frequency in patients infected with the human immunodeficiency virus (HIV). Deficiency of the von Willebrand factor cleavage protease, ADAMTS13, has been implicated as the cause of TTP. However, the pathophysiology of HIV-associated TTP and the thrombotic potential in these patients are not known. This article provides not only an overview of the literature regarding HIV-associated TTP, but also presents new data on this disease. We propose a mechanism for the initial onset of HIV-associated TTP that includes the release of extreme amounts of von Willebrand factor and the downregulation of ADAMTS13 and/or the production of autoantibodies to ADAMTS13.

Keywords

Thrombotic thrombocytopenic purpura, HIV, ADAMTS13, anti-ADAMTS13 antibodies, von Willebrand factor

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Thrombotic thrombocytopenic purpura (TTP) is an acute prothrombotic disorder resulting from a deficiency of the von Willebrand factor cleavage protease ADAMTS13.[1] The enzyme ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 repeats) cleaves the peptide bond between Tyr-842 and Met-843 of the mature subunit of von Willebrand factor (VWF) and prevents the interactions of the largest VWF multimers with platelets. In the plasma of patients with TTP, ultra large VWF multimers (ULVWF) have been observed that provoke widespread microvascular thrombosis.[2] TTP is characterised by microvascular platelet aggregation and thrombus formation resulting in thrombocytopenia, microangiopathic haemolytic anaemia, variable renal and neurologic dysfunction and fever.[3] Infection with the human immunodeficiency virus (HIV) is postulated as a direct precipitant of TTP, presumably through infection of vascular endothelial cells resulting in dysfunction, localised thrombin generation and consumption of ADAMTS13.[4] HIV-associated TTP was first described in 1987 by Jokela et al.[5] Since then, several case studies have been reported. The occurrence of TTP in association with HIV infection is now well recognised.[6,7]

Prevalence of HIV-associated Thrombotic Thrombocytopenic Purpura

Although congenital TTP is a very rare disease, the incidence of HIV-associated TTP is much higher. A group in South Africa estimated the incidence of TTP in HIV-infected individuals to be 15–40 times that in non-infected individuals.[8] Also, more than 80 % of TTP cases in South Africa are found to be HIV-related and it is expected that the incidence of HIV-associated TTP will continue to rise.[9] HIV-associated TTP cases were also described in other countries, such as the UK and Italy.[10–12]

Features and Treatment of HIV-associated Thrombotic Thrombocytopenic Purpura

HIV-associated TTP is a haematologic disorder observed in patients infected with HIV. As with congenital non-HIV-associated TTP, this syndrome is characterised by microangiopathic haemolytic anaemia, thrombocytopenia, renal dysfunction, fluctuating neurological abnormalities and fever. Treatment regimens include highly active antiretroviral therapy (HAART), infusion of fresh frozen plasma, plasma exchange, steroids and immunomodulatory agents.

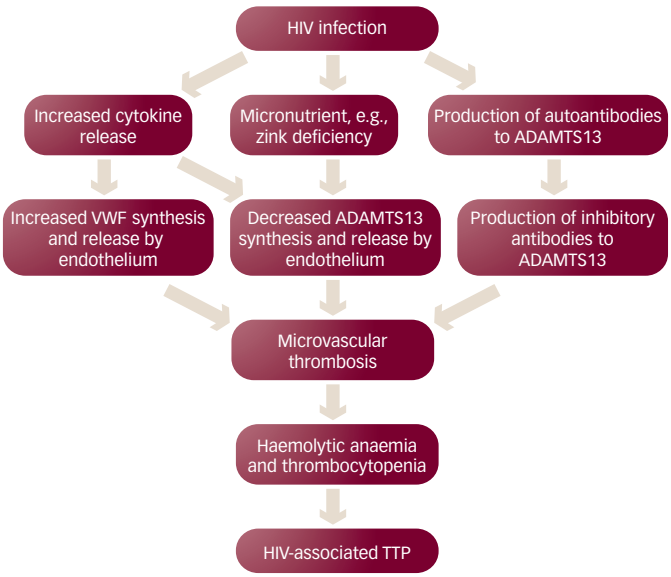
One case study stressed the importance of CD4 count and viral loads in HIV-associated TTP.[11] The patient presented with a normal CD4 count, a high viral load and a suboptimal response to plasma exchange while remission was obtained with combined antiretroviral therapy (cART). Other case studies confirmed that there may be a causative link between TTP and advanced HIV infection, since all patients presented with extremely low CD4 and CD8 counts.[12] This is not always the case in our experience (unpublished data). The treatment strategy in the case described by Miller et al.[11] included fresh plasma and a corticosteroid, but only after plasma exchange did the platelet count start to rise and the steroid was tapered; cART treatment was started afterwards. Another case study described a patient with myocardial injury in HIV-associated TTP who responded poorly to plasma infusion and steroid therapy, but recovered fully following plasma exchange.[9]

Table 1: ADAMTS13 Levels, Activities and Autoantibodies, VWF Levels and Tissue Factor Levels of Citrated Plasma from Patients with TTP, HIV-positive People on cART and HIV-positive People not on cART

Test	Unit	TTP (Mean±SD)	HIV+ on cART (Mean±SD)	HIV+ not on cART (Mean±SD)	Normal Range
ADAMTS13 levels	ng/ml	144±109	934±245	627±109	520–1,060
ADAMTS13 activity	ng/ml	366±97	735±230	421±260	481–785
ADAMTS13 autoantibodies	% of positive patients	50	50	83	none
VWF antigen levels	%	494±130	317±187	767±378	50–150
Tissue factor levels	% of patients with increased levels	36	50	87	none

ADAMTS13 = a disintegrin and metalloprotease with thrombospondin type 1 repeats; cART = combination antiretroviral therapy; SD = standard deviation; TTP = thrombotic thrombocytopenic purpura; VWF = von Willebrand factor.

Figure 1: Proposed Mechanism for the Initial Onset of HIV-associated Thrombotic Thrombocytopenic Purpura



ADAMTS13 = a disintegrin and metalloprotease with thrombospondin type 1 repeats; TTP = thrombotic thrombocytopenic purpura; VWF = von Willebrand factor.

A recent study of 24 patients with HIV-associated TTP emphasises the importance of the prompt initiation/re-initiation of cART in parallel with plasma exchange and steroid treatment that lead to prompt remission.[10] Adjunct immunomodulatory agents such as rituximab have been used with some success in refractory cases.[10] It has been reported that patients with HIV-associated TTP seem to be far more responsive to plasma infusion treatment regimens than their HIV-negative counterparts, suggesting a different pathophysiology.[13]

Pathophysiology of HIV-associated Thrombotic Thrombocytopenic Purpura

The pathophysiology and initial onset of TTP in HIV-positive patients are not fully understood. In 1994, Cruccu et al. suggested that the aetiology of thrombocytopenia in HIV-infected patients may be due to direct infection of megakaryocytes by the virus, immune-mediated destruction, impaired haematopoiesis, toxic effects from medications and microangiopathic anaemia syndromes.[12] It is now generally accepted that HIV-associated TTP is an acquired form of ADAMTS13 deficiency. Acquired ADAMTS13 deficiency is often due to the presence of autoantibodies directed against ADAMTS13.[14,15] These autoantibodies to ADAMTS13 play a pivotal role in the pathogenesis of acquired TTP. By decreasing the function of ADAMTS13, the autoantibodies impair the cleavage of ULVWF into smaller sizes, leading to the formation of platelet-VWF thrombi in the microcirculation. Only limited data are available regarding the presence of autoantibodies in HIV-associated TTP patients. In one study, only 38 % of patients with reduced ADAMTS13 activity had inhibitory antibodies. This was measured by using the residual collagen binding assay and performing mixing studies with normal plasma.[14] The same group also found only a mild reduction of ADAMTS13 levels in patients with HIV-associated TTP and an increase in VWF levels in all patients.

We measured ADAMTS13 levels, activity and autoantibodies in 40 patients diagnosed with HIV-associated TTP at the Haematology Clinic of the Universitas Hospital in Bloemfontein, South Africa. As a control group, we used the plasma of 104 HIV-positive persons visiting the local primary healthcare clinic. The control HIV group was divided into two groups, depending on whether they were on cART or not. The ADAMTS13 levels and autoantibodies were measured using the respective enzyme-linked immunosorbent assay (ELISA) kits from American Diagnostica (US). The ADAMTS13 activities were measured using the Actifluor™ ADAMTS13 fluorescence resonance energy transfer (FRET) assay (also from American Diagnostica). We also measured the VWF levels using antibodies from Dako (South Africa) and tissue factor levels in plasma with an ELISA kit from American Diagnostica. The results are summarised in *Table 1*.

We found that the ADAMTS13 levels extensively decreased in TTP patients. HIV infection on its own did not seem to have a significant influence on ADAMTS13 levels. However, HIV-positive persons who were not on cART seemed to have slightly lower ADAMTS13 levels than those who were on cART, although the levels were still in the normal range. It is possible that less metalloproteases such as ADAMTS13 are synthesised in these patients, since micronutrient deficiencies are widespread and compound the effects of HIV disease.[16] Our findings regarding ADAMTS13 levels differed from those of another group in South Africa. They detect normal ADAMTS13 levels in one-third of adult cases with HIV-associated TTP.[8] The activities of ADAMTS13 that we measured using the FRET assay did not correlate with the ADAMTS13 levels in all groups. However, the lowest activities were found in the TTP patients. The assay might not be as suitable as the ADAMTS13 levels to diagnose the presence of HIV-associated TTP. We also found that only 50 % of our TTP patients presented with autoantibodies to ADAMTS13. This correlates with what is reported in the literature.[14] An interesting finding was that most of the HIV-positive persons who were not on cART also presented with high amounts of autoantibodies against ADAMTS13. We thus suggest that antibody formation against ADAMTS13 is a result of the weakened immune system in HIV. Similar to what is reported in the literature, we found increased VWF levels in patients with HIV-associated TTP.[8] The VWF antigen levels were

greatly increased in HIV-positive patients who were not on cART. This could be due to an HIV-associated inflammatory state or concomitant infection. Similar to the VWF levels, we found that most (87 %) of the HIV-positive persons who were not on cART had increased tissue factor levels, which is known to be a risk factor for thrombosis. We found that only 36 % of the TTP patients presented with increased tissue factor levels. This is probably because these patients were all on cART.

We propose a mechanism for the initial onset of HIV-associated TTP (see *Figure 1*). HIV type 1 (HIV-1) infection is associated with inflammation and elevated levels of inflammatory cytokines such as gamma interferon and tumour necrosis factor alpha (TNF-α) and beta (TNF-β).[17] It is known that inflammatory cytokines, such as TNF-α and interleukin-1, 6 and 8, have profound stimulatory effects on the endothelial release of ULVWF[18] In addition, the synthesis of ADAMTS13 that cleaves the ULVWF is inhibited.[19] This might ultimately lead to the deficiency of ADAMTS-13 and the overexpression of ULVWF, resulting in the initiation of TTP. Furthermore, HIV-infection is associated with widespread micronutrient deficiencies that might cause decreased ADAMTS13 synthesis.[16] Autoantibodies to ADAMTS13 are also present in HIV-positive patients as a result of the weakened immune system. An inhibitory antibody might also cause the initial onset of HIV-associated TTP.

Laboratory Diagnosis of HIV-associated Thrombotic Thrombocytopenic Purpura

The diagnosis of TTP rests on evidence of microangiopathic haemolytic anaemia (MAHA) and thrombocytopenia in the absence of disseminated intravascular coagulation and other known causes of thrombotic microangiopathy. Highly specific diagnostic tools such as plasma levels of ADAMTS13 are recommended, but are not always routinely available for immediate clinical diagnosis. The presence of schistocytes on a blood smear is the morphologic hallmark of the disease. A schistocyte count of 1 % or more in conjunction with thrombocytopenia suggests TTP if other causes of MAHA have been excluded.[20] We suggest the use of ADAMTS13 levels as a confirmatory test of HIV-associated TTP. The measurement of ADAMTS13 activity levels using the FRET assay is expensive and does not necessarily confirm the disease. The exact role of inhibitory autoantibodies to ADAMTS13 remains uncertain and further investigation is required.

Conclusion

HIV-associated TTP is a heterogeneous disorder and its initial onset is still not clear. We propose that infection with HIV might trigger the disease through the inflammatory process. Inflammatory cytokines stimulate the release of extreme amounts of VWF.[17] Extremely high levels of VWF were found in the plasma of HIV-positive persons who are not on HAART as well as in TTP plasma. The cytokines also downregulate the release of the VWF-cleaving protease ADAMTS13.[19] We found very low levels of ADAMTS13 in TTP plasma. This might be sufficient to cause thrombotic microangiopathy and precipitate an acute episode of TTP even in the presence of normal cleaving protease activity. Furthermore, the release of VWF may be sufficient to overwhelm the capacity of the cleaving protease and result in a 'consumptive deficiency', as speculated by Gunther et al.[14] An important finding in HIV-associated TTP that differs from acquired TTP is the fact that not all patients present with autoantibodies to ADAMTS13. More specific methods might be needed to identify autoantibodies, especially inhibitory autoantibodies, in these patients. Inhibitory antibodies to ADAMTS13 might also trigger the disease. We could finally show that neither HIV-infection per se, nor the use of HAART, has any effect on ADAMTS13 levels, making it a useful diagnostic tool to diagnose HIV-positive patients with TTP. The increased tissue factor levels in HIV-positive persons who are not on cART indicate the thrombotic potential in HIV that might also contribute to the initial onset of HIV-associated TTP. ■

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Comparison of ADAMTS13 and Von Willebrand factor levels and activities, and plasminogen levels, in plasma products currently available for the treatment of thrombotic thrombocytopenic purpura in South Africa

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ABSTRACT

Objective: Thrombotic thrombocytopenic purpura (TTP) results from a deficiency in the Von Willebrand factor (VWF) cleaving protease, ADAMTS13. Treatment involves plasma exchange (PEX) therapy with either fresh frozen plasma (FFP), cryosupernatant (CSP) or solvent/detergent-treated plasma (SDP), available in South Africa as Bioplasma FDP. The aim of the study was to generate in vitro data on these products, and to explore possible differences between the products that may offer treatment advantages.

Methods: Twenty samples per product (FFP, CSP and Bioplasma FDP) were analysed for levels and activities of ADAMTS13 and VWF. Plasminogen levels, a proposed physiological back-up system for ADAMTS13, were also determined. FFP and CSP samples were subanalysed according to ABO blood group. Samples were analysed by means of commercially available ELISA assays.

Results: All samples had normal/high ADAMTS13 activity (Median activity for SDP = 94.0%, CSP = 80.5%, FFP = 122.0%) and plasminogen levels. The VWF content was mostly normal for Bioplasma FDP, typically deficient for CSP and mostly deficient for FFP, which was an unexpected finding. Depending on the parameter, Bioplasma FDP was the most standardised, with coefficients of variation (CV) from 14.1% to 27.3%, while FFP showed great inter-individual variation (CV 24.6% to 208.6%). Statistically significant differences were found across products ($P \leq 0.0095$), and ABO blood groups ($P = 0.0001$).

Conclusion: All three products can be used for the treatment of TTP. The choice of product depends on the need for additional viral safety, costs, product availability and the perceived impact of within-product variations.

1. Introduction

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening condition that belongs to a diverse group of disorders, collectively known as thrombotic microangiopathy (TMA) syndromes [1]. Despite their diversity, these disorders are all characterised by widespread microvascular occlusion in several organs [1].

Central to the pathophysiology of TTP is a deficiency of ADAMTS13 (a disintegrin and metalloprotease with thrombospondin Type I repeats, 13th member), the plasma protease that functions to cleave ultra-large Von Willebrand factor (ULVWF) multimers into smaller, less adhesive forms [2]. Unusually large VWF multimers accumulate and result in abnormal platelet aggregation and thrombus formation, with subsequent thrombocytopenia and an associated fragmentation haemolysis [3]. Plasmin, the major enzyme of the fibrinolytic system, has been identified as a possible back-up mechanism for ADAMTS13, and the potential therapeutic importance of fibrinolysis was recently demonstrated in a murine model of acquired TTP [4].

Acute idiopathic TTP is the most common form of TTP. It is characterised by autoantibodies, usually IgG, directed against ADAMTS13 [2]. HIV-associated TTP differs from acute idiopathic TTP in that autoantibodies to ADAMTS13 are often not detected [5]. The incidence of TTP in HIV-positive individuals is considered to be up to 40 times greater than that in the general population [6]. As a country with one of the world's highest HIV prevalence rates, HIV-associated TTP is of particular importance in South Africa [7]. Although the association between HIV and TTP has been evident since the 1980s, the exact pathogenesis remains unclear and is considered to be a multifactorial process [6]. Gunther et al. [8] and Meiring et al. [5] summarised the suspected pathogenesis of HIV-associated TTP to be a combination of

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endothelial cell damage with loss of its inherent anticoagulant properties, excessive VWF release, and reduction in ADAMTS13 activity, whether it be secondary to consumption, inhibition or proteolytic inactivation [8].

Daily plasma exchange (PEX) or, alternatively, large volume plasma infusion, forms the mainstay of therapy in TTP [2,9] and has reduced mortality rates by more than 70% [2]. Proposed explanations for the efficacy of PEX include removal or dilution of inhibitor autoantibodies and ULVWF multimers, together with replacement of the deficient ADAMTS13 [10].

Plasma products used for this purpose in South Africa include fresh frozen plasma (FFP), cryosupernatant (CSP) and solvent/detergent-treated plasma (SDP), locally available as Bioplasma FDP [2]. Many international laboratory and clinical studies have compared and assessed the content and in vivo effectiveness of the different products [10–20]. The general conclusion drawn from these comparative studies was that despite documented differences among the different plasma products, no one product has been proven superior in the treatment of TTP [11].

No data have been published on the ADAMTS13, VWF or plasminogen content of the products locally available in South Africa. In addition, no clinical trials comparing the efficacy of these plasma products in the setting of HIV-associated TTP have been conducted. Considering the lack of empirical data on the composition of products available locally, and keeping in mind that South Africa has a different disease profile with most of its TTP cases being associated with HIV [6,7], an ideal treatment regimen is yet to be defined.

The aim of this study was to generate empirical in vitro data on the products available locally (FFP, Bioplasma FDP and CSP) to confirm their in vitro equivalence and utility for local use, and also to explore any possible differences in the products that may offer treatment advantages in the setting of HIV-associated TTP. This study specifically compared the levels and activities of the plasma proteins and enzymes relevant in the treatment of HIV-associated TTP, namely ADAMTS13, VWF and plasminogen.

2. Materials and methods

2.1. Selection and grouping of samples

Testing-segments of the FFP and CSP units issued in December 2015, still frozen at below −18 °C, were collected from the Universitas Academic Hospital blood bank. In order to maintain the cold-chain, the samples were subsequently stored at −80 °C in the Department of Haematology and Cell Biology until batch testing commenced in January 2016.

The segments were subdivided according to the different ABO blood groups. Five samples each of blood groups A and B, and 10 blood group O samples were included for each of the blood products, giving a total of 10 Group O and 10 non-group O samples per product. Due to cost constraints and limited availability, samples from group AB products were not included in the study. The SDP tested in the study was Bioplasma FDP, a product of the National Bioproducts Institute (NBI). The NBI provided 40 200 mL bottles of Bioplasma FDP from separate lots for analysis, which included two bottles per lot from 20 different lots.

2.2. Sample testing

The ADAMTS13 antigen levels and activities and plasminogen levels were measured using the applicable commercially available ELISA kits (Technozym ADAMTS13 Antigen, Technozym ADAMST13 Activity and Technozym Glu-Plasminogen; Technoclone; Vienna, Austria) according to the manufacturer's instructions [21–23].

Von Willebrand factor testing included two ELISA-based assays to determine VWF antigen (VWF:Ag) level and collagen-binding (VWB:CB) activity. These assays were performed as previously described by Meiring et al. [24]. The ristocetin co-factor assay (VWF:RCo), based on ristocetin-induced platelet agglutination, was done on the PAP-8E aggregometer (Bio/Data Corporation; Horsham, PA, USA). Multimeric analysis of VWF involved electrophoretic separation of VWF multimers on a 0.65% agarose gel, followed by rabbit anti-human VWF/horseradish peroxidase binding, and finally luminographic visualisation with the aid of x-ray film and cassettes [24]. Densitograms of the multimer patterns were plotted using a gel documentation system (Syngene G Box, Syngene; Frederick, MD USA).

2.3. Data analysis

Data were analysed by the Department of Biostatistics, University of the Free State. The results of the various tests were described using ranges, medians, means, standard deviations (SDs), coefficients of variation (CVs) and analysis of variance (ANOVA).

For each plasma product, the various measurements – ADAMTS13 antigen, ADAMTS13 activity, Glu-plasminogen, VWF:Ag, VWF:RCo, VWF:CB and VWF multimers – were reported as proportions below/within/above the recommended reference ranges supplied with the package inserts [21–23]. These proportions of below-/within-/above-range readings were then compared across the various plasma products by means of chi-square cross tabulation with Fisher's exact *P*-value. The coefficients of variance (CVs) were calculated and compared to available biological variation database values [25–28].

2.4. Ethical considerations

Approval to conduct the research was obtained from the Ethics Committees of both the Faculty of Health Sciences, University of the Free State (ECUFS NR 191/2015) and the South African National Blood Service (SANBS) (2015/25).

3. Results

The basic descriptive statistics of the comparison between the different plasma products are shown in Table 1 and Fig. 1. Table 2 summarises the comparison of the proportions of below-/within-/above-range readings across the various plasma products for the different parameters tested and Table 3 shows the *P*-values of these comparisons.

Fig. 1 illustrates the deficiency in ADAMTS13 antigen levels (Fig. 1a) contrasted to the high ADAMTS13 activity levels (Fig. 1b) for FFP. CSP showed deficiency in VWF antigen and activity levels (Fig. 1c–e). FFP, which still contained the cryoprecipitate fraction with VWF, showed deficiency of VWF activities (Fig. 1d and e). The distribution of Glu-plasminogen is illustrated in Fig. 1f.

Table 2 shows that Bioplasma FDP samples were nearly consistently within the reference ranges for most of the parameters tested. CSP had predominantly normal ADAMTS13 levels and activities, with an absence of VWF levels and activities. FFP samples showed variation, while normal Glu-plasminogen levels were observed in all three plasma products. Table 3 demonstrates statistically significant differences in at least one plasma product for almost every parameter tested, particularly when Bioplasma FDP was compared with each of the other two plasma products.

The proportions of below-/within-/above-range readings of the blood group O and non-O CSP and FFP samples for the various parameters tested, are summarised in Table 4. Table 5 shows the *P*-values of the blood group comparisons for the different parameters. When all the results for a particular parameter fell within the same category (below/within/above the reference range), no *P*-values were calculated for the

Table 1
Comparison of the minimum (min) and maximum (max) values, medians, means, lower and upper quartiles (LQ and UQ), standard deviations (SD) and coefficient of variance (CV%) for the levels and activities of ADAMTS13 and VWF, and the antigen levels of Glu-plasminogen in the different plasma products.

Variable	Normal range [21–23]	Min	LQ	Median	UQ	Max	Mean	SD	CV%
Bioplasma FDP (n = 20)									
ADAMTS13 Antigen	0.6–1.6 μg/mL	1.13	1.58	1.98	2.70	2.70	2.07	0.56	27.34
ADAMTS13 Activity	40–130 %	62.00	82.50	94.00	104.00	127.00	92.95	15.67	16.86
VWF:Ag	51–143 %	54.00	76.50	87.50	95.50	122.00	86.15	16.21	18.81
VWF:RCo	50–150 %	39.00	54.50	58.00	68.00	81.00	61.00	10.21	16.74
VWF:CB	49–157 %	38.00	57.50	75.00	83.00	100.00	71.30	18.82	26.39
Glu-plasminogen	60–250 μg/mL	92.00	98.00	113.00	122.00	143.00	122.45	15.85	14.10
CSP (n = 20)									
ADAMTS13 Antigen	0.6–1.6 μg/mL	0.20	0.31	0.84	1.33	1.73	0.86	0.51	59.69
ADAMTS13 Activity	40–130 %	60.00	72.50	80.50	97.00	142.00	87.05	22.56	25.92
VWF:Ag	51–143 %	0.00	0.00	0.00	0.00	15.00	1.60	3.80	237.69
VWF:RCo	50–150 %	0.00	0.00	0.00	0.00	10.00	0.50	2.24	447.21
VWF:CB	49–157 %	0.00	0.00	0.00	0.00	17.00	1.00	3.80	379.75
Glu-plasminogen	60–250 μg/mL	71.00	91.50	100.50	138.50	194.00	113.35	32.34	28.53
FFP (n = 20)									
ADAMTS13 Antigen	0.6–1.6 μg/mL	0.23	0.29	0.32	0.46	1.56	0.49	0.39	78.96
ADAMTS13 Activity	40–130 %	63.00	100.50	122.00	160.00	160.00	124.70	33.29	26.69
VWF:Ag	51–143 %	22.00	45.00	55.50	70.00	95.00	57.05	20.59	36.09
VWF:RCo	50–150 %	0.00	0.00	0.00	0.00	75.00	12.85	26.80	208.58
VWF:CB	49–157 %	0.00	0.00	0.00	0.00	84.00	13.35	27.78	208.09
Glu-plasminogen	60–250 μg/mL	73.00	87.50	95.00	117.50	163.00	105.70	26.01	24.61

CSP = cryosupernatant; FFP = fresh frozen plasma; VWF:Ag = Von Willebrand factor antigen; VWF:RCo = VWF ristocetin co-factor; VWF:CB = VWF collagenbinding activity.

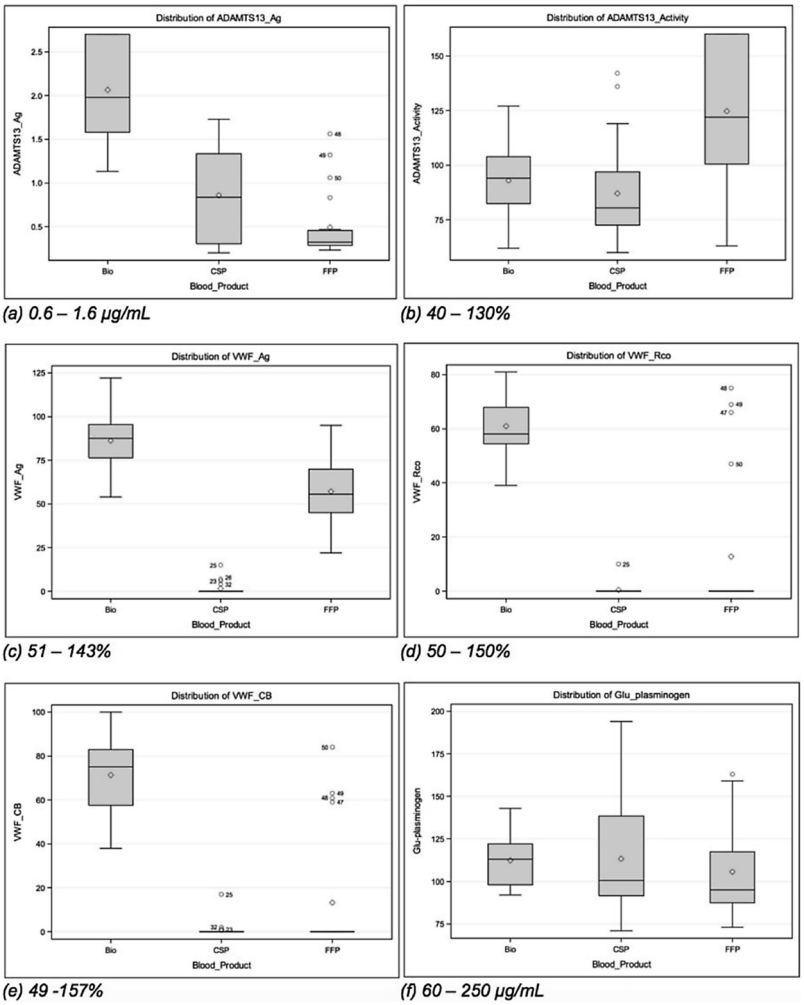

Fig. 1. The analysis of variance (ANOVA) procedure illustrating the distribution of the different test parameters for each plasma product. The normal reference range [21–23] for each test is indicated below the box plot.

Table 2
Number of below-/within-/above-range readings of the different plasma products for the various parameters tested.

Plasma product	Range reading	Parameters tested					
		ADAMTS13 Ag	ADAMTS13 Act	VWF:Ag	VWF:RCo	VWF:CB	Glu-plasminogen
Bioplasma FDP (n = 20)	Below	0	0	0	2	4	0
	Within	5	20	20	18	16	20
	Above	15	0	0	0	0	0
CSP (n = 20)	Below	7	0	20	20	20	0
	Within	12	18	0	0	0	20
	Above	1	2	0	0	0	0
FFP (n = 20)	Below	16	0	9	17	16	0
	Within	4	11	11	3	4	20
	Above	0	9	0	0	0	0

Ag = antigen; Act = activity; VWF:Ag = Von Willebrand factor antigen; VWF:RCo = VWF ristocetin co-factor; VWF:CB = VWF collagen-binding activity; CSP = cryosupernatant; FFP = fresh frozen plasma.

data. No significant differences between blood group O and non-O CSP samples were found. Although the differences in VWF:RCo and VWF:CB levels between blood group O and non-O FFP samples were not statistically significant, it is worth noting that the four non-O samples with normal values were all of blood group B.

Fig. 2 shows the VWF multimer patterns of the Bioplasma FDP samples of which 18 (90.0%) yielded results that were comparable to normal plasma controls. The VWF multimer patterns of the CSP samples are represented in Fig. 3. Three (15.0%) CSP samples contained intermediate multimers, while in the remainder, only small multimers and a complete absence of the larger forms were observed. The VWF multimer patterns of FFP samples, shown in Fig. 4, demonstrated great inter-individual variation across the different samples. The patterns that were comparable to normal plasma all occurred in blood group B samples.

4. Discussion

The rationale behind the use of plasma therapy as the cornerstone in the management of TTP, includes the removal or dilution of inhibitor autoantibodies and ULVWF multimers, and replacement of the deficient ADAMTS13 [1]. Although the exact pathogenesis of HIV associated TTP is still unclear, excessive VWF release and reduction in ADAMTS13 activity, whether secondary to consumption, inhibition, or proteolytic inactivation, are evident [8]. Consequently, the ideal plasma product to use in PEX/plasma-infusion therapy would be one with normal/high ADAMTS13 levels and activity, and absent/low levels of the large and ULVWF multimers. In addition, adequate levels of plasminogen, the circulating zymogen of plasmin that is a proposed back-up mechanism for ADAMTS13, can also be considered beneficial [4].

One of the major benefits of pooling plasma products is standardisation of the content [29]. Not surprisingly, Bioplasma FDP, which is derived from pooled fresh human plasma, showed the least variation for all the parameters tested, with CVs ranging from 14.1% to 27.3% (Table 1).

Several studies have confirmed that SDP retains normal in vitro levels and activities of selected plasma proteins, including plasminogen and ADAMTS13 [29–34]. The adequate levels and activities of ADAMTS13 and normal levels of plasminogen found in this study (Table 2) confirm that Bioplasma FDP is on par with international SDP, and would provide consistent replacement of these important enzymes if used as replacement fluid. The VWF multimer patterns were equivalent to that of normal plasma controls, showing absence of only the largest forms (Table 4; Fig. 2). Bearing in mind that only the ultra-large forms are implicated in the pathogenesis of TTP [3], presence of the large multimers would not be regarded as detrimental.

When considering the large volume of plasma required for the treatment of an acute episode of TTP, the additional benefit of viral inactivation provided by the solvent/detergent process may be considered essential. In fact, both the British Committee for Standards in

Haematology (BCSH) guidelines for the use of FFP, cryoprecipitate and CSP [35], and South African clinical guidelines for the use of blood products [36], suggest the use of pathogen inactivated plasma in patients likely to receive large or repeated doses of plasma.

CSP is FFP from which the precipitable cryoproteins (Factor VIII, VWF, Factor XIII, fibronectin and fibrinogen) have been removed [35]. As expected, in 17 of the 20 samples tested, VWF multimer patterns revealed complete absence of large VWF multimers and intermediate forms (Table 4; Fig. 4). Although these results could be anticipated, CSP should still contain ADAMTS13 [35]. Several studies confirmed the preservation of ADAMTS13, not only in CSP, but also FFP and SDP [10,20,31]. Seven of the CSP samples tested had deficient ADAMTS13 antigen levels, yet normal enzyme activity. This was also the case with 16 of the FFP samples tested. ADAMTS13 activity levels actually exceeded the upper limit of the assay's detection range in six of these 16 FFP samples (Fig. 1a and b). A possible explanation could be that partial degradation of the enzyme may result in loss of the specific epitope required to bind to the anti-ADAMTS13 monoclonal antibody in the antigen assay without impairing enzyme function. The importance of this finding is uncertain, as activity assays may have more clinical significance than antigen assays [37].

When analysing all 20 FFP samples as a single cohort, significant variation was found across the different FFP samples with regard to all the parameters tested. To a certain extent, these variations may be

Table 3
Fisher's exact *P*-values for the comparisons across the different plasma products for the various parameters tested.

Comparison	ADAMTS13 Ag	ADAMTS13 Act	VWF:Ag	VWF:RCo	VWF:CB	Glu-plasminogen	VWF multimer patterns
Bioplasma FDP vs FFP	< 0.0001*	0.0012*	< 0.0012*	< 0.0001*	0.0004*	0.1085	< 0.0001*
Bioplasma FDP vs CSP	< 0.0001*	0.4872	< 0.0001*	< 0.0001*	< 0.0001*	0.1085	< 0.0001*
FFP vs CSP	0.0095*	0.0310*	0.0001*	0.2308	0.1060	0.1085	0.2308

*Statistically significant difference, $P < 0.05$; Ag = antigen; Act = activity; VWF:Ag = Von Willebrand factor antigen; VWF:RCo = VWF ristocetin co-factor; VWF:CB = VWF collagen-binding activity; CSP = cryosupernatant; FFP = fresh frozen plasma.

Table 4
Proportions of below-/within-/above-range readings of blood groups O and non-O CSP and FFP samples for the various parameters tested.

Plasma product	Blood group	Range reading	Parameters tested					
			ADAMTS13 Ag	ADAMTS13 Act	VWF:Ag	VWF:RCo	VWF:CB	Glu-plasminogen
CSP	O	Below	1	0	10	10	10	0
		Within	8	9	0	0	0	10
		Above	1	1	0	0	0	0
	Non-O	Below	6	0	10	10	10	0
		Within	4	9	0	0	0	10
		Above	0	1	0	0	0	0
FFP	O	Below	10	0	10	10	10	0
		Within	0	1	0	0	0	10
		Above	0	9	0	0	0	0
	Non-O	Below	6	0	4	7	6	0
		Within	4	10	6	3	4	10
		Above	0	0	0	0	0	0

Ag = antigen; Act = activity; VWF:Ag = Von Willebrand factor antigen; VWF:RCo = VWF ristocetin co-factor; VWF:CB = VWF collagen-binding activity; CSP = cryosupernatant; FFP = fresh frozen plasma.

Table 5
Fisher's exact *P*-values for the comparison between blood groups O and non-O FFP and CSP samples for the various parameters tested.

Comparison	ADAMTS13 Ag	ADAMTS13 Act	VWF:Ag	VWF:RCo	VWF:CB	Glu-plasminogen
CSP: O vs non-O	0.0573	1.000	ND	ND	ND	ND
FFP: O vs non-O	0.0867	0.0001*	1.000	0.0867	0.2105	ND

*Statistically significant difference, $P < 0.05$; ND = not determined; Ag = antigen; Act = activity; VWF:Ag = Von Willebrand factor antigen; VWF:RCo = VWF ristocetin co-factor; VWF:CB = VWF collagen-binding activity; CSP = cryosupernatant; FFP = fresh frozen plasma.

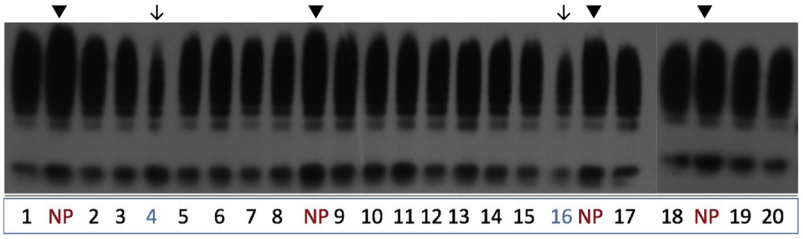

Fig. 2. VWF multimer patterns of all 20 Bioplasma FDP samples. The arrows heads (▼) indicate normal plasma controls. Except for two samples, indicated by arrows (↓), all Bioplasma FDP samples had VWF multimer patterns comparable to normal plasma controls.
NP = normal plasma.

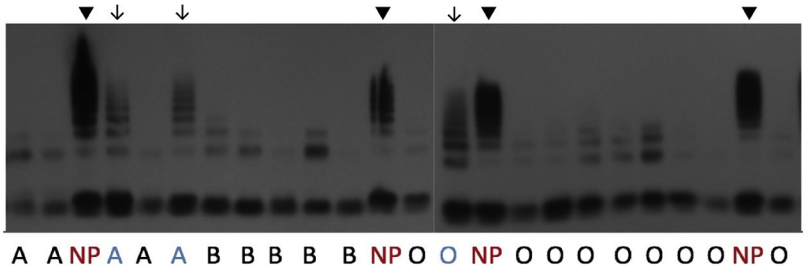

Fig. 3. VWF multimer patterns for CSP samples 1–20. The arrows heads (▼) indicate normal plasma controls. Only three samples, indicated with arrows (↓), still contained intermediate multimers, the remaining samples showed complete absence of the larger forms, with only small VWF multimers left. A = blood group A; B = blood group B; O = blood group O; NP = normal plasma.

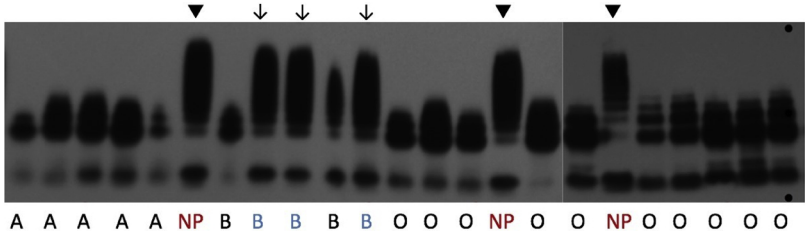

Fig. 4. VWF multimer patterns of all 20 FFP samples. The arrows heads (▼) indicate normal plasma controls. Note the great inter-individual variation across the different samples: some patterns (all from ABO blood group B), indicated by arrows (↓), are comparable to that of the normal plasma controls, showing absence of only the largest multimer forms, while others show absence of the large and intermediate forms. A = blood group A; B = blood group B; O = blood group O; NP = normal plasma.

attributed to the different ABO blood groups. However, with inter-individual variations evident even among samples of the same blood group category (O versus non-O), additional factors should be considered.

This higher inter-individual variation was not completely unexpected, considering that FFP is prepared from single-donor whole-blood units following application of physical separation methods [36]. Variations across the different FFP samples could therefore reflect biological variations in the donors. However, the between-subject variation (CVG) for almost all the parameters tested in this study still

Table 6
Between-subject biological variation (CV_G) of the different parameters tested in fresh frozen plasma (FFP) samples, compared to previously published results.

Between-subject biological variation (CV_G)				
Test	Blood group			Previously published findings
	All (n = 20)	Non-O (n = 10)	O (n = 10)	
ADAMTS13 Act	26.69	8.19	20.50	9.63 (CV_{TG}: 9.63) [27]
ADAMTS13 Ag	78.96	22.30	7.82	6.28 (CV_{TG}: 10.70) [27]
VWF:Ag	36.09	29.77	39.29	31.71 [25] 27.30 [26] 22.59 ($CV_{TG:}$ 22.87) [27]
VWF:RCo	208.58	131.93	ND	18.5 (CV_{TG}: 19.83) [27]
VWF:CB	208.09	131.52	ND	ND
Glu-plasminogen	24.61	22.74	14.89	10.5 [28]

ND = not determined.

exceeded that reported in the literature (Table 6) [25–28].

Unlike CSP, the cryoprecipitate fraction, rich in high molecular weight VWF multimers, is still included in the units of FFP. Yarranton et al. [10] confirmed that the whole spectrum of VWF multimers of all weights are present in FFP. Therefore, the most surprising result in our study was the absence of high, and even intermediate, molecular weight multimers found in 85% (17/20) of all the FFP samples tested. We speculate that to an extent, these variations might be attributed to, among others, pre-analytical variables, including handling, transport and storage of blood products after donation, or specimen handling, transport or storage.

Finally, the choice of blood product is also determined by cost. Table 7 summarises the cost per unit and PEX of the different blood products [38,39] at the time of finalising this report

(July 2018). On average, even when fewer units are required, FFP is still less economical at R14 944.90 (US$1 117.36) per exchange, compared to R12 228.96 (US$913.89) and R10 559.20 (US$790.42) per exchange for Bioplasma FDP and CSP, respectively [40].

5. Limitations

Cost constraints limited the number of samples tested per studied plasma product. As a result, the small sample sizes, and in particular, the small numbers representing the different ABO blood groups, reduce the likelihood of statistically significant results and necessitate cautious interpretation.

Plasma products in South Africa are stored at below −18 °C for up to one year [36]. The 2018 BCSH guidelines recommend storage at ≤ −25 °C for up to 36 months to preserve the activity of labile coagulation factors. (A) The International Society of Blood Transfusion (ISBT) considers storage temperatures between −18 °C and −25 °C acceptable for up to three months [41]. Differences in South African practice may be explained by the fact that plasma products are in high demand with very limited supply and average storage times almost never exceed a three month period.

Quality monitoring of standard FFP in the United Kingdom also entails measurement of factor VIII levels which need to be at least 0.7 IU/mL in at least 75% of units, immediately after being thawed [42]. Coagulation factor content of locally available plasma products is also tested for internal quality control purposes. According to the package insert, Bioplasma FDP® contains a minimum of 0.4 IU/mL of each coagulation factor [43]. Average coagulation factor levels for FFP are available on the Western Province Blood Transfusion Service (WPBTS) website and are also published in the clinical guidelines for the use of blood products in South Africa, available from the South African National Blood Service (SANBS) and WPBTS [36]. Average factor VIII levels are reported as 0.85 IU/mL [36]. As per the Standards of Practice for Blood Transfusion in South Africa, 7th ed.(2016), random sampling and quality control testing of blood products are routinely performed according to local establishments' quality manuals [44]. The focus on ADAMTS13, VWF and plasminogen levels in our study, was not to ascertain quality in general, but rather to assess suitability of the different products for the treatment of TTP in South Africa.

6. Conclusions

All 60 samples had normal/high ADAMTS13 activity and normal Glu-plasminogen levels. With a few exceptions, Bioplasma FDP sample results were consistently within the normal reference ranges for all the parameters tested. As expected, CSP had deficient/absent VWF levels and activities, with absence of the large and intermediate multimer forms. No significant difference between blood group O and non-O CSP samples were found.

Surprisingly, the majority of FFP samples also showed reduced/absent VWF activities with abnormal multimer patterns. Those FFP samples with near-normal VWF activities and multimer patterns were all of blood group B, while the FFP samples with above-normal ADAMTS13 activities were all representative of blood group O. These findings suggest that non-O FFP may be more beneficial in the treatment of bleeding diatheses, whereas group O FFP may be preferable in the treatment of TTP – a hypothesis that requires further investigation.

Theoretically, all three plasma products meet the minimum criteria for a replacement fluid, namely that it contributes to improved VWF cleavage, whether secondary to replacement of ADAMTS13 or its purported back-up mechanism, plasminogen, without adding additional pathogenic ULVWF multimers. One can therefore conclude that any one of these plasma products would be equally appropriate for the treatment of HIV-associated TTP. The choice of product would depend on factors such as the need for additional viral safety, financial expenditure, product availability and the perceived practical impact of within-product variations.

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Table 7
Price per unit and estimated price per exchange (calculated for a 60 kg patient with an estimated plasma dose of 40 mL/kg) for the different plasma products according to the 2018 South African public sector price lists [38,39].

Product	Unit volume	2018 price including value-added tax	Estimated price per PEX
Bioplasma FDP®	200 mL	R1 019.08 (US$76.14)*	R12 228.96 (US$913.89)
FFP	240–300 mL	R1 494.49 (US$111.74)	R14 944.90 (US$1 117.36)
CSP	190–310 mL	R1 055.92 (US$79.03)	R10 559.20 (US$790.42)

*Calculation based on ZAR (South African rand) versus US$ (United States dollar) exchange rate of approximately R13.38 per dollar on 10 July 2018 [40]; PEX = plasma exchange; FFP = fresh frozen plasma; CSP = cryosupernatant.

Competing interests

The authors have no conflict of interest to declare.

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High levels of von Willebrand factor and low levels of its cleaving protease, ADAMTS13, are associated with stroke in young HIV-infected patients

S-35

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Background **Stroke associated with human immunodeficiency virus infection may occur through a variety of mechanisms. Von Willebrand factor is a marker of endothelial dysfunction, and is elevated in human immunodeficiency virus infection. High levels of von Willebrand factor, a protein involved in platelet adhesion and aggregation, and low levels of ADAMTS13, a metalloproteinase that cleaves von Willebrand factor, have been associated with an increased risk of thrombosis.**

Aim **To investigate the role of von Willebrand factor and ADAMTS13 in the pathogenesis of human immunodeficiency virus-related stroke in young patients.**

Methods **A case-control study ($n = 100$) comprising three participant groups: human immunodeficiency virus-positive antiretroviral therapy-naïve young strokes ($n = 20$), human immunodeficiency virus-negative young strokes ($n = 40$), and human immunodeficiency virus-positive antiretroviral therapy-naïve nonstroke controls ($n = 40$). von Willebrand factor and ADAMTS13 levels were measured in plasma samples collected five- to seven-days poststroke.**

Results **Human immunodeficiency virus-positive stroke participants had higher von Willebrand factor levels than human immunodeficiency virus-negative strokes (173·5% vs. 135%, $P = 0·032$). They tended to have higher levels of von Willebrand factor than human immunodeficiency virus-positive nonstroke controls (173·5% vs. 129%, $P = 0·061$). Human immunodeficiency virus-positive stroke participants had lower levels of ADAMTS13 than human immunodeficiency virus-positive nonstroke controls (0% vs. 23·5% $P = 0·018$) most likely due to the effect of the acute stroke. However, in the nonstroke group, these levels were significantly reduced compared with population norms. von Willebrand factor levels in all human immunodeficiency virus-positive participants were negatively correlated with CD4 counts.**

Conclusions **Stroke in human immunodeficiency virus infection is associated with a prothrombotic state, characterized by elevated von Willebrand factor and low ADAMTS13 levels.**

Key words: HIV infection, stroke, von Willebrand factor

Introduction

Stroke in the setting of HIV infection may occur through a number of mechanisms. These include HIV-associated vasculopathy, opportunistic infections, cardio-embolism, and disorders of hemostasis (1–3).

Von Willebrand factor (VWF) and its cleaving protein, ADAMTS13, are involved in hemostasis. VWF is vital for platelet adhesion and initiates platelet plug formation at sites of vascular injury. It is secreted by platelets and damaged endothelial cells (4). ADAMTS13 is synthesized by the liver. It cleaves the ultra large VWF multimers into smaller multimers. Uncleaved large multimers may promote active thrombosis if present in excess (5,6). High VWF and low ADAMTS13 levels may therefore be associated with an increased risk for thrombosis and stroke (7,8). Low activity of ADAMTS13 due to blocking antibodies has been described in HIV-associated thrombotic thrombocytopenia purpura (TTP) (9,10).

Little is known about the role of these proteins in HIV-related stroke. We therefore sought to test the hypothesis that HIV-associated strokes would be related to elevated VWF and low ADAMTS13 levels.

Patients and methods

Twenty consecutive ART-naïve HIV-positive (HIV+ve) ischemic stroke patients and 40 HIV-negative (HIV–ve) ischemic stroke patients, matched for age, were enrolled from the Groote Schuur young stroke cohort. Acute ischemic stroke participants in the Groote Schuur young stroke cohort were recruited between August 2010 and August 2012 from the stroke unit and acute medical admissions of four hospitals in Cape Town, South Africa. Forty healthy HIV+ve controls matched for age were enrolled from a primary health care HIV clinic in the same community. All stroke participants were investigated and treated according to standard clinical protocols. The study was approved by the GSH/University of Cape Town Human Research Ethics Committee (HREC ref: 178/2010), and informed consent was obtained from all participants.

VWF and ADAMTS13 levels were measured in plasma samples collected five- to seven-days after stroke onset in cases, or on the day of enrolment of controls. The timing of collection was standardized to minimize fluctuations during the acute phase of stroke. VWF and ADAMTS13 levels were assessed using an antigen-based enzyme-linked immunosorbent assay (ELISA) in the Haemostasis Laboratory at the University of the Free State, South Africa. VWF levels were measured using antibodies from Dako (Glostrup, Denmark). VWF levels were calculated from a standard curve using the WHO 6th FVII/VWF standard.

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ADAMTS13 levels were measured using an Imubind ADAMTS13 ELISA kit from American Diagnostica (Stamford, CT, USA), according to the manufacturer's instructions. We used a normal range for both tests of 50–150%. CD4 T-lymphocyte counts (CD4) were obtained for all HIV+ve participants.

Mann–Whitney *U* tests were used for between-group comparisons. Spearman's Rank Order correlation was used to relate VWF levels to CD4 counts.

Results

All three participant groups were comparable with respect to age and gender. The median age of participants was 35 years (*IQR* 30 – 38). The median CD4 count of the HIV+ve stroke participants was not significantly different from that of HIV+ve controls ($P = 0{\cdot}058$) (Table 1).

No stroke etiology could be identified in nine (45%) of the HIV+ve and 17 (43%) of the HIV–ve stroke participants. Cardioembolic disease, neurosyphilis, and hypertensive small vessel disease were identified as etiological factors in the remaining cases. Six of the HIV+ve strokes were polymerase chain reaction (PCR) positive for varicella zoster (VZV) in the cerebrospinal fluid, but none had clinical features of encephalitis or meningitis.

HIV+ve strokes had elevated VWF levels compared with HIV–ve strokes ($P = 0{\cdot}032$). There was a trend to higher levels of VWF in HIV+ve strokes compared with HIV+ve nonstroke controls ($P = 0{\cdot}061$; see Fig. 1b). ADAMTS13 levels were low in both HIV+ve and HIV–ve stroke groups and were not significantly different between the two. ADAMTS13 levels were lower in the

Table 1 Results

n	HIV+ stroke 20	HIV– stroke 40	HIV+ controls 40	HIV+ stroke vs. HIV– stroke 20 vs. 40	HIV+ stroke vs. HIV+ controls 20 vs. 40
Age (median)	35	36·5	33·5	$P = 0{\cdot}16$	$P = 0{\cdot}86$
Age IQR	29·5–36·8	30·5–41	29·3–38		
Female (%)	60	52·5	55	$P = 0{\cdot}78$	$P = 0{\cdot}93$
Black African n (%)	16 (80)	24 (60)	40 (100)	$P = 0{\cdot}21$	$P = 0{\cdot}017$
VWF median (%)	173·5	135	129	$U = 263$	$U = 280{\cdot}5$
VWF IQR (%)	118·3–215·5	107·5–168	94·8–175	$P = 0{\cdot}032$ $r = 0{\cdot}28$	$P = 0{\cdot}061$ $r = 0{\cdot}24$
ADAMTS13 median (%)	0	0	23·5	$P = 0{\cdot}71$	$U = 262$
ADAMTS13 IQR (%)	0–1·5	0–6	0–240·8		$P = 0{\cdot}018$ $r = 0{\cdot}31$
n	20	40	39	20 vs. 40	20 vs. 39
CD4 count median (cells/mm^3)	234	NA	383	NA	$U = 271{\cdot}5$
CD4 count IQR (cells/mm^3)	100·8–381·3		205–520		$P = 0{\cdot}058$ $r = 0{\cdot}25$

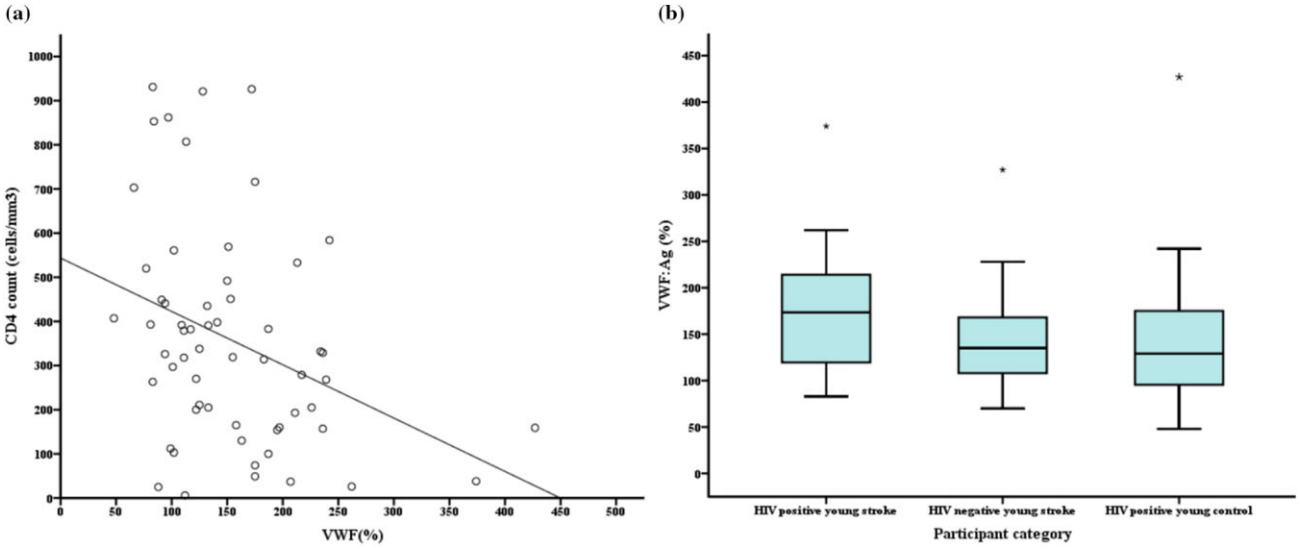

Fig. 1 (a) VWF levels and CD4 count correlation in all HIV-positive participants: VWF levels in HIV+ve participants were negatively correlated with CD4 count [Spearman's Rank Order correlation $r_s(59) = 0{\cdot}36$, $P = 0{\cdot}006$]. (b) Intergroup VWF levels: HIV+ve stroke participants had higher plasma levels of VWF compared with HIV–ve stroke participants ($P = 0{\cdot}032$). There was a trend to higher levels of VWF in HIV+ve strokes compared with HIV+ve nonstroke controls ($P = 0{\cdot}061$).

HIV+ve stroke group compared with the HIV+ve nonstroke controls ($P = 0{\cdot}018$). However, the ADAMTS13 levels of the latter group were still lower than that of the defined normal range.

CD4 counts were negatively correlated with VWF levels in all HIV+ve participants (strokes and nonstroke controls; $P = 0{\cdot}006$; see Fig. 1a). CD4 counts were not correlated with ADAMTS13 levels in HIV+ve participants.

Discussion

In this study, performed within a few days of an acute ischemic stroke, stroke in HIV-infected participants was associated with a prothrombotic state with high levels of VWF and low levels of ADAMTS13. The question is whether these changes occurred as a consequence of the stroke, as part of an acute phase reaction, or whether they preceded the stroke and contributed to its cause. We cannot definitively answer this question. However, the finding of a negative correlation between CD4 counts and VWF levels in the HIV+ve participants shows that VWF rises with advancing immunosuppression. At a certain critical prothrombotic level, this may precipitate strokes in susceptible individuals. Endothelial dysfunction is an important feature of HIV infection and becomes more severe in advanced HIV where it is associated with increased cardiovascular risk (11). VWF is released by platelets and endothelial cells. It is likely that in HIV infection VWF levels are elevated as a result of both endothelial dysfunction and chronic low grade activation of the procoagulant pathways. In our study, the median VWF levels were significantly higher in the HIV+ve stroke group compared with the HIV−ve group. There was a trend to higher VWF in HIV+ve strokes compared with HIV+ve controls, but there was no significant difference between the HIV−ve strokes compared with HIV+ve controls. Therefore, the elevated VWF is not solely the consequence of the stroke.

With regard to ADAMTS13 levels, these were markedly reduced in both HIV+ve and HIV−ve stroke participants. It is likely therefore that this indicates a nonspecific poststroke effect. However, ADAMTS13 levels were reduced to 23% of the population mean in the HIV+ve nonstroke controls (Table 1). This suggests ADAMTS13 is reduced with HIV infection. It may be that the low levels poststroke mask a low prestroke level due to a floor effect. This, in combination with high VWF levels, may place patients at higher risk of stroke. A prothrombotic state with auto-antibodies to ADAMTS13 has previously been described in the condition of HIV-associated TTP (10).

A potential limitation of this study is the heterogeneity of stroke etiology. However, the strokes were most commonly idiopathic. Furthermore, the relevance of a positive VZV PCR in HIV stroke is still debated. Studies using measurements of these factors at several time points poststroke would help to differentiate causal from consequential effects.

In summary, we have shown that VWF levels are elevated in HIV+ve stroke patients and that ADAMTS13 levels are low in HIV+ve patients without stroke. These findings support the role of both VWF and ADAMTS13 in the pathogenesis of HIV-related stroke. These factors are likely to contribute to the increased cerebrovascular risk associated with HIV infection.

Authors' contributions

S. A., M. C., and A. S. contributed to the conception and design of the study. S. A. and A. S. performed the main data collection. All authors contributed to writing and critical review of the manuscript, and gave their final approval of the version to be published.

Ethics approval

The Human Research Ethics Committee of the University of Cape Town/Groote Schuur Hospital approved this study (HREC ref: 178/2010).

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Case Report

Valproic Acid Associated Platelet Dysfunction: Case Report

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SUMMARY

Valproic acid is commonly used as an anticonvulsant. It has been shown to inhibit the secondary phase of platelet aggregation. This can be reflected in increased bleeding times and haemorrhage. We describe a case of a 56-year-old male with a history of bleeding during a previous operation. He had valproic acid associated platelet dysfunction.

Keywords: Valproic acid; Platelet aggregation; Platelet function tests; Preoperative care; Haemorrhage.

INTRODUCTION

Preoperative evaluation of bleeding disorders relies heavily on history taking. The first step is to establish if the patient has had a history of major surgery and/or trauma and whether there was any significant bleeding associated with these events. If previous major surgery and/or trauma had not been associated with significant bleeding then surgery can proceed. When the patient has had no history of major surgery or trauma, history still remains important to establish the likelihood that the patient has an acquired bleeding disorder or the likelihood of an inherited bleeding disorder in the patient and/or in close relatives. Drug history must be considered when one is evaluating for an acquired bleeding disorder.

CASE REPORT

A 56-year-old male with recurrent meningioma was preoperatively screened for a bleeding tendency after he gave a history of bleeding during a previous operation for the meningioma. He was on valproic acid for seizure prophylaxis. The full blood count was normal: white cell count (WCC) 9.0, haemoglobin (Hb) 14 and platelet count (Plt) 213; as were prothrombin time (PT) and activated partial thromboplastin time (aPTT). A prolonged PFA-100 bleeding time of 15 minutes led to further investigations. Von Willebrand screening tests; von Willebrand factor antigen (VWF:Antigen), von Willebrand factor-collagen binding activity (VWF:CBA) & von Willebrand factor ristocetin cofactor (VWF:RCo) assay, factor VIII (FVIII) level, and the multimer pattern were all normal. Platelet aggregometry on platelet-rich plasma showed lack of aggregation with arachidonic acid (AA) but there was aggregation with adenosine diphosphate (ADP), epinephrine (EPI), Collagen (Col), and ristocetin (Figure 1). These findings were suggestive of a drug induced platelet dysfunction. Since valproic acid was the only drug which the patient was taking, it was stopped for two weeks under close monitoring for seizures. The bleeding time and platelet function tests were then repeated. The bleeding time normalised and the platelet function tests were all normal. The patient proceeded with the operation which was uneventful.

DISCUSSION AND CONCLUSION

There are no standard guidelines on the testing and interpretation of platelet function tests [1] but lack of aggregation with only AA acid is found in the

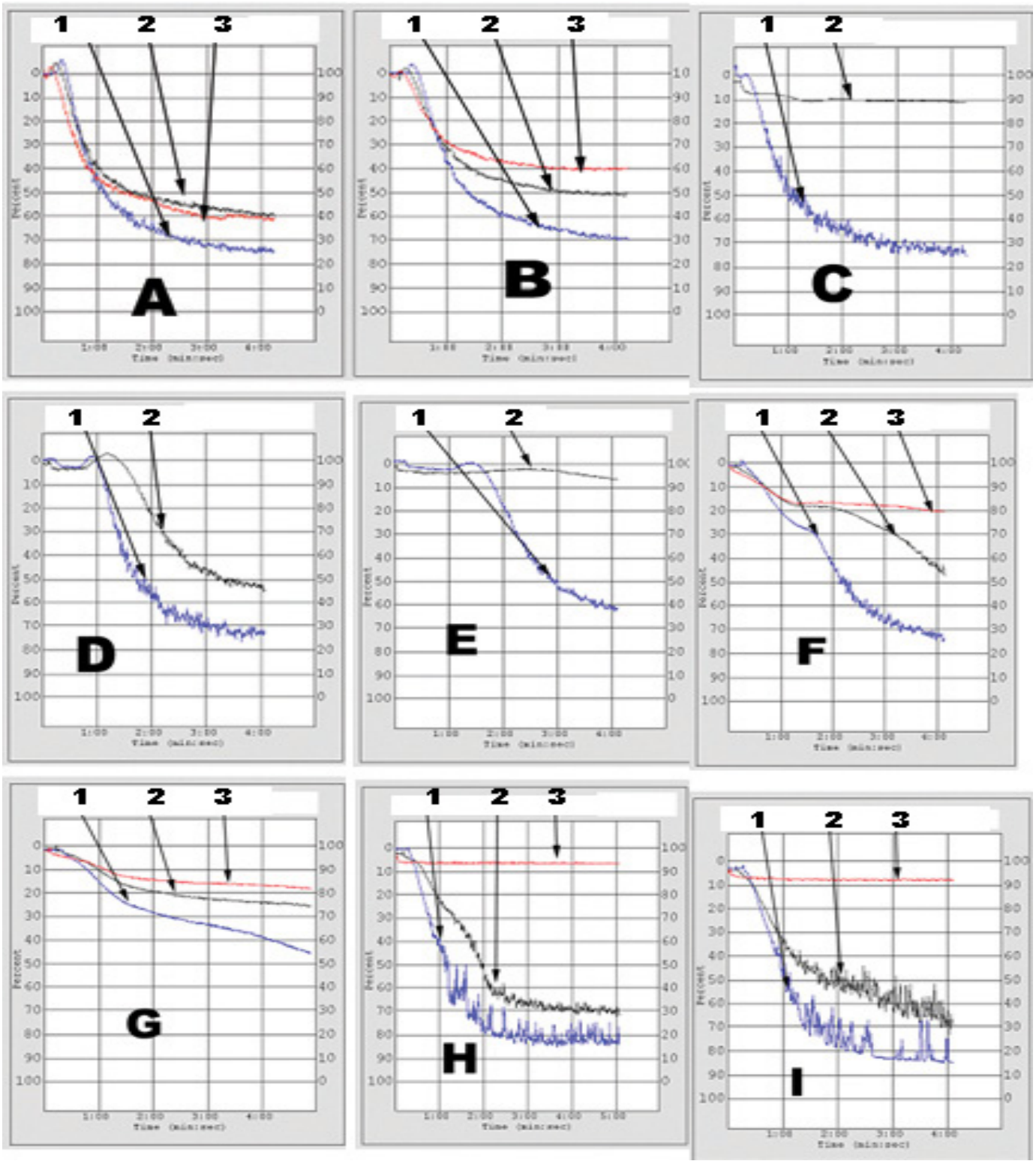

Figure 1. Aggregometry results. (A and B) adenosine diphosphate (ADP); (A) is control and (B) is patient's sample. Traces 1, 2 and 3 represent high, medium and low concentrations of ADP respectively. Graph (B) shows aggregation with ADP. (C) Arachidonic acid (AA); Trace 1 is control and Trace 2 is patient's sample. There was no aggregation with AA. (D and E) Collagen (Col); (D) is control and (E) is patient's sample. Traces 1 and 2 represent high and low concentrations of Col. (E) shows aggregation with Col. (F and G) epinephrine (EPI); (F) is control and (G) is the patient's sample. Traces 1, 2 and 3 represent high, medium and low concentration of EPI. (G) shows aggregation with EPI. (H and I) ristocetin; (H) is control and (I) is patient's sample. Traces 1, 2 and 3 represent high, medium and low concentrations of ristocetin respectively. (I) shows aggregation with ristocetin.

so called "aspirin-like defect" [1] of platelets which is seen in patients taking aspirin and other drugs. The lack of aggregation with AA implies inability by the platelets to produce thromboxane A2 which is required for the AA agonist to induce platelet aggregation. Platelet aggregation testing with AA has been suggested as the ideal test to perform before all other aggregation tests to screen for induced platelet dysfunction. [2] It should of course be noted that the impedance-based whole blood platelet aggregation test may give different aggregometry patterns to the ones obtainable by the method we used (optical-based platelet-rich plasma test). [3]

An association between valproic acid and excessive bleeding during surgery has been well described. [4-5] Valproic acid may affect both platelet count and function (thereby prolonging bleeding time) and coagulation factors such as fibrinogen and factor VIII. [6-7]

This case emphasises the role of a proper medication history in the workup of a patient with a bleeding diathesis.

FOOTNOTES

Conflicts of interest: The authors declare no competing conflicts of interest

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