
**Sequencing of exon 28 of Von Willebrand
factor in five patients with type 2 Von
Willebrand disease**

Maliengoane Sylvia Mothabeng
B.Med.Sc Hons (Haematology)
University of the Free State

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Faculty of Health Sciences
University of the Free State
Bloemfontein

Study Leader: Prof. S M. Meiring

Bloemfontein

Co-Study Leader: Dr A. de Kock

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Declaration

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Maliengoane Sylvia Mothabeng

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Abbreviations

ADAMTS13	A Disintegrin And Metalloproteinase with a ThromboSpondin type 1 motif, member 13
ADP	Adenosine diphosphate
Ag	Antigen
APTT	Activated partial thrombin time
Bp	Base pair(s)
BS	Bleeding score
CB	Collagen binding
CB/Ag	Collagen binding/VWF antigen
cDNA	Complementary deoxyribonucleic acid
CK	Cystine knot
DDAVP	Desmopressin (1-deamino-8-D-arginine vasopressin)
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBC	Full blood count
FVIII	Factor VIII
FVIII/Ag	Factor VIII/VWF antigen
g	Gravity
GP	Glycoprotein
h	Hour
HMW	High molecular weight
Ig	Immunoglobulin
ISTH	International society on thrombosis and haemostasis
Kb	Kilo bases
LMW	Low molecular weight
MgCl₂	Magnesium chloride
Min	minute

mRNA	Messenger ribonucleic acid
PCR	Polymerase chain reaction
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PT	Prothrombin time
PT-VWD	Platelet type Von Willebrand disease
RCo	Ristocetin cofactor
RCo/Ag	Ristocetin cofactor assay/VWF antigen
RIPA	Ristocetin induced platelet aggregation
rpm	Revolutions per minute
Sec	Second
TBE	Tris Boric acid and EDTA
TTP	Thrombotic thrombocytopenic purpura
ULVWF	Ultra large von Willebrand factor
VWD	Von Willebrand disease
VWF	Von Willebrand Factor
VWF:Ag	Von Willebrand Factor antigen assay
VWF:CB	Collagen binding assay
VWF:FVIII B	FVIII binding activity
VWF:RCo	Ristocetin cofactor assay
VWFpp	Von Willebrand Factor propeptide
WHO	World health organization

Chapter 1: Introduction

Von Willebrand disease (VWD) is a common bleeding disorder caused by either quantitative (type 1 and 3) or qualitative (type 2) defects of von Willebrand factor (VWF) (Pasi 2005). Prevalence of VWD has been estimated to be 0.6-1.3% in the general population, occurring more in women than in men with a ratio of 7:3 (Dietrich 2007). VWF is a multimeric adhesive protein which plays an important role in primary haemostasis by promoting platelet adhesion to sub-endothelium at the site of vascular injury (Castaman *et al.* 2003). VWF also transports coagulation factor VIII (FVIII) to the site of injury to perform its role in coagulation and it also protects FVIII from premature proteolysis in the circulation (Budde and Schneppenheim 2001). VWF is synthesized and stored in the endothelial cells and megakaryocytes (Denis *et al.* 2008) and secreted into the plasma upon stimulation. In the plasma, VWF multimers are subjected to cleavage by the metalloprotease, ADAMTS-13 (Reininger 2008a). The human VWF gene has been localized to chromosome 12 and a partial pseudogene on chromosome 22. The 52 exons span 178kb, approximately 0.1 % of human chromosome 12. The exons range from 40bp to 1.4 kb for the largest exon (exon 28) (Mancuso *et al.* 1989). Mutations in the VWF gene result in the most common bleeding disorder VWD (Pasi 2005).

The classification of VWD distinguishes between quantitative (type 1 and type 3) and qualitative (type 2) forms. Type 1 is characterized by a partial deficiency of VWF and type 3 is a complete absence of VWF. The functional type 2 defects are divided in four subcategories types 2A, 2B, 2M, and 2N. Type 2A VWD is characterised by the loss of high and intermediate VWF multimers. This might be a result of increased proteolysis of VWF or defective synthesis due to mutations in the A2 domain of VWF gene (Michiels *et al.* 2006). Missense mutations have been reported in the D2, A1 and A2 Domain in the VWF gene (Franchini 2006). In type 2B VWD, missense mutations in the D3, A1 and A2 domains of VWF gene result in a gain in function of VWF

protein. This means that VWF has an increased affinity for binding to platelet GPIb causing spontaneous adhering of VWF to platelets. As a result patients with type 2B VWD have premature clearance of platelets in the circulation causing thrombocytopenia (Franchini 2006). Type 2M VWD is caused by decreased affinity for VWF to bind to platelets. This is caused by mutations in the region of the platelet binding site. These mutations result in lessened interaction between VWF multimers and platelets despite the presence of all sizes of multimers (Wilde 2007), (Michiels *et al.* 2005). Mutations in exon 28 have been found in most patients with type 2M VWD. Type 2N VWD is caused by mutations in the FVIII binding region of VWF (Budde and Schneppenheim 2001). Majority of the mutations causing type 2N are located on the D domain with fewer located on the D3 domain of the VWF gene. Recently, many studies worldwide have been conducted on finding mutations in exon 28 of the VWF gene. However none have been done on South African populations.

The diagnosis of VWD usually requires a panel of tests. Despite all these tests, the diagnosis and classification of VWD often remains a challenge. Identification of mutations that cause functional defects of VWF (type 2 VWD) is needed to improve the diagnosis of the disease. Mutations that cause functional abnormalities of VWF occur mostly in exon 28 of the VWF gene. Exon 28 primarily encodes the platelet glycoprotein Ib (GPIb) and collagen binding domain of VWF (A1 domains) and the ADAMTS13 cleavage domain (A2 domains). To date, no single test is available that provides appropriate information about the various functions of VWF. Several analyses therefore are required to diagnose VWD. These tests are also subjected to pitfalls and it is important to take the pitfalls in to consideration when diagnosing VWD. Identification of VWF mutations is important to improve the diagnosis of VWD and therefore for predict better treatment choices for patients.

Our specialised Haemostasis laboratory is the only laboratory in South Africa that performs all the diagnostic tests needed for a proper diagnosis of VWD. By adding mutation analyses to our comprehensive VWD testing process, will

not only improve diagnosis of the disease but will also allow us to construct a VWD registry in our country.

In this study we aim to setup a routine method to use in order to search for mutations in exon 28 of the VWF gene. We searched for mutations in exon 28 of 5 patients with functional defects of VWF (two with type 2M, two with type 2B and one with type 2A VWD).

Chapter 2: Literature review

This literature review will be divided in 3 parts. In this first part I will describe the VWF gene and protein. The second part will describe VWD and the third part will discuss the molecular diagnoses of VWD.

1. Von Willebrand factor

Von Willebrand factor is a large multimeric glycoprotein that is essential for platelet dependent primary haemostasis particularly in the microvasculature where high fluid shear forces are present (Perutelli and Molinari 2007). It also acts as a carrier for coagulation factor VIII (FVIII). It transports FVIII to the site of injury to perform its role in coagulation and it also protects FVIII from premature proteolysis in the circulation (Budde and Schneppenheim 2001).

1.1. *Von Willebrand factor gene*

The human VWF gene is localised on chromosome 12p13.2. The gene spans 178 kilobase pairs with 52 exons as shown in Figure 1. A non-coding pseudo gene on chromosome 22 is highly homologous with the VWF gene and spans from exon 23 to 34 (Castaman *et al.* 2003). The exons of the VWF gene range in length from 40 to 1379 base pairs, while the introns range from 97 to 19.9 kilobase pairs. The first 17 exons of the VWF gene, encodes both the signal peptide and propeptide while the other 35 exons encode for the mature subunit of VWF (Mancuso *et al.* 1989).

The AT rich area in the promoter region is similar to a TATA box but with the atypical sequence TAATTA which is found 32 base pairs upstream of the transcription initiation site. An untranslated 5' leader sequence is encoded by exon 1 and the second exon encodes most of the signal peptide beginning with the ATG translation initiation codon (Standen and Peake 1991). The largest exon of VWF gene is exon 28 containing 1379 bases that encode for the A1 and A2 domains of the protein (Sadler 1998).

1.2. VWF protein

The premature VWF protein contains a 22 amino acid signal peptide, a 7441 amino acid pro-peptide and a mature VWF molecule containing 2050 amino acids (Castaman *et al.* 2003). The mature protein contains a clustering of cysteine residues on the amino and carboxy terminals, with a total of 169 cysteine residues throughout the VWF protein (Ruggeri 2001). It consists of A, B, C, and D domains as shown in Figure 1. The various protein domains are responsible for different binding functions of the mature molecule.

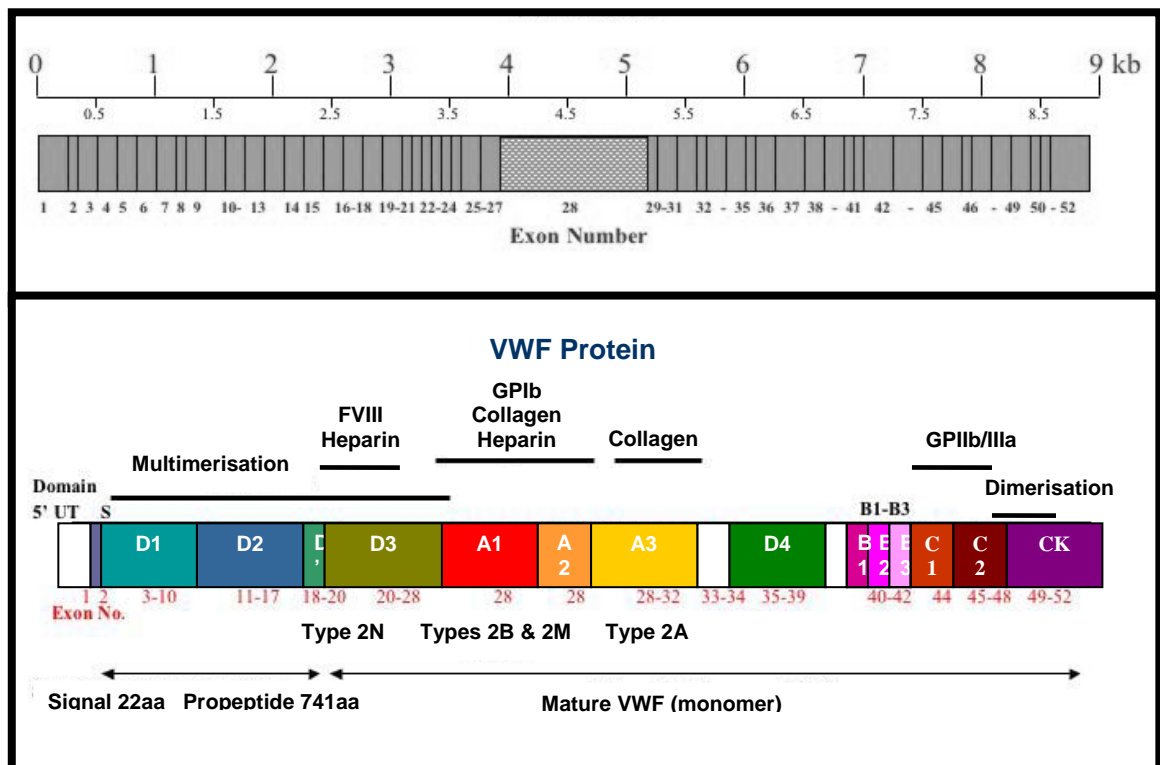


Figure 1: The human VWF mRNA and protein

(Adapted from <http://www.vwf.group.shef.ac.uk/pictures.html>)

The A domain is divided into three domains A1, A2 and A3 domains. The A1 domain of VWF has binding sites for glycoprotein Iba (components of the Gplb-IX-V complex), heparin and collagen type VI (Budde and Schneppenheim 2001). The A2 domain contains the cleavage site for the metalloprotease ADAMTS13 (Reininger 2008a). Because of its low stability, the A2 domain is able to regulate the following processes: exposing the ADAMTS13 cleavage site on the A2 domain during high shear, docking of

ADAMTS13 on Ultra large VWF (ULVWF) and platelet adhesion onto ULVWF. The A3 domain binds collagen types I and III. It also regulates the A1 domain (Chen and Lopez 2006).

The C domain consists of the C1, C2 and CK domains. The C1 domain includes an Arg-Gly-Asp (RGD) sequence that binds to platelet integrin $\alpha_{IIb}\beta_3$. Cysteine residues appear to be paired in disulfide bonds in the secreted protein (Sadler 1998). Platelet integrin $\alpha_{IIb}\beta_3$ does not bind to VWF when the platelets are inactive. However, upon activation $\alpha_{IIb}\beta_3$ has a high affinity to bind to fibrinogen and VWF (Sadler 1998).

The D domain consists of the D1, D2, D3 and D' domains. The binding site for FVIII is located on the D'-D3 domains, which also bind P-selectin. This is how the newly secreted ultra large VWF anchors itself to the activated endothelial cells. It also promote the stretching of VWF as to expose the ADAMTS13 cleavage site on the A2 domain (Reininger 2008a). Cysteine residues appear to be paired in disulfide bonds in the secreted protein (Sadler 1998).

1.3. Von Willebrand factor synthesis

VWF is synthesized and stored in the endothelial cells and megakaryocytes. It is synthesised as a proVWF monomer with a large propeptide attached onto the N-terminal as illustrated in Figure 2. Within the endoplasmic reticulum (ER), two proVWF monomers form a dimer through forming disulfide bonds at the C-terminal (cystine knot (CK) domains). The propeptide folds towards the D domain and binds to the D3 domain by forming a disulfide-linked intermediate (Sadler 2005).

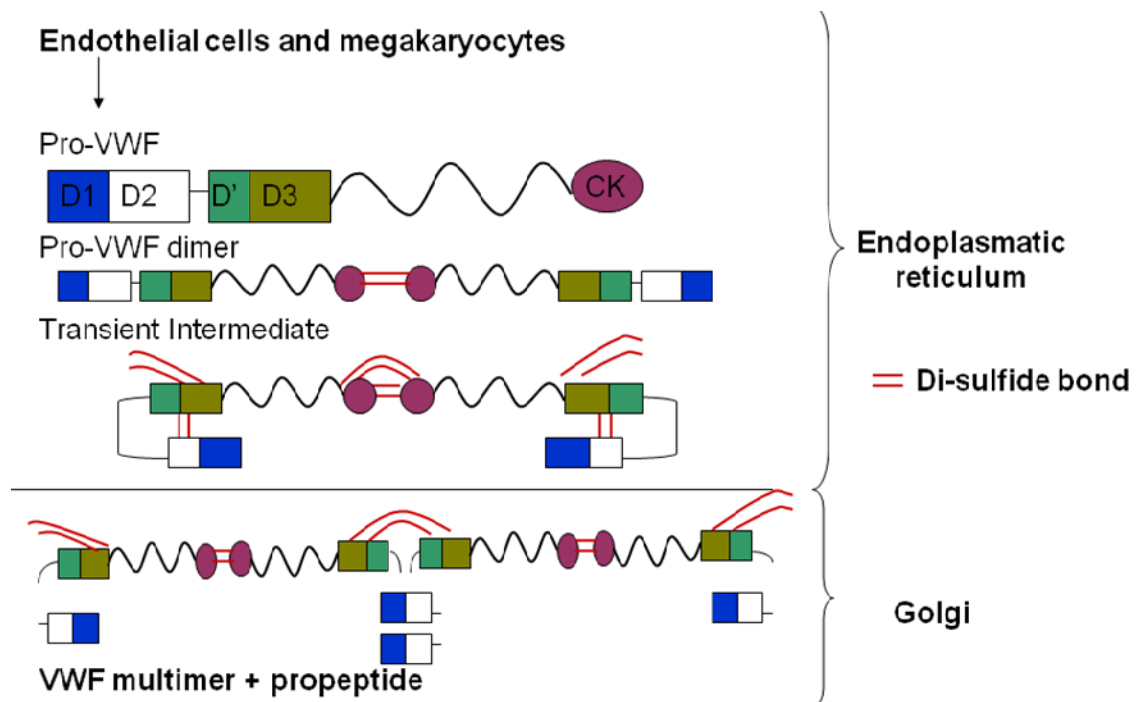


Figure 2: Illustration of the synthesis of VWF

While transferring to the Golgi apparatus, the propeptide is cleaved off from VWF dimers and the dimers binds to each other through di-sulfide bonds between the D'–D3 domains. Both mature VWF and the propeptide are secreted into the plasma. The VWF propeptide (VWFpp) seems to play a vital role in the assembly of VWF multimers, as well as in determining the survival of VWF in plasma. The large VWF multimers are secreted immediately if required or packed and stored as ultra large VWF (ULVWF) into the endothelium Weibel-Palade bodies or platelet α -granules where it can be released after stimulation by regulatory proteins. VWF multimers range in molecular weight from 0.5×10^6 to over 10×10^6 Da (Denis *et al.* 2008). Plasma VWF has a half life of 8-14 hours (h) with a concentration of $10 \mu\text{g/ml}$ (Sztukowska *et al.* 2008).

1.4. Glycosylation of VWF

The mature subunit of VWF is extensively glycosylated amounting to 19% of the total molecular weight (Gallinaro *et al.* 2008). The glycosylation process includes the incorporation of the 12 N-linked and 10 O-linked oligosaccharide chains onto a mature VWF molecule (Titani *et al.* 1986). Glycosylation

influences the functional and structural integrity of VWF. However the function of these oligosaccharides still remains uncertain. It has been shown that they protect VWF from proteolytic degradation. It preserves the structure of VWF and it influences the interaction of VWF with platelets and collagen (Millar and Brown 2006). Glycosylation of VWF also influences the plasma levels of VWF (Denis *et al.* 2008).

1.5. Effect of ABO bloodgroup on VWF

The ABO blood group system consists of the carbohydrates A, B, and H antigens. A variation on a common precursor side chain with either N-acetylgalactosamine or D-galactose, results in an A or B antigen. Individuals with blood group O that contain no A or B antigens, have 25% lower VWF levels in plasma than non-O individuals (Gallinaro *et al.* 2008). A study by Sukhu *et al.* (2003) also showed that individuals with blood group O have lower levels of VWF as compared to non-group O individuals. Previous studies were done only on Caucasian populations. The study of Sukhu *et al.* (2003) includes sample groups of African and Indian populations in South Africa. Interestingly they showed that the African individuals with blood group O showed the lowest VWF levels of the group. This indicates that not only does the blood group play a vital role in diagnosis of VWD but also the ethnicity of the patient has to be considered in the diagnosis of VWD. The low VWF levels associated with blood group O individuals is attributed to the accelerated clearance of VWF from plasma (Gallinaro *et al.* 2008). It is also shown the VWF is more susceptible to proteolysis of ADAMTS13 in bloodgroup O individuals (Sousa *et al.* 2007).

1.6. Clearance

The decreased half life of VWF can be determined by calculating the ratio of VWF_{pp} to the mature VWF concentration in plasma as described by Sztukowska *et al.* (2008). Previously it was thought that VWF was cleared using the scavenger receptor pathway that is found mainly in hepatocytes, renal cells, and endothelial cells. But a massive molecule like VWF is

cleared by macrophages and not by the lipoprotein scavenger pathway that normally clears coagulation proteins. Macrophages target the VWF-FVIII complex, since inactivation of macrophages results in increased VWF and FVIII levels (van Schooten *et al.* 2008). The VWF clearance pathway is vulnerable to pharmacologic disturbances thus it could be useful to prolong the half life of VWF or infused FVIII (Gilbert 2008). There are a number of proposed pathways that seem to have an influence on the clearance of VWF. These include the presence of sialyl groups that protect VWF from premature clearance, the presence of O-linked glycans that improve the survival of VWF (Denis *et al.* 2008), as well as the presence of specific mutations (R1205H) that increase the clearance rate of VWF (Lenting *et al.* 2004)

1.7. Role of VWF in primary haemostasis

Platelets are the cellular components in haemostasis that have the capability to act in response to blood flow or lesions in the endothelium. To accomplish this, platelets have subcomponents that interconnect with other cellular components and coagulation proteins in the blood (Schmugge *et al.* 2003). Interaction between circulating platelets and VWF does not seem to occur in the absence of injury. However vascular damage causing endothelium exposure induces the binding of VWF to the endothelium and to platelets at high shear rate with the affinity to retain them at the site of injury (Sadler 1998). The physiological function of VWF relies critically on the molecular size of VWF to mediate platelet adhesion and aggregation in primary haemostasis (Hassenpflug *et al.* 2006). The α -granules of platelets only release VWF when platelets are activated. Thus, circulating VWF in plasma is derived mostly from endothelial cells. The large multimers are the most haemostatic active (Ruggeri 2007). They are also more receptive to high shear stress because they are more flexible. Platelets bind to collagen type I and III but this interaction is dependent on the FVIII-VWF complex and also on fibronectin (Houdijk *et al.* 1985). Previously it was thought that VWF does not facilitate platelet adhesion at low shear rates however this is not true. Interaction between fibrinogen and platelet integrin α IIb β 3 receptor facilitates

this process (Perutelli and Molinari 2007). But it is in high shear stress situations where VWF plays an integral part.

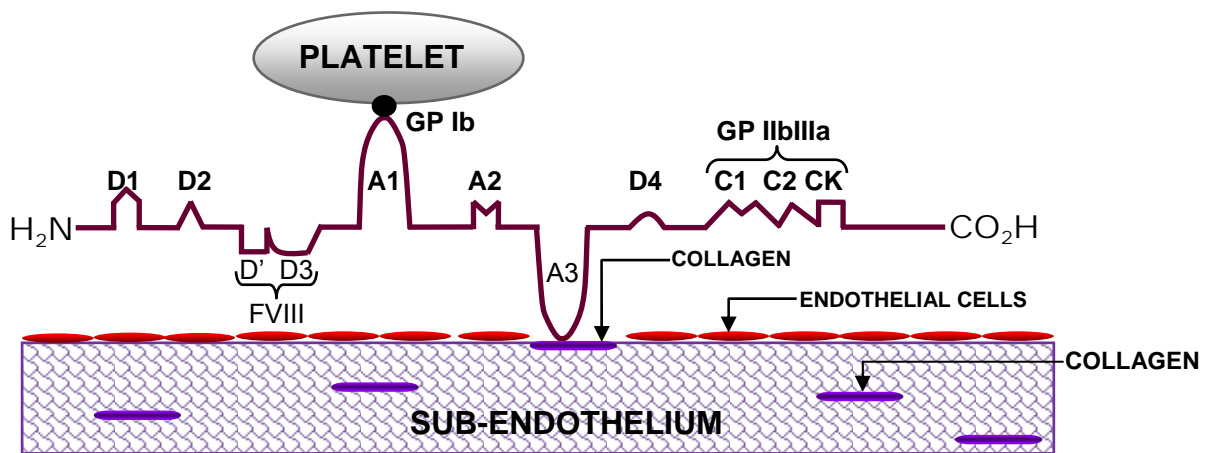


Figure 3: Interaction between VWF A3 domain and collagen

Under high shear stress situations, platelets agglutinate by the interaction of GPIb α to VWF (Perutelli and Molinari 2007). The A3 domain of VWF is the binding site for collagen (type I and III) as seen in Figure 3 (Reininger 2008a) (Reininger 2008b). Interaction between the A1 domain of VWF and GPIb α (of the GPIb-IX-V complex) receptor of platelets initialises the process of platelets rolling, thereafter GPIIa/IIIb receptors arrest platelets (Budde and Schneppenheim 2001). The bond between GP Ib and VWF is not strong enough to sustain an irreversible effect thus the platelets roll on the vessel wall in the direction of the flow (Ruggeri 2001).

2. Von Willebrand disease

The condition now known as Von Willebrand disease (VWD) the most common bleeding disorder, was first described by the Finnish physician, Eric Adolf von Willebrand in 1926, from members of a family in the Åland Islands who inherited the disease in an autosomal dominant pattern (McDonald 2007).

2.1. Prevalence of VWD

The prevalence VWD has been estimated at 0.6-1.3% in the general population, occurring more in women than in men with a ratio of 7:3 (Dietrich

2007). Diagnosis of VWD in African American women as compared to Caucasian women was found to be 1.3% and 15.9% respectively (Dietrich 2007). Chile and Venezuela are the only two developing countries where the prevalence of VWD has been documented. In parts of Chile the prevalence has been recorded as 113 per million and in Zulia Venezuela only 43 per million (Srivastava 2005). The South Africa Haemophilia Register in 1998 showed a VWD prevalence of 321 in a general population of approximately 36 million people (9 per million) (Srivastava *et al.* 1998). Bird (1996) reported that 60% of the VWD patients tested in the Western Cape South Africa had type 1 VWD, 10% with type 2 and 30% with type 3 VWD. However their sample of patients was relatively small. VWD is clearly under diagnosed in South Africa and studies are lacking regarding statistical prevalence of VWD in populations in South Africa.

2.2. *Inheritance of VWD*

Von Willebrand disease is an autosomally inherited disorder caused by a deficiency or abnormality of VWF. Type 1, 2A, 2B and 2M are inherited in an autosomal dominant pattern where as types 3 and 2N have autosomal recessive inheritance (Federici and Mannucci 2007).

2.3. *Clinical symptoms*

The clinical manifestations of the different types of VWD range from mild to severe bleedings. Excessive mucocutaneous bleedings are the most common symptom affecting the quality of life of the patients. Patients with VWD may also experience easy bruising and prolonged bleeding from cuts or post operative bleeding. The most common diagnostic symptom is bleeding after dental extraction. Clinical manifestations in women may only be menorrhagia. It is thus important to assess the history of the menstrual cycles in these patients (Federici 2006).

2.4. *Diagnosis of VWD*

Diagnosis cannot be made accurately without the presence of phenotypic information. There are two stages in the diagnosis of VWD: (i) the screening of patients at risk of VWD by using the bleeding score and laboratory tests as well as family and clinical history; (ii) specific laboratory test are applied to diagnose VWD and specify the type and subtype. However, the uncertainty of the link between clinical manifestations, laboratory assays and VWF functionality often resulting in difficult diagnosis (Pasi 2005).

Clinical history of familial bleeding tendencies is an essential part in the proper diagnosis of VWD. Drugs like aspirin inhibit platelets aggregation and it is therefore important to also take drug intake history into account. Such drugs can influence the bleeding intensity of a patient already suspected of having VWD (McDonald 2007). The influence of other external factors like stress, exercise, oral contraceptives, pregnancy, and ABO bloodgroup also have an influence on the levels VWF in plasma resulting in either a false negative or false positive result.

2.5. *The Bleeding Score (BS)*

By using the bleeding score (BS) correctly an experienced haematologist can easily determine the extent of hereditary or acquired bleeding tendency scaling them into different categories namely:

- Very mild: Patients with one or two indistinct minor bleeding tendencies.
- Mild: Patients with one or two noticeable mucocutaneous bleeding episodes such as recurrent episodes of epistaxis, and/or prolonged menstruation.
- Moderate: Patients with inherited bleeding tendency that usually presents in early childhood that is characterized by recurrent mucocutaneous bleeds, excessive bleeding after a tooth extraction, or surgery or bleeding that needed medical attention and/or FVIII/VWF concentrate.

- Pronounced: Frequent mucocutaneous bleeds that presented in early childhood including soft tissue, muscle bleeds and need for prophylactic treatment with FVIII/VWF concentrate.

2.6. Preliminary screening tests

The screening tests include a full blood count (FBC), the bleeding time, prothrombin time (PT) and activated partial thromboplastin time (APTT) and factor VIII coagulant activity.

The full blood count (FBC) is used to assess the platelet count and morphology. Bleeding time is an important screening test, but the reproducibility and sensitivity are questioned. This test should be performed by a qualified laboratory technician using standardised instruments. It is important to take note that aspirin should not be taken at least 10 days prior to the tests being performed as it influences the bleeding time (Kessler 2007). Prolonged PT or APTT can suggest coagulation factor deficiency. VWD patients might have a normal or a prolonged APTT.

The platelet function analyser, PFA 100 is used to mimic high shear conditions of primary haemostasis. The PFA 100 has a high sensitivity to detect VWD types 1, 2A, 2B, 2M and 3. The reason being that the PFA100 is sensitive to the loss of VWF especially the HMW multimers (Favaloro 2006b). However the PFA 100 results can be influenced by other factors including low hematocrit, low platelet counts and platelet dysfunction. This means that the PFA 100 is neither predictive nor specific for any disorder including VWD (Favaloro 2006c).

The one stage assay for the determination of FVIII activity is used as a screening test for bleeding disorders. Decreased FVIII activity might be seen in severe cases of type 2N, type 1, type 2A and type 3 VWD since decreased FVIII activity levels also indicates decreased levels of VWF. The usefulness of this assay in the screening of VWD is still disputed as a normal

activity of FVIII does not rule out the presence of VWD. Likewise a decreased activity of FVIII does not indicate VWD (Lippi *et al.* 2007).

2.7. Laboratory tests used in the diagnosis of VWD

The following tests are used in our laboratory to diagnose VWD: VWF antigen, ristocetin cofactor assay, collagen binding assay, VWF multimer analysis, ristocetin induced platelet agglutination and the FVIII binding assay.

The VWF antigen assay (VWF:Ag) measures the levels of VWF in the patients plasma but provides no information about the quality and functionality of the VWF (Lippi *et al.* 2007). The VWF:Ag assay is needed to discriminate between qualitative and quantitative VWD. It is an ELISA (Enzyme linked immunosorbent assay) method that is sensitive and easy to standardize. Recently a trend towards automated methods has increased allowing for more rapid results. It is vital to note that the results obtained from the VWF:Ag cannot be used alone to diagnose VWD. Functional assays need to be done for proper diagnosis.

The ristocetin cofactor assay (VWF:RCo) assesses functional VWF activity using ristocetin and is still one of the preferred standard functional assays. This assay assesses the interaction between platelets and VWF by using ristocetin as an agonist (Castaman *et al.* 2003). Formalin fixed donor platelets are used to eliminate the possibility of binding defects in the patient's own platelets. The VWF:RCo assay thus evaluates the platelet binding function of VWF.

The collagen binding activity of the VWF (VWF:CB) assesses the functional ability of the high molecular weight (HMW) multimers of VWF to adhere to collagen. This is an ELISA based assay, where the patient VWF binds to a collagen-coated ELISA plate. The adhesion is measured using a peroxidase linked antibody. This assay can distinguish between type 1 and type 2 VWD. This assay also provides useful functional information on VWF (Budde and

Schneppenheim 2001). The usefulness of the collagen binding assay is its ability to detect defects in the VWF multimers. This implies that type 2A and type 2B will give lower VWF:CB results due to the absence of the HMW multimers (Favaloro 1999).

VWF multimer analysis is a time consuming and laborious assay. The latest methods reduced the turnaround time from 5 to 3 days. It is a Western blotting process onto nitrocellulose or nylon filters. The discontinued use of radioactive material rendered the method insensitive to very low levels of VWF (Budde *et al.* 2006). The molecular weight of normal VWF ranges from 800 to 20000 kDa, which can be examined using agarose gel electrophoresis. Low resolution agarose gels differentiate between low intermediate and high VWF multimers. In types 1, 2M and 2N VWD the full range of multimers are present, whilst in types 2A and 2B the high and sometimes intermediate multimers are absent as illustrated in Figure 4.

Densitometry of the multimer patterns is used for quantification and comparison of VWF multimer patterns (Meiring *et al.* 2005b). See Figure 5.

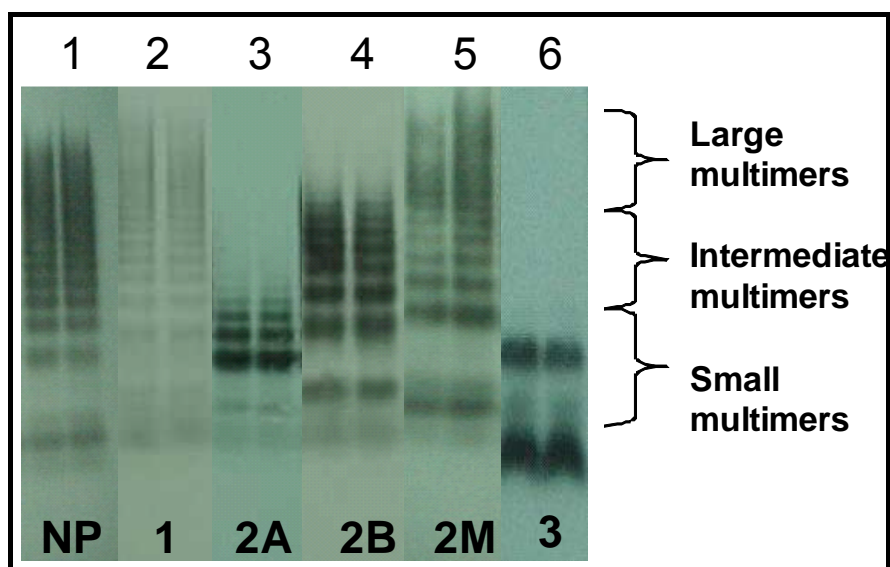


Figure 4: Multimer patterns from a 0.65% agarose gel showing the different types of VWD (Meiring *et al.* 2005a).

(Lane 1: normal plasma; lane 2: type1 VWD; lane 3: type 2A VWD; lane4: type 2B VWD; lane 5: type 2M VWD; lane 6: type 3 VWD)

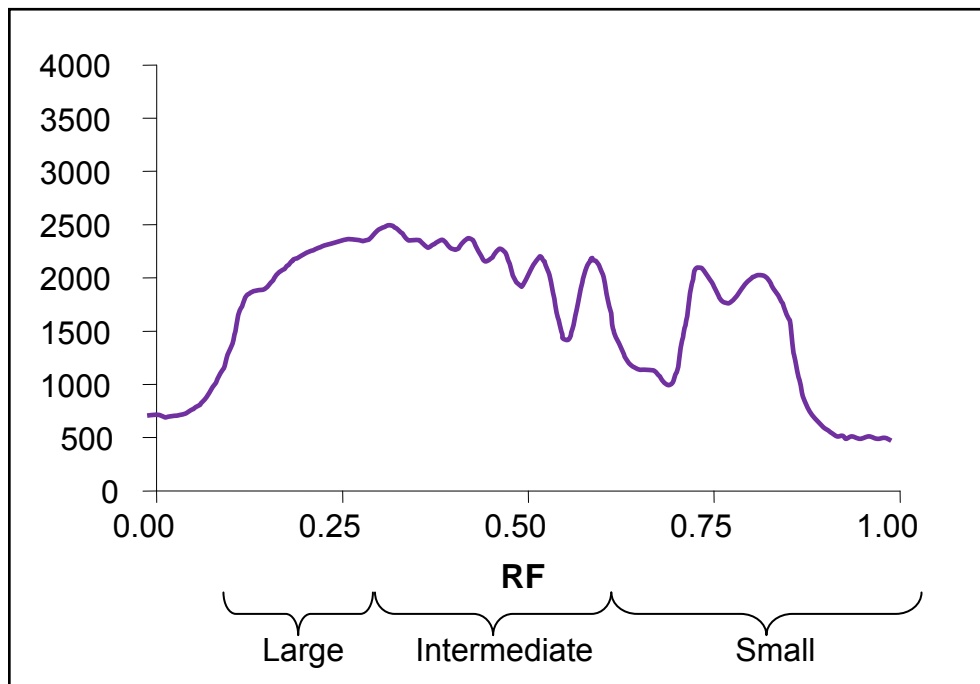


Figure 5: Densitometric tracing of the multimer analysis in normal plasma

The ristocetin induced platelet agglutination (RIPA) measures the binding of the patient's VWF to his/her own platelets by using different concentrations of ristocetin as agonist. The results are shown as the concentration of ristocetin able to induce 30% agglutination of platelets (Castaman *et al.* 2003). In Figure 6, the percentage agglutination is plotted on the Y axis and the ristocetin concentration on the X axis. Within a normal RIPA a ristocetin concentration of 0.9 mg/ml is needed to obtain 30% agglutination, while with an increased RIPA only 0.4 mg/ml is needed to obtain 30% agglutination. With a decreased RIPA a max agglutination of only 22% is reached at 1.4 mg/ml ristocetin. The normal range for the RIPA is found to be between 0.8 and 1.2 mg/ml ristocetin (Castaman *et al.* 2003).

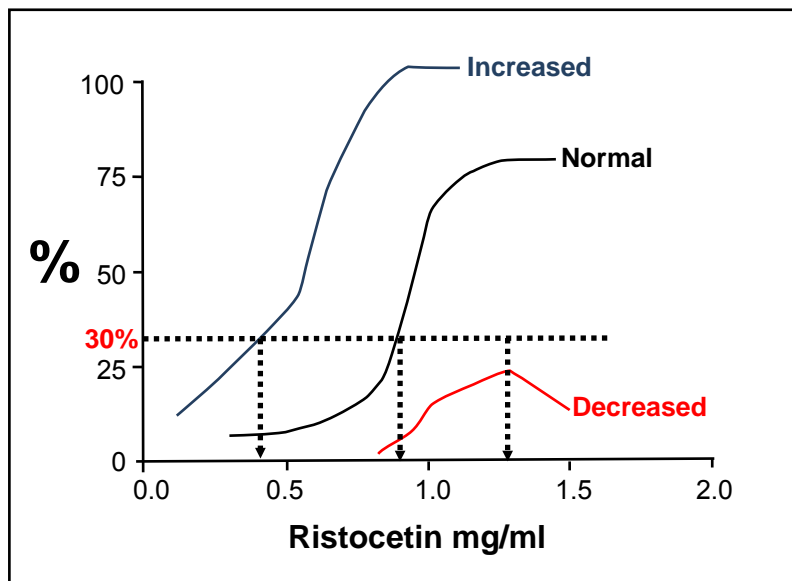


Figure 6: A RIPA graph showing the maximum amplitude reached using different concentrations of ristocetin in three different patients

FVIII binding assay (VWF:FVIII) measures the binding affinity of VWF to FVIII, this is also an ELISA assay. The microtiter plates are coated with anti-VWF antibody after which the patient's plasma is added. The FVIII bound to the plate is then removed with high ionic strength and an excess amount of recombinant FVIII is added to bind to the patients VWF on the plate and assayed. This test is used to discriminate between haemophilia A and type 2N VWD (Castaman *et al.* 2003).

A new suggested pathogenic mechanism in VWD is the reduction of VWF half life. During multimerization, the propeptide is cleaved off the VWF. It is secreted in equal concentration with VWF (Budde and Schneppenheim 2001). The decreased half life of VWF can be determined by calculating the ratio of VWFpp to VWF concentration in plasma. This is used to diagnose patients with VWD characterised reduced survival of VWF (Sztukowska *et al.* 2008). This can be important differentiating type 1 patients with increased clearance as to classical type 1. But this can also be extended to other subtypes of VWD that have increased clearance.

2.8. Laboratory diagnosis of von Willebrand disease

The classification of VWD distinguishes between partial quantitative (type 1), qualitative (type 2), and total quantitative (type 3) deficiency of VWF. Quantitative defects are divided in four subcategories types 2A, 2B, 2M, and 2N VWD (Casana *et al.* 1998).

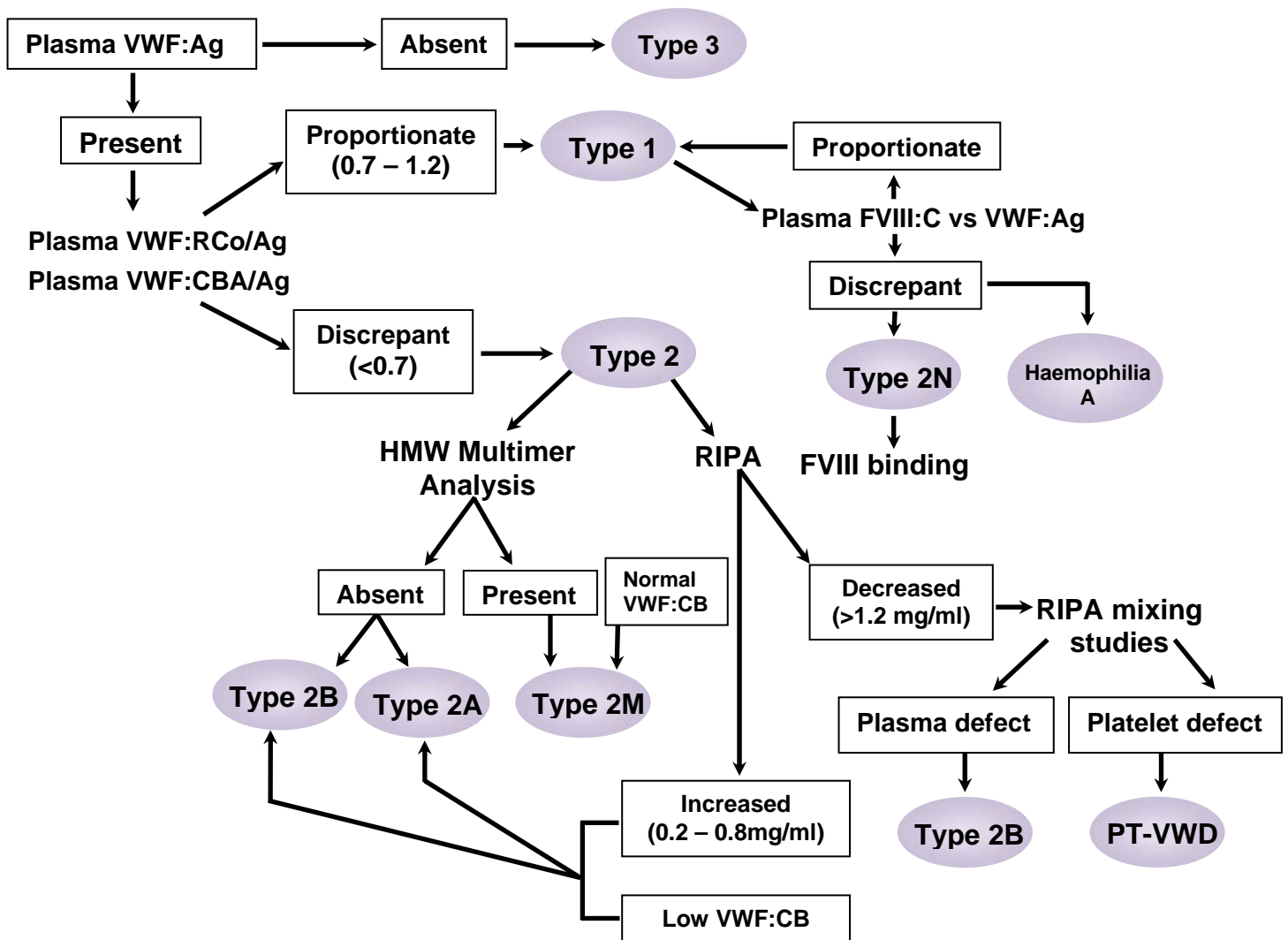


Figure 7: Flow diagram used as a tool in the diagnosis of VWD subtypes

Our VWD testing facility uses the flow diagram in Figure 7 in their diagnosis of VWD. Type 3 VWD is diagnosed when very low or unquantifiable VWF:Ag levels is found. Type 1 VWD is diagnosed where there is a proportionate reduction of both VWF:Ag and VWF:RCo with a RCo/Ag ratio

more than 0.7 as well as VWF:CB and VWF:Ag with a CB/Ag ratio more than 0.7. Type 2 is diagnosed where the RCo/Ag or the CB/Ag ratio is less than 0.7. The RIPA test discriminates between type 2B VWD, type 2A or type 2M VWD. In type 2B an increased RIPA will be found while a decreased RIPA is indicative of a type 2A or 2M VWD. The VWF:CB are low in type 2A and type 2B VWD, while the levels are normal in type 2M VWD. The absence of the large and intermediate multimers is seen in type 2A VWD while the absence of only the large multimers is seen type 2B VWD. Multimers of all sizes are present in type 2M VWD, but the density profile differs from that of normal plasma. More smaller and less larger multimers are seen in type 2M VWD. Type 2N VWD can be suspected when the ratio between FVIII and VWF:Ag levels are disproportionate and the FVIII levels are less than 20%. Diagnosis of type 2N should be confirmed by the FVIII binding assay.

2.9. *Challenges in the laboratory diagnosis of VWD*

Several analyses (tests) are required to diagnose VWD and it is important to take the inadequacies of these tests in consideration in the diagnosis of VWD. To date, no single test is available that provides appropriate information about the various functions of VWF. Great caution must be used in the diagnosis of VWD when interpreting laboratory results. A combination of both the screening and diagnostic tests results must be interpreted for proper diagnosis of VWD. Misdiagnosis is likely to occur due to lack of specific testing. For example, results based only on FVIII levels may lead to misdiagnosis of a type 2N VWD patient as a haemophilia A patient. Results obtained only from platelet aggregation studies can easily misdiagnose VWD as Bernard Soulier syndrome and type 2B VWD can be misdiagnosed as immune mediated thrombocytopenia (Adil and Qureshi 2008). The availability of tests for the diagnosis of VWD differs from centre to centre. Limited tests are available for the diagnosis of VWD in developing countries. The VWF:Ag and VWF:RCo assay being the primary and only tests available in most centres in developing countries (Srivastava 2005).

Low VWF levels might indicate VWD but cannot distinguish between the different subtypes. Type 2 VWD patients may have VWF levels that are within normal range. In a survey done in 2005 by the United Kingdom's National External Quality Assessment Scheme (UK NEQAS), it was reported that out of the 186 centres that participate in the VWF:Ag survey, only 26% use ELISA method, 61% use immunoturbidometric assays and 1% use immunoelectrophoresis. There are nine different antibody sources used by the different centres in the ELISA and the results obtained from the three methods were comparable (Kitchen *et al.* 2006).

VWF:RCo is a functional assay that measures the interaction of VWF to platelet GPIIb using the antibody and therefore lack physiological analogue. Platelet agglutination studies are not reproducible and also not sensitive for the presence of large multimers. Furthermore no consistency is obtained where the activity levels are lower than 11U/dL (Kitchen *et al.* 2006).

The collagen binding assay assesses the ability of VWF to bind to collagen. A disadvantage of this method is that the sensitivity depends on the preferred type of collagen used and its source. There is still an ongoing debate on the best type of collagen to be used in this method (Kessler 2007). The ELISA based VWF activity test seems to have no correlation with VWF:RCo in types 2A, 2B and 2M VWD (Kitchen *et al.* 2006). Due to the pitfalls in the laboratory diagnosis, molecular diagnosis is used in industrialised countries to assist in the diagnosis of VWD. Before the molecular diagnosis is discussed, it is necessary to first describe the different types and subtypes of VWD in more detail.

Quantitative VWD

2.9.1. Type 1

Type 1 VWD is the most common type of VWD, which constitutes about 60-80% of cases and is inherited as an autosomal dominant trait with partial penetrance. The VWF molecule is functionally normal, in type 1 VWD the quantity of VWF in plasma is deficiently low. This results in a

20 – 50% reduction in VWF antigen and activity levels. Other laboratory findings indicate normal multimer distribution and normal or impaired RIPA (Wilde 2007). Complications in the diagnosis of type 1 VWD is due to the fact that individuals with blood group O have a lower VWF levels than non O individuals. Platelet VWF also influences the classification of VWD as patients with low platelets VWF levels have a more prolonged bleeding time in contrast to patients with normal platelets VWF levels (Franchini 2006).

The diagnosis of type 1 VWD has been disputed regarding the pathophysiology of the disease. The influence of external factors on the levels of VWF such as stress, pregnancy as well as genetic factors such as the influence of the ABO blood group, alterations in the VWF sequence such as the Tyr1584Cys mutation and polymorphisms in the platelet integrin may influence the bleeding tendency of patients with type 1 VWD. Unidentifiable mutations amount for up to 50% of all cases diagnosed as type 1. This indicates that these individuals have certain mutations that are not identified in the integral parts of the VWF gene and thus not linked to the phenotype (Keeney *et al.* 2008).

2.9.2. Type 3

Type 3 VWD is indicated by extremely low or undetectable levels of VWF, it is the most severe recessive form of the disease. It is caused by the inheritance of two null VWF genes. It is also accompanied by low levels of FVIII. Laboratory diagnosis indicates no or very low levels of VWF:Ag, VWF:RCo and FVIII levels of less than 10% (Lillicrap 2007). Patients with type 3 have severe bleeding tendencies resulting in not only mucocutaneous bleeds but also hemarthroses and hematomas. A reduction or termination in mRNA expression caused by deletions, compound heterozygous mutations and nondeletions has been reported in such patients. Patients on FVIII-VWF concentrate treatment may develop alloantibodies against VWF which may be due to large deletions in the VWF gene (Franchini 2006). The prevalence of type 3 varies from

1/million to 1/500000 but in the Arab nations the prevalence is higher. This is due to inter family marriages that results in a less diverse gene pool. The genotype includes gene insertions, nonsense mutations, gene deletions, alterations to mRNA splicing and missense mutations. This often results in the removal of cysteine residues that most likely in the severe form, inhibits the cells from releasing VWF in the plasma (Lillicrap 2007).

Qualitative VWD

2.9.3. Type 2A

Type 2A is the most frequent subtype among type 2 VWD. A loss of high and intermediate VWF molecular weight multimers, low RCo/Ag ratio, prolonged bleeding time and low CB/Ag ratio are typical characteristics of type 2A VWD. The loss of high VWF multimers and the increased triple structure is as a result of an increase in proteolysis secondary to mutations in the A2 domain of VWF gene (Michiels *et al.* 2006). Type 2A mutations maybe divided into two groups. Group 1 mutations result in malfunctioning of intracellular transport as well as failure of assembly, storage and secretion of normal VWF multimers. Group 2 mutations cause increased proteolysis of VWF multimers by circulating ADAMTS13. Another reason for the loss of HMW multimer in type 2A is due to defects in post-translation processes such as dimerization or polymerization. Missense mutations have been reported in the D2, A1 and A2 domains of the VWF gene (Franchini 2006). Laboratory findings in patients with type 2A VWD consists of:

- prolonged BT
- constantly low RCo/Ag ratio
- low CB/Ag ratio
- absence of high molecular weight multimers but in accordance to severity the absence of intermediate multimers as well
- decreased RIPA (Michiels *et al.* 2005)

2.9.4. Type 2B

The inheritance is autosomal dominant but cases of recessive inheritance have been reported. Laboratory tests for type 2B report normal VWF:Ag, low VWF:RCo, increased RIPA and low VWF:CB levels in the absence of large multimers. The missense mutations in the D3, A1 and A2 domains of the VWF gene result in a gain of function of VWF protein. This means that VWF has an increased affinity for binding to platelets GPIb causing spontaneous adhering of VWF to platelets. As a result, patients with Type 2B VWD have premature clearance of platelets in the circulation that leads to thrombocytopenia (Franchini 2006). It thus seems that in type 2B VWD, the loss in the large multimers is due to the clearance of VWF from the plasma. The large multimers are the most haemostatically active and bind almost spontaneously to platelet GPIb. Laboratory investigations that distinguish between type 2B and type 2A show that type 2B differs from type 2A only by high molecular weight multimers being absent and that the RIPA is increased (Michiels *et al.* 2005). This illustrates the importance of a proper diagnostic tool, since both this subtypes have similar results and only specialised tests can distinguish between types 2A and 2B.

2.9.5. Type 2M

The 2M is characterised by decreased affinity of VWF binding to platelets resulting from mutations in the region of the GPIb binding site. This causes a lessened interaction between the VWF multimers and platelets, even with the presence of the large multimers (Wilde 2007). Although there are several individual cases of rare mutations the majority of the mutations are characterised into three groups. Group 1 are variants that are characterised by the presence of large multimers and a reduction in the ristocetin cofactor activity. Identified mutations causing this variant include small deletions and missense mutations concentrated in the VWF A1 domain. This domain includes a platelet GP Ib binding site and these mutations irreversible amend the contact between VWF and platelets. In second group the multimer patterns show a slight reduction in the high

molecular weight multimers with a reduction in platelet dependent functions. The multimer patterns may be indicative of a type 2A VWD but the functional effects indicate that these mutations are suitably considered type 2M VWD. In group 3 mutations the presence of ultra large multimers in plasma and the decreased VWF activity and antigen indicate type 2M VWD (Vicenza). The multimer patterns of platelet VWF Vicenza are similar to plasma multimer patterns of patients that have been infused with desmopressin. The exact molecular cause of type 2M Vicenza has not yet been described. Other rare forms of type 2M VWD include variants that are characterised with uncleaved propeptide from VWF and variants with slightly modified VWF multimer structures mutations for this have not yet been clearly identified (Mohlke *et al.* 1999). Type 2M laboratory investigations yield a normal multimer patterns and normal FVIII and VWF:Ag levels with a decreased RIPA and VWF:RCo levels and mostly normal VWF:CB activity (Michiels *et al.* 2005). There are exceptions of rare type 2M VWD that have previously described in literature (Ribba *et al.* 2001). These are characterised by decreased binding to the sub-endothelium matrix including collagen caused by mutations in the A3 domain. There is slight or no effect on the binding of VWF to GPIb but the CB/Ag ratio is low. These cases can be discriminated from the type 2B and type 2A because of the presence of the large multimers, while the RIPA is not increased (Favaloro 2007).

2.9.6. Type 2N

When the ratio of VWF:Ag to FVIII is reduced, a type 2N VWD is suggested. Further investigations using the factor VIII binding assay (VWF:FVIII B) can confirm a diagnosis of type 2N resulting from mutations in the FVIII binding region of VWF. The recessive inheritance of the FVIII binding defect may be either homozygous, compound heterozygous or a null allele. Consequently type 2N may be diagnosed as haemophilia A or it may also have a low VWF:Ag levels (Budde and Schneppenheim 2001). The majority of mutations causing type 2N are located on the D domain with fewer located on the D3 domain of the VWF gene. These

mutations are responsible for the severity and functional defects of type 2N VWD. Mild FVIII deficiency is caused by a specific mutation (R854Q) resulting in very low VWF:FVIII activity. Mutation E787K causes severe pseudo-haemophilia with very low FVIII levels as much as 1IU/dl (Schneppenheim and Budde 2005).

2.9.7. Platelet type VWD

Platelet type VWD (PT-VWD) is a rare autosomal dominant bleeding disorder, characterised by increased affinity of platelet GPIb for normal VWF. The characteristic of PT-VWD are quite similar to that of type 2B VWD. Therefore most patients with PT-VWD can be misdiagnosed as type 2B (Favaloro 2006a). The GPIb gene is located on chromosome 17 and mutations on this gene cause PT-VWD (Franchini *et al.* 2008). The two types can be distinguished by RIPA mixing studies. When plasma of a type 2B VWD patient is added to normal platelet rich plasma, it induces aggregation. But when plasma of a PT-VWD patient is added there is no effect (Miller *et al.* 1983). This confirms that the defect in the patients with type 2B VWD is in the plasma, while the defect in PT-VWD is in platelets. It is important to clearly distinguish between the two types because the treatment and management is different.

2.10. Treatment of VWD

Desmopressin (1-deamino-8-D-arginine vasopressin, DDAVP) is intended to stimulate VWF secretion by endothelial cells. The binding of DDAVP onto V2 receptors on membranes of endothelial cells stimulates the secretion of VWF by activating the cyclic adenosine monophosphate (c-AMP)-mediated signalling. This results in a three to five fold increase in the baseline levels of FVII and VWF. The recommended dose by intravenous infusion or subcutaneous injection in adults is a standard dose of 300µg. In children a 150µg intranasal spray is recommended (Federici 2008).

When choosing a treatment method for patients with VWD, it is vital to take the following into account (Pasi *et al.* 2004):

- the character of the bleeding incidence

- the subtype of VWD and the FVIII and VWF levels
- the bleeding history of the patient and the reaction to treatment
- the levels of VWF and FVIII after treatment with DDAVP
- presence of inhibitors
- side effects of treatment

Type 1 VWD is usually treated with DDAVP where a single dose can be administered to treat bleeds as well as minor procedures. Water retention is a major side effect of DDAVP so patients' fluid intake needs to be monitored. For poor responders to DDAVP or those that cannot tolerate DDAVP, it is advisable to use factor VIII concentrate that contains VWF. Cryoprecipitate can be used as one of the treatment methods to increase VWF levels. The use of desmopressin in patients with type 2A, type 2M and type 3 VWD is generally unsuccessful (Wilde 2007). The results obtained in patients with type 2B treated with DDAVP is controversial. It is contradictory to use DDAVP as the released VWF is defective and can stimulate thrombocytopenia (Adcock *et al.* 2006).

VWF/FVIII concentrates are suggested in patients with, type 2A, type 2M, type 3 VWD and especially in type 2B because DDAVP can cause transient thrombocytopenia. VWF/FVIII concentrates may be used in patients with type 1 VWD who do not respond well to DDAVP or those who have contraindications to its use (Federici and Mannucci 2007).

3. Molecular diagnostics

Molecular diagnosis cannot be considered on its own. It is used to validate and confirm the results obtained from laboratory VWD diagnostic testing. It is of special importance in type 3 VWD where genetic counselling and pedigree studies are valuable. Molecular diagnosis is also important in distinguishing between haemophilia A and type 2N VWD and also between VWD type 2B and platelet type pseudo-VWD (Keeney *et al.* 2008). Mutation detection in type 3 has been clearly described. In many patients with type 1 VWD, no mutations

could be found in the VWF gene. This suggests that other factors (like ABO bloodgroup) can influence the VWD phenotype (McDonald 2007).

Quantitative VWD (type 1 and 3 VWD) are usually caused by defects in the promoter region resulting from frameshift or nonsense mutations and large deletions. Qualitative VWD on the other hand is caused mostly by missense mutations.

The analysis of molecular testing results is complicated by the degree of homology of the VWF gene and the presence of a pseudo gene. However, the use of primer specific PCR improved the result analysis. Another complication in result analysis is the presence of multiple exon polymorphisms in the VWF gene. This can be resolved by *in vitro* expression of the mutations to elucidate their consequence trigger (Pruthi 2006).

Since this dissertation deals with mutations in type 2 VWD, I will describe these mutations in more detail in the next section.

3.1. Mutations in type 2 VWD

Mutations that cause human type-2 VWD occur mostly in exon 28 of the VWF gene. This exon primarily encodes the mature VWF's A1 and A2 domains. These two domains contain the VWF binding sites for platelet glycoprotein Ib complex, sulfatides, collagen, and heparin. Mutations in these sites, almost always result in type 2A, 2B or 2M VWD. The first VWD mutation to be reported was also found in exon 28 (Federici and Mannucci 2007). There are 81 coding region single nucleotide polymorphisms (cSNPs) found in the VWF gene coding region, with 32 resulting in amino acid substitution (James and Lillicrap 2006). VWF is thus highly polymorphic that makes it difficult to detect mutations due to the variation in the VWF sequence. All mutations that cause type 2B VWD are also found in exon 28. A gain of function has been proved in more than 20 different amino acid substitutions in the A1 domain as shown in Figure 8. The majority of mutations responsible for type 2M VWD are also located in the A1 domain but are localised separate from the ones that cause type 2B therefore explaining the phenotypic loss of function (James and Lillicrap 2006).

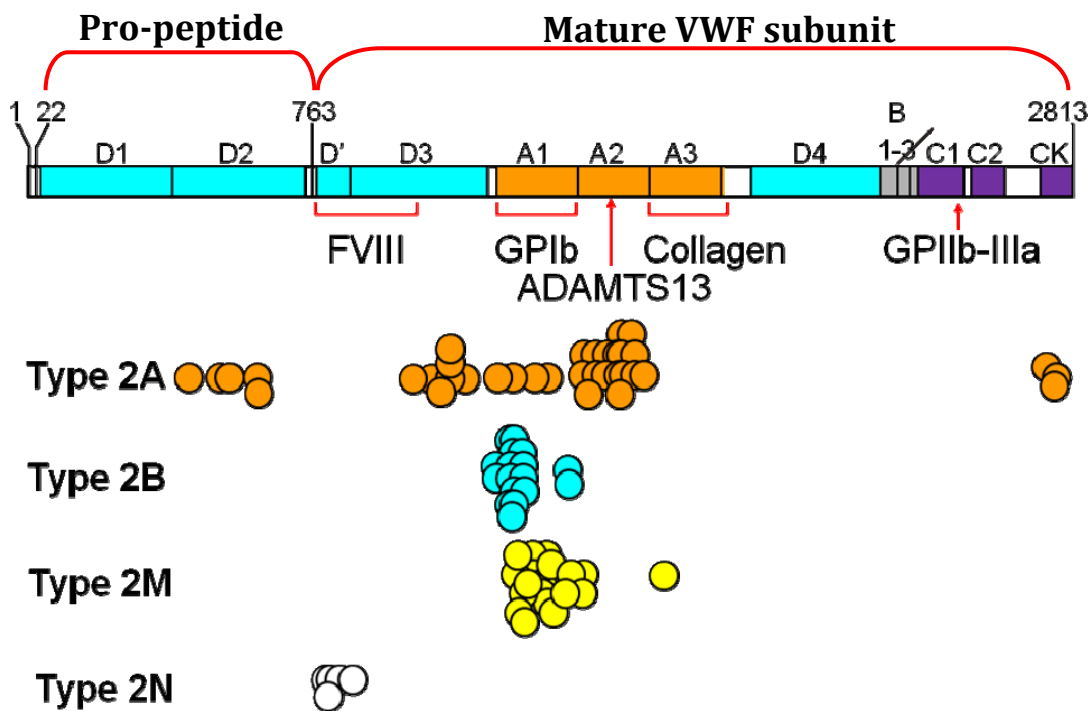


Figure 8: Distribution of mutation on the VWF gene causing specific subtypes of type 2 VWD.

Table 1 summarises the number of reported mutations in exon 28 of the VWF gene according to the ISTH VWF database. Genetic analysis of exon 28 on chromosome 12 is conducted by using two or more sequencing reactions with specific primers to cover the whole exon that spans 1.37kb (James and Lillicrap 2006). Mutations in exon 28 affect mostly the A1 and A2 domains, but also the D3 and A3 domains. It is thus important to study mutations found in these domains encoded by exon 28 as they result in different functional and qualitative abnormalities in VWF (Casana *et al.* 2001).

Table 1: Number of mutations in exon 28 of VWF per subtype of VWD report to date

Subtype of VWD	Number of mutations in exon 28
Type 1	33
Type 3	19
Type 2	
Type 2A	77
Type 2B	56
Type 2M	18
Unclassified	
Unclassified	14
Total	220

The majority of reported mutations in type 2A are found in the A1 domain of VWF. This is the domain that contains the binding site for GPIb of platelets and also collagen. The high molecular weight multimers are lacking in type 2A VWD, which results from mutations in the VWF gene. The distribution of mutations found in type 2A VWD patients is shown in Table 2.

Table 2: Distribution of mutations in type 2A VWD

Exon	Domain	Number of reports
12-16	D2	8
26	D3	1
28	A2	70
51	CK	1
52	CK	6

There are 86 mutations reported in type 2A VWD. These mutations occur between exons 12 and 52. The majority are found in exon 28 (81%). The most commonly reported mutation in type 2A VWD is the R1597W mutation. This mutation is also found in exon 28. Table 3 provides a summary of mutations reported up to date in exon 28 of type 2A VWD patients. The R1597W mutation causes increased proteolysis of VWF multimers by

circulating ADAMTS13 (Franchini 2006). Patients with this mutation exhibit a high sensitivity of VWF multimers to proteolysis after secretion in plasma. When treated with DDAVP, patients with this mutation exhibit a temporary increase in large multimers associated with temporary normalization of bleeding time and correction of VWF:RCo values (Federici *et al.* 2004).

Table 3: Most frequently reported mutations in type 2A VWD

Amino acid substitution	Nucleotide alteration	Number of reports	% of the 86 Type 2A
G1505E	4514G>A	2	2
S1506L	4517C>T	8	9
R1597W	4789C>T	9	11
R1597Q	4790G>A	4	5
G1609R	4825G>A	3	3
I1628T	4883T>C	2	2

All mutations reported in patients with type 2B are located within exon 28. The majority of these affect the A1 domain. Type 2B is diagnosed when VWF has an increased affinity for binding to platelets GPIb causing spontaneous adhering of VWF to platelets. Some missense mutations in the D3, A1 and A2 domains of the VWF gene result in a gain in function of the VWF protein. As a result patients with Type 2B VWD have premature clearance of platelets in the circulation causing thrombocytopenia (Franchini 2006). There are 54 reported type 2B mutations. The most frequently reported mutation in type 2B VWD found in exon 28, is the R1306W mutation seen in Table 4. This mutation affects the A1 domain of VWF and shows diverse clinical as well as laboratory phenotypic characteristic. These characteristics include (i) inconsistency of VWF levels, (ii) inconsistency of multimer patterns, (iii) changes in the quantity of abnormal VWF that binds to GPIb α , (iv) inconsistent incidence of thrombocytopenia and (v) bleeding tendencies vary even in direct family members (Szanto *et al.* 2007). Type 2B VWD patients with this specific mutation in can easily be misdiagnosed.

The importance of combining molecular diagnosis with the routine diagnostic tests is thus critical in this case.

Table 4: Most frequently reported mutations in type 2B VWD

Amino acid substitution	Nucleotide alteration	Number of reports	% of the 54 Type 2B
R1306W	3916C>T	10	19
R1308C	3922C>T	6	11
V1316M	3946G>A	9	17
P1337L	4010C>T	2	4
R1314Q	4022G>A	7	13

The most complex type 2 VWD subtype is type 2M VWD. Up to date 23 mutations have been reported of which only two have been reported more than once. The majority of these mutations are also located in exon 28. These mutations affect the D', D3 and A1 domains. Decreased affinity of VWF to platelet binding is the result of secondary mutations in the GPIb binding region and causes type 2M VWD. This leads to a weak interaction between VWF multimers and platelets (Wilde 2007), despite the presence of large multimers. Type 2M mutations are located among exons 17, 27, 28 and 52. Table 5 shows only the two type 2M mutations that are reported more than once. The V1279I mutation is linked to the allele that does not co-segregate with the phenotype and this mutation is also present in the pseudogene. The reason is that the presence of this mutation in the pseudogene is due to inter-chromosomal conversion between the chromosomes 12 and 22 (Casana *et al.* 2001). Another mechanism that could cause this mutation is that a part of the pseudo gene of no more than 135bp is copied into the VWF gene. Not only does this hypothesis insinuate that a relationship exists between the VWF gene and the pseudogene it also indicates that the pseudogene may play a part in the development of mutations in VWF gene.

Table 5: Most frequently reported mutations in type 2M VWD

Amino acid substitution	Nucleotide alteration	Number of reports	%of the 23 Type 2M
V1279I	3835G>A	2	9
I1425F	4273A>T	2	9

3.2. *Nomenclature used in molecular diagnosis*

The Von Willebrand factor subcommittee of the Scientific and Standardization Committee of the ISTH recommends that numbering of VWF cDNA start from the initiator codon ATG as +1. This means that the numbering starts 250 nucleotides downstream from the transcription initiator site. Both the original and mutated nucleotides are specified subsequent to the nucleotide position number to eliminate confusion with the amino acid sequence. Mutation substitutions are denoted with a “> or →” between the nucleotides for example 4803A>T or 4803A→T. Polymorphism substitutions are denoted with a “/” symbol between the nucleotides for example 4803A/T.

Regarding the amino acid sequences, it is recommended that numbering should start at the initiator methionine as the +1 position. Single letter amino acid nomenclature is preferred over the previously used three letter amino acid codes. In mutations the wild type amino acid is given first followed by the position then the mutant amino acid. In polymorphisms the wild type and the mutant amino acids are given before the position (Goodeve and Peake 2001).

4. VWD in South Africa

Our Specialised Haemostasis laboratory is the only laboratory in South Africa that does all the screening and specialised tests necessary for the proper diagnosis of VWD. The majority of the laboratories rely only on the screening tests such as VWF:Ag and VWF:RCo to diagnose VWD. Mutational analysis of the VWF gene will complement our routine diagnostic tests. It will be used as a

confirmatory test especially in patients with inconspicuous phenotypes. Mutational analyses on the VWF gene have never been done on populations from Africa. Mutational studies on VWF will not only improve our knowledge on our patients' phenotype but also put us in par with other laboratories around the globe in the field of VWD diagnosis. This study is thus the first to undertake mutation analysis is done on patients with VWD in South Africa.

5. Aim of project

The aim of this study was to search for mutations in exon 28 of VWF in five patients with type 2 von Willebrand disease, and thus also to set up the technique for molecular diagnosis of type 2 VWD in South Africa.

Chapter 3: Methodology

1. METHODS

1.1. *Ethical consideration*

Ethical approval was received from the Ethics Committee of the University of the Free State (ETOVS: 253/05B). Patients from our Haemophilia Treatment Centre clinic were invited to participate in the study and the patients were given an informed consent form to sign.

1.2. *Population*

Blood samples from five patients previously diagnosed with type 2 VWD were collected (two patients with type 2M, two with type 2B and one with type 2A VWD).

1.3. *Sample collection*

For the genetic analysis we obtained 5 ml venous blood in EDTA-treated sample tubes and four 5ml tubes containing 3.2% tri-citrate were drawn for the laboratory diagnosis. One 5ml citrate tube of each patient was centrifuged at 800g for 10min to obtain platelet poor plasma (PPP) and the PPP was stored at - 80°C until assayed.

1.4. *Functional and antigenic assays*

The following laboratory tests were done in our specialised haemostasis laboratory:

- Von Willebrand Factor Antigen (VWF:Ag)
- The collagen binding activity of Von Willebrand Factor (VWF:CB)
- Function of VWF activity using ristocetin cofactor (VWF:RCo)
- Ristocetin induced platelet aggregation (RIPA)
- Multimeric analysis

The VWF:Ag levels were measured with an ELISA assay as described by Meiring *et al.* (2005). In short, a multi well plate was coated with a goat anti

VWF antibody. The patient's platelet poor plasma was added and thereafter a peroxidase conjugated polyclonal antibody to VWF was added. A coloured reaction in the presence of a peroxidase substrate indicates VWF presence in plasma. A standard curve was constructed from using known VWF concentrations of the international standards of FVIII and VWF (WHO). The VWF activity was read off from the standard curve and results were reported as percentages.

The collagen binding assay was also measured with an ELISA assay as described by Meiring *et al.* (2007). In short, a multi well plate was coated with collagen type III (Type X collagen, Sigma, USA). The patient's plasma was added and thereafter a peroxidase conjugated polyclonal antibody to VWF. A colour change with a peroxidase substrate indicated VWF binding to collagen. The international standard for VWF and FVIII of the WHO were used to construct a standard curve with known collagen binding concentrations. The collagen binding activity of each patient was read off from the standard curve. The results were reported as percentages.

The VWF:RCo was done using a ristocetin cofactor assay kit (Helena Laboratories, France). Plasma is added to formalin fixed platelets that are induced to agglutinate using an antibiotic ristocetin. The test was done on the Chronolog 560 CA aggregometer (Chronolog, USA). The ristocetin cofactor activity of each patient was read off from the standard curve constructed with the calibrator from the kit. The results were also reported as percentages.

The RIPA was done according to the method described by Meiring *et al.* (2005a). Two of the 5ml citrated tubes that were drawn from the patient centrifuged at 200g for 5min to obtain platelet rich plasma (PRP) and the PRP collected. The remaining blood was centrifuged at 800g to obtain platelet poor plasma (PPP). Ristocetin concentrates ranging from 0.1mg/ml to 2.5mg/ml were added to the PRP, while the PPP was used as a blank. A Chronolog 560 CA aggregometer (Chronolog, USA) was used to measure the percentage platelet agglutination in the presence of different ristocetin

concentrations. The results were reported as the concentration of ristocetin (mg/ml) where 30% agglutination of platelets occurs. In type 2A and type 2M VWD one expects a decreased response to ristocetin, thus more than 1.2mg/ml of ristocetin is required to induce 30% agglutination. While in type 2B an increased response to ristocetin is expected, thus less than 0.8mg/ml of ristocetin is required to generate 30% agglutination.

The multimer analysis was done using a Western blot technique where horizontal agarose (0.65%) electrophoresis is followed by the transfer of the VWF onto a polyvinylidene fluoride (PVDF) membrane. The multimer pattern of VWF was visualised by immunolocalisation and luminographic detection and no radioactivity was used. The multimer density profiles were determined using the Synergene Gel documentation system (Vacutec, USA). This method is also described by Meiring *et al.* (2005b).

1.5. *Molecular analysis*

- **DNA extraction**

Blood from the EDTA tube was used as a source for DNA. DNA extraction was done according to the manufacturer's instructions using the Promega Wizard™ Genomic DNA Purification Kit (Promega Corporation Madison, USA). In short, 300 µl EDTA blood was added to 900 µl red cell lysis buffer. The supernatant was removed from the centrifuged samples and the samples were then vortexed. Added 300 µl of white cell lysis and 100 µl protein precipitate to the samples and vortexed. The samples were centrifuged and the supernatant was transferred to a new tube. The DNA was precipitated using 100 µl iso-propanol and centrifuged. The DNA was rehydrated with 100 µl rehydration buffer and the isolated DNA was stored at -70°C until use.

- **PCR amplification**

A fragment of 1514 bp containing exon 28 of the VWF gene was amplified by polymerase chain reaction (PCR) using primers seen in Table 6. Exon 28 is 1379bp long and thus the largest of the 52 exons of VWF. This makes sequencing of the entire exon difficult. To overcome this problem we decided to amplify the exon using four fragments ranging from 487bp to 770bp. The fragments not only make the sequencing data more manageable but also allows for a more precise amplification of exon 28. We used primers that are designed in such a way to anneal at sequence mismatches between the VWF wild type and the pseudogene. These primers also allows for repeat overlapping sequencing of some regions in the exon that can be reanalysed to verify the sequencing data.

The PCR reactions were optimised using the primers in Table 6. The red nucleotides in Table 6 represent areas where there are nucleotide mismatches between wild type and the pseudogene. We used gradient PCR to establish the optimum annealing temperatures for the primers. The maximum temperatures also help the primers to bind strongly on the correct place on the DNA strands. After amplification the fragments were visualised using a 3% agarose gel shown in Figure 9. The reactions also included a blank. The PCR products from fragment 1, 2, 3 and 4 yielded products of 487, 700, 770 and 649 base pairs respectively in size. The total reaction volume of each reaction was 50 µl, containing 200 ng genomic DNA, 10 pmol primer (Whitehead scientific), 45µl Platinum PCR Supermix containing 22 U/ml of platinum Taq DNA polymerase, 1.65mM MgCl₂, 55mM KCl and 220 µM dNTP product of Invitrogen, Life technologies (Carlsbad, CA).

Table 6: Primers used for PCR of exon 28 (Penas *et al.* 2005)

Primer	Primer sequence (5'-- 3')	size
VW-F1	AGA AGT GTC CAC AGG TTC TTC	487bp
VW-R1	AGA TTT GGA ACA GTG TGT ATT TCA AGA CCT	
VW-F2	AGA AGT GTC CAC AGG TTC TTC	700bp
VW-R2	GCA CGA AGG CCT TGT TCT CAG	
VW-F3	AAG CAG GCC CCT GAG AAC AA	770bp
VW-R3	ATA CCA GGT GCA GGG GAG AG	
VW-F4	TGG TTC TGG ATG TGG CGT TC	649bp
VW-R4	TCT TGG CAG ATG CAT GTA GC	

The PCR conditions for optimal results were as follows:

Phase 1: 1 cycle: 94°C for 5 minutes

Phase 2: 30 cycles: 94°C for 30 sec; 58°C for 30 sec; 72°C for 2 minutes

Phase 3: 1 cycle: 72°C for 10 minutes.

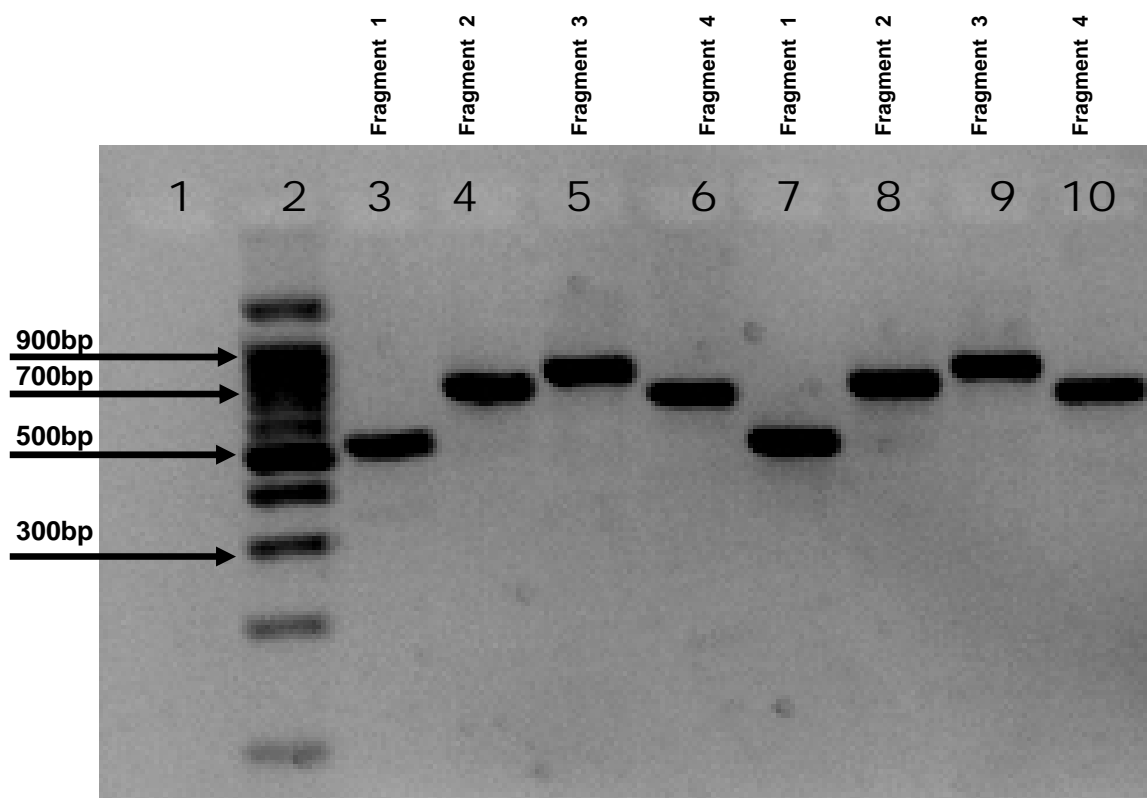


Figure 9: A 3% agarose electrophoresis gel of the different fragments of Exon 28

(Lane 1: blank negative control, Lane 2: 100bp molecular weight ladder, Lane 3 and 7: fragment 1 (487bp), Lane 4 and 8: fragment 2 (700bp), Lane 5 and 9: fragment 3 (770bp), Lane 6 and 10: fragment 4 (649bp)).

- **Gel electrophoresis**

A 3 % agarose gel was used to separate the PCR products. The PCR product was mixed with loading buffer (0.25% bromophenol blue in 40% sucrose solution) on para-film. The samples and a molecular weight marker were loaded onto the solidified gel in separate slots and electrophoresis at 100 volts for 1 to 2 hours in 1 x TBE buffer to separate the fragments. The DNA fragments were visualized using a UV transilluminator (see Figure 9).

- **DNA purification**

When the presence of a single bands of correct sizes were confirmed, the PCR product was purified using a High Pure PCR Product Purification Kit (Roche Applied Science, Germany). This was done to remove excess primers, dNTP's, and Taq. In short the PCR product was added to the 500 µl binding buffer and centrifuged using a collection tube and the flow through was discarded. Added 250 µl wash buffer to the filter tubes and centrifuged. This process was repeated twice. The flow-through solution was discarded. Added 100 µl elution buffer to the filter tube that is connected to a 1.5ml micro-centrifuge tube and centrifuged to elude the pure DNA solution. The purified PCR product was stored at -20°C until use.

A 3 % agarose gel was used to separate the purified PCR products. This was to insure that no loss of product during purification occurred.

- **Sequencing**

We used both the forward and reverse primers (Whitehead Scientific) from the PCR for sequencing. The purified PCR products were sequenced using the ABI Prism Big Dye Terminator Ready reaction kit v3.1 (Applied Biosystems, California, USA) protocol. The sequencing thermo cycle conditions for optimal results were as follows:

Phase 1: 1 cycle: 96°C for 1 minute

Phase 2: 25 cycles: 94°C for 10 sec; 58°C for 5 sec; 60°C for 4 minutes

The reaction volume was 20 µl, containing: 10µl purified PCR product, 3.2 pmol primer, 2.5 X Ready reaction mix and 5 X BigDye sequencing buffer

(Applied Biosystems, California, USA). This is in accordance with manufacturer's protocol. The matrix of the sequencer was changed to obtain optimal results.

The sequence reaction products were purified using Ethanol/Sodium acetate precipitation in micro-centrifuge tubes according to the protocol of the ABI Prism Big Dye Terminator Ready reaction kit v3.1 (Applied Biosystems, California, USA), with a slight modification. In short, we add 3µl of 3M sodium acetate (pH 5 kept at 5°C) to a 0.6ml Eppendorf tube. We added 62.5µl of 95% ethanol that was kept at -20°C and also 14.5µl sterile distilled water. We then added 20µl of the sequencing product to the mix and vortexed it for 30 seconds followed by centrifuged for 10 seconds. The mixture was kept at room temperature for 30 min and then centrifuged for a further 30 min at 13500g. The supernatant was aspirated then 250µl of 70% alcohol was added to the pellets and the samples were vortexed for 2 min and centrifuged for 10 min again. The supernatant was aspirated and the samples were left to dry at 90°C for 1 min.

- **Re-suspending the samples with polymer and denaturing**

Hi-Di Formamide (Applied Biosystems, California, USA) was added to the samples and vortexed for 1 minute, then centrifuged at maximum speed for 30 seconds. The samples were denatured at 95°C for 2min and chilled on ice for 5 min. The samples were then vortexed for 1 min and centrifuged at high speed for an additional 30 seconds. The re-suspended products were kept in the dark until loaded onto the sequencer.

- **Capillary electrophoresis**

After denaturing, the samples were run on an ABI Prism 310 Genetic Analyser (Applied Biosystems, California, USA) using POP-6 polymer. We used run module Seq POP6(1 mL) E with the following configurations: POP-6 polymer, 1-mL syringe, 61 cm 50 µm capillary. We used mobility file DT310POP6{BDv3}v2.mob. The pGEM -3Zf(+) double stranded DNA control template was sequenced in all the reactions as a quality control. The

control template and primers were provided with the sequencing reaction kit. The reference sequence of the control is available in the BigDye Terminator v3.1 sequencing kit protocol available from the manufacture. Figure 10 shows the sequence results of the control template.

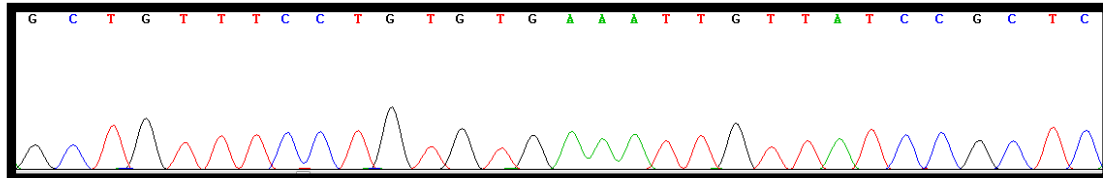


Figure 10: Control sequence

- **Data Analysis**

After capillary electrophoresis, the sequencing results were analyzed using the Sequencing Analysis Software version 2.1 (Applied Biosystems). The sequencing results were compared with the VWF reference sequence (NM_000552.3) in GenBank using LALIGN software available online at http://www.ch.embnet.org/software/LALIGN_form.html. The sequences were transcribed to the protein sequences using ExPASy software also available online at <http://au.expasy.org/tools/dna.html>. The protein sequences from the patients were then compared to the VWF protein reference sequence (NP_000543.2). If any amino acid change was observed, the change was compared to previously reported polymorphisms or mutations stipulated by the VWF database of the International Society of Thrombosis and Haemostasis (ISTH) (<http://www.vwf.group.shef.ac.uk/>).

No statistical analysis was required in this study. A report for the molecular diagnosis was constructed using the recommendations noted in literature review (Keeney *et al.* 2008).

Chapter 4: Results

1. Patient demographics

From the five VWD patients tested, two have been diagnosed with type 2M, two with type 2B and one with type 2A VWD. The results of each patient are listed below.

- **Patient 1 results**

Patient 1 is a Coloured male and is diagnosed with type 2B VWD with the laboratory results shown in Table 7. The results show FVIII levels and VWF:Ag levels within the normal range. This excludes the diagnosis of a type 1 and type 3 VWD. The decreased RCo/Ag and CB/Ag ratio are indicative of a type 2 VWD. The increased RIPA is suggestive of a type 2B VWD subtype. The absence of large multimer patterns validates the diagnosis to be type 2B VWD subtype. RIPA mixing studies using normal plasma can differentiate between PT-VWD and type 2B VWD. Diagnoses of PT-VWD can be confirmed by molecular studies of the GPIb gene. Diagnosis of type 2B can be confirmed by molecular analysis of VWF gene more especially exon 28. The densitogram also shows the comparison between the normal and the patient's plasma multimers. This also confirms a type 2B diagnosis.

Table 7: Laboratory analysis of patient 1 including multimer patterns and densitogram

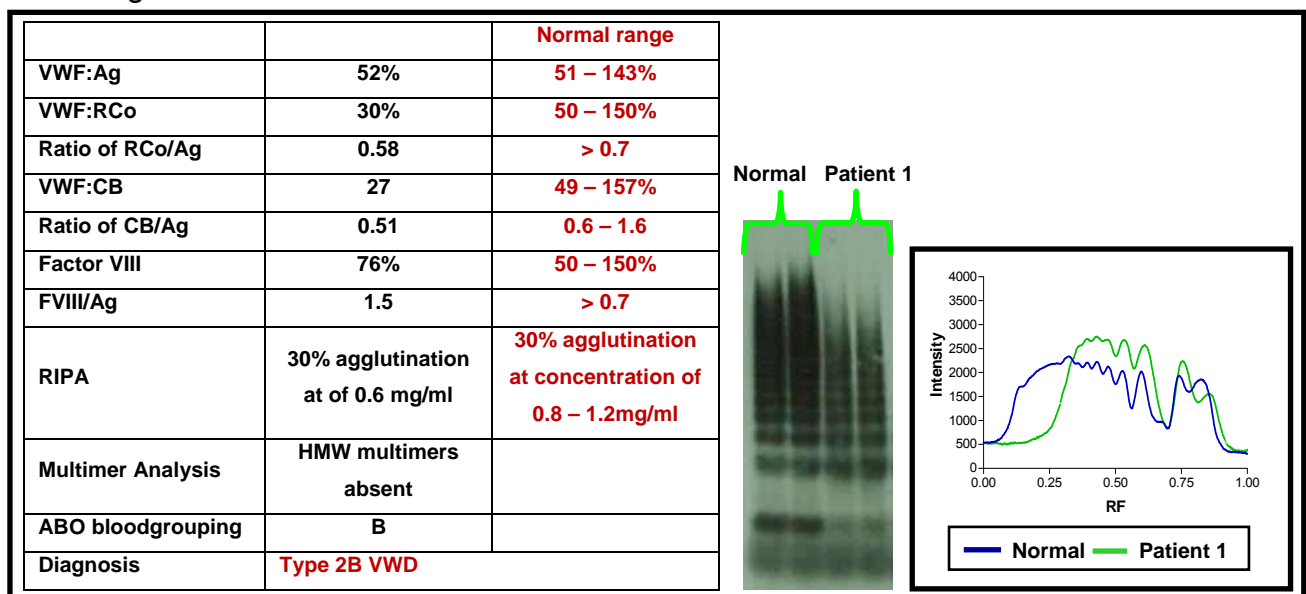


Table 8: Genetic analysis results of patient 1

Patient No:	1		
	Exon 28		
Exon studied	Exon 28		
Diagnosis	Type 2B		
VWF ref number	NM_00552.3		
VWF protein ref	NP_000543.2		
Polymorphisms	Nucleotide alteration	Amino acid change	Mode of inheritance
	4641C/T	T1547	Heterozygous
Mutations	4141G/A	T/A1381	Heterozygous
	3922C>T	R1308T	Heterozygous

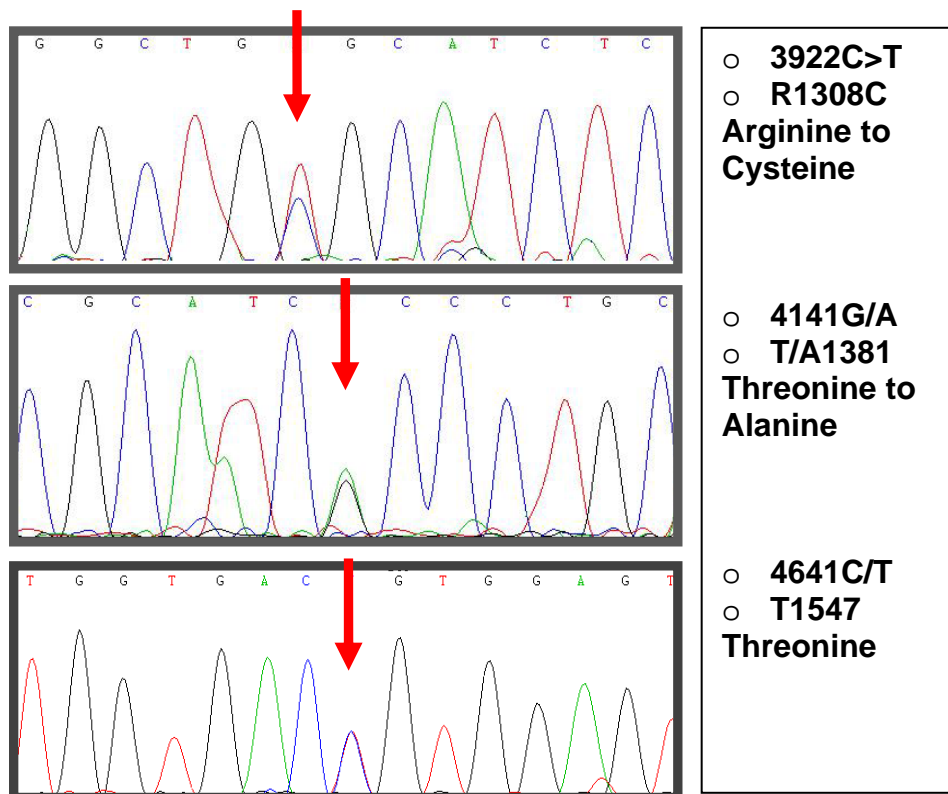


Figure 11: Sequencing results of patient 1

Two polymorphisms and one mutation were detected. One heterozygous polymorphism, the alteration of a thymine (T) to a cytosine (C) at codon number 4641 in one chromosome. This results in no amino acid substitution at position 1547 of the protein where the threonine (T) remains unchanged. The other heterozygous polymorphism is an alteration of an adenine with a guanine at codon 4141 in one chromosome. This results in an amino acid change from a

threonine (T) to alanine (A) at position 1381. These polymorphisms have previously been described by in the literature and are in the VWF database at <http://www.ragtimedesign.com/vwf/polymorphism/>. One heterozygous mutation was found in this patient. The sequencing results are also shown in Figure 11. This mutation results from a cytosine (C) to a thymine (T) nucleotide alteration at codon 3922 in one of the chromosomes. This produces an amino acid change from an arginine (R) to a cysteine (C) at amino acid number 1308. This mutation also has been reported previously in literature and is also in the VWF database <http://www.ragtimedesign.com/vwf/mutation/>.

• **Patient 2 results**

Patient 2 is an African female and is a diagnosed with type 2A VWD. The laboratory results are shown in Table 9. The VWF:Ag presence excludes type 3 VWD diagnosis. The low VWF:Ag levels suggests either a type 1 or type 2 VWD. However the possibility of type 2 is enhanced by the RCo/Ag and CB/Ag ratios that are less than 0.7. The decreased RIPA suggests either type 2A or 2M. The absence of large and intermediate multimers indicates the diagnosis of type 2A. The densitogram results (see below) confirm a type 2A diagnosis.

Table 9: Laboratory analysis of patient 2 including multimer patterns and densitogram

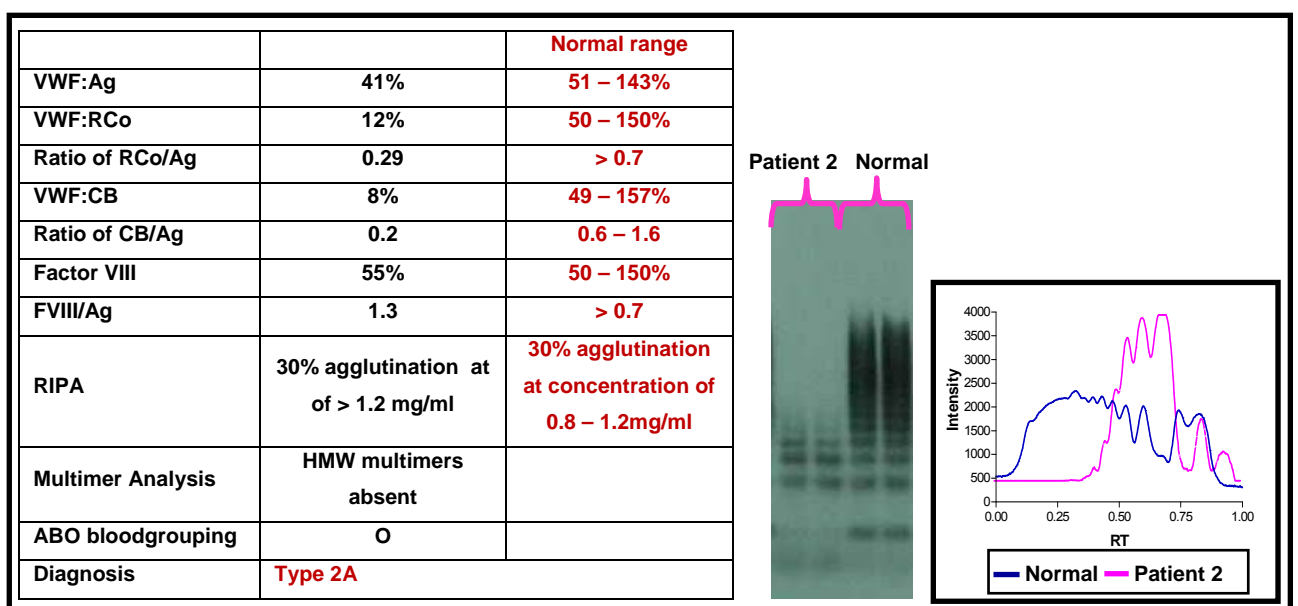


Table 10: Genetic analysis results of patient 2

Patient No:	2		
Exon studied	Exon 28		
Diagnosis	Type 2A		
VWF ref number	NM_00552.3		
VWF protein ref	NP_000543.2		
	Nucleotide alteration	Amino acid change	Mode of inheritance
Polymorphisms	4641C/T	T1547	Heterozygous
Mutations	4508T>C	L1503P	Heterozygous
	4517C>T	S1506L	Heterozygous

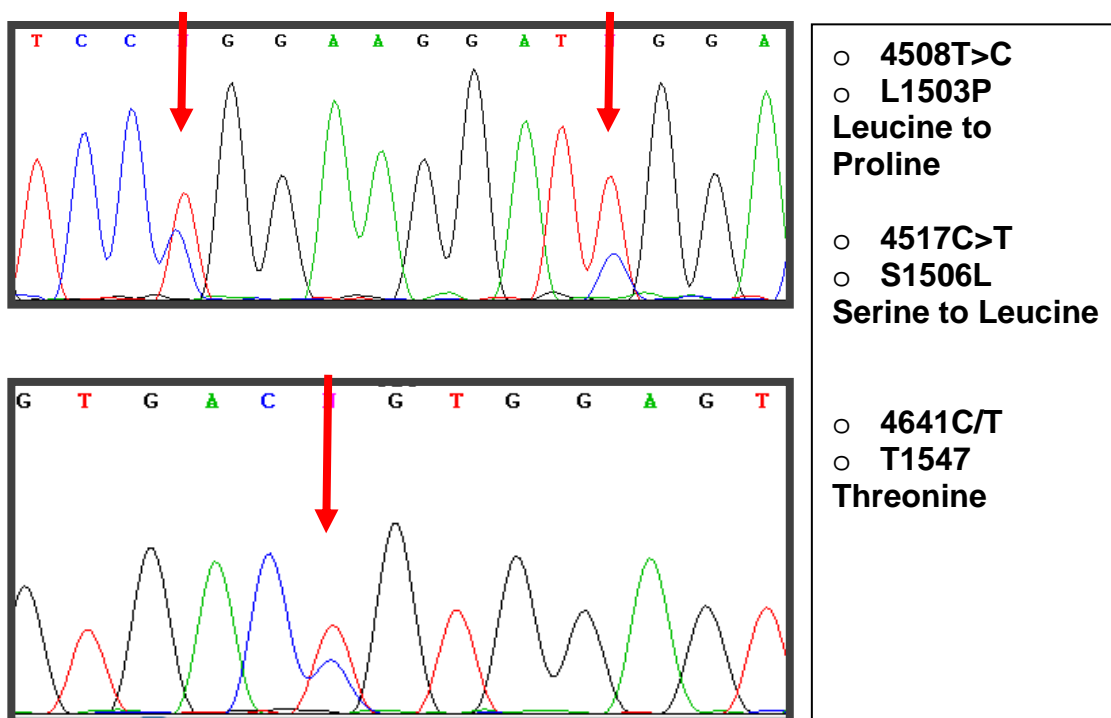


Figure 12: Sequencing results of patient 2

One polymorphism and two mutations were detected in this patient. One heterozygous polymorphism, the alteration of a thymine (T) to a cytosine (C) at codon number 4641 in one chromosome was detected. This results in no amino acid substitution at position 1547 of the protein where the threonine (T) remains unchanged. This polymorphism has previously been described by in literature and is in the VWF database. The first mutation is heterozygous and results from a thymine (T) to cytosine (C) nucleotide alteration at codon 4508 in one of the chromosomes. This produces an amino acid change from a leucine (L) to a

proline (P) at amino acid number 1503. The other mutation is also heterozygous and results from a cytosine (C) to a thymine (T) at codon 4517 in one chromosome. This produces as amino acid change from a serine (S) to a leucine (L) at amino acid number 1506. Both these mutation have been reported previously in literature and in the VWF database <http://www.ragtimedesign.com/vwf/mutation/>. Table 10 is a summary of the mutational analysis and shows a part of the sequence of patient 2.

• **Patient 3 results**

Patient 3 is a Caucasian male and is diagnosed with type 2M VWD. The laboratory results are shown in Table 11. The low VWF:Ag levels suggest either a type 1 or type 2 VWD. The RCo/Ag ratio is less than 0.7 suggesting a type 2 VWD; however the CB/Ag ratio is 1.3. The decreased RIPA suggests either type 2A or 2M VWD. The presence of large multimer patterns as seen on the x-ray film and the presence of normal VWF:CB levels indicate the diagnosis of type 2M VWD. Comparing the normal and patient plasma multimers on a densitogram (see below) confirms the type 2M diagnosis.

Table 11: Laboratory analysis of patient 3 including multimer patterns and densitogram

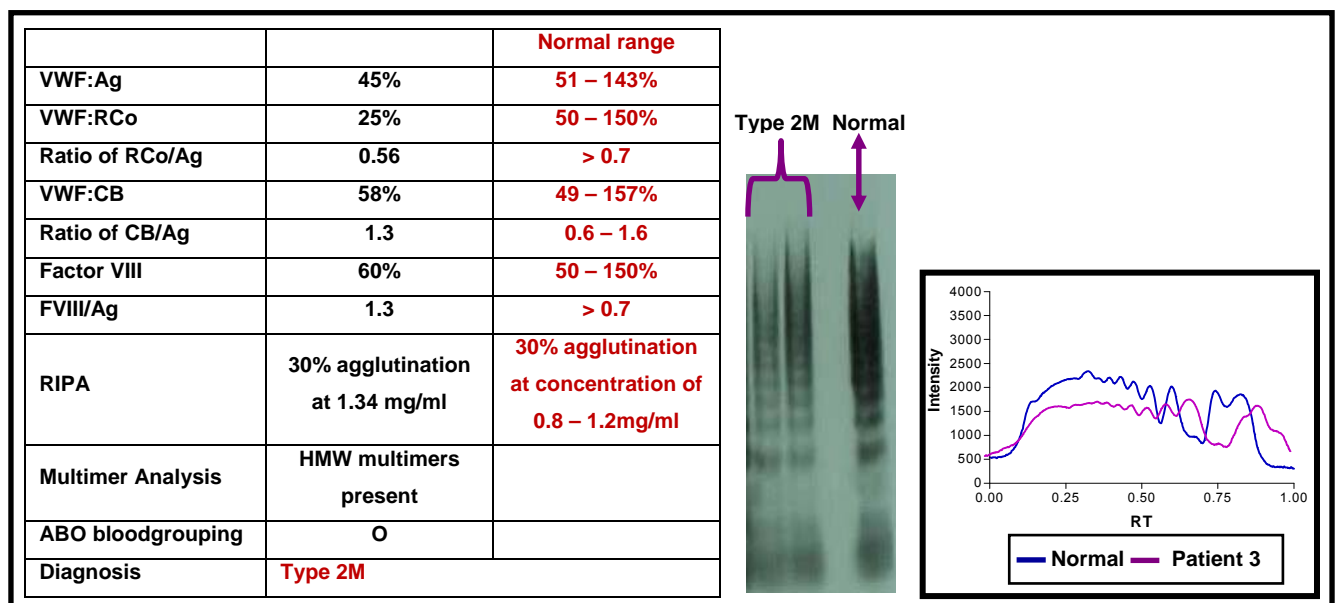


Table 12: Genetic analysis of patient 3

Patient No:	3		
Exon studied	Exon 28		
Diagnosis	Type 2M		
VWF ref number	NM_00552.3		
VWF protein ref	NP_000543.2		
	Nucleotide alteration	Amino acid change	Mode of inheritance
Polymorphisms	4141G>A	A/A1381	Homozygous
	4414C>G	D/D1472	Homozygous
	4641C>T	T1547	Homozygous
Mutations			

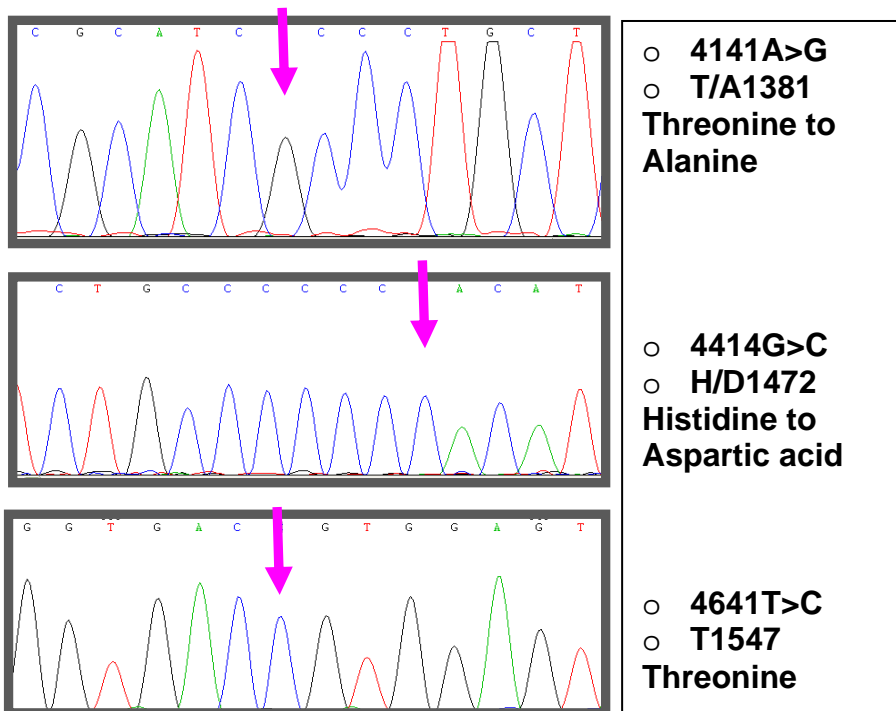


Figure 13: Sequencing results of patient 3

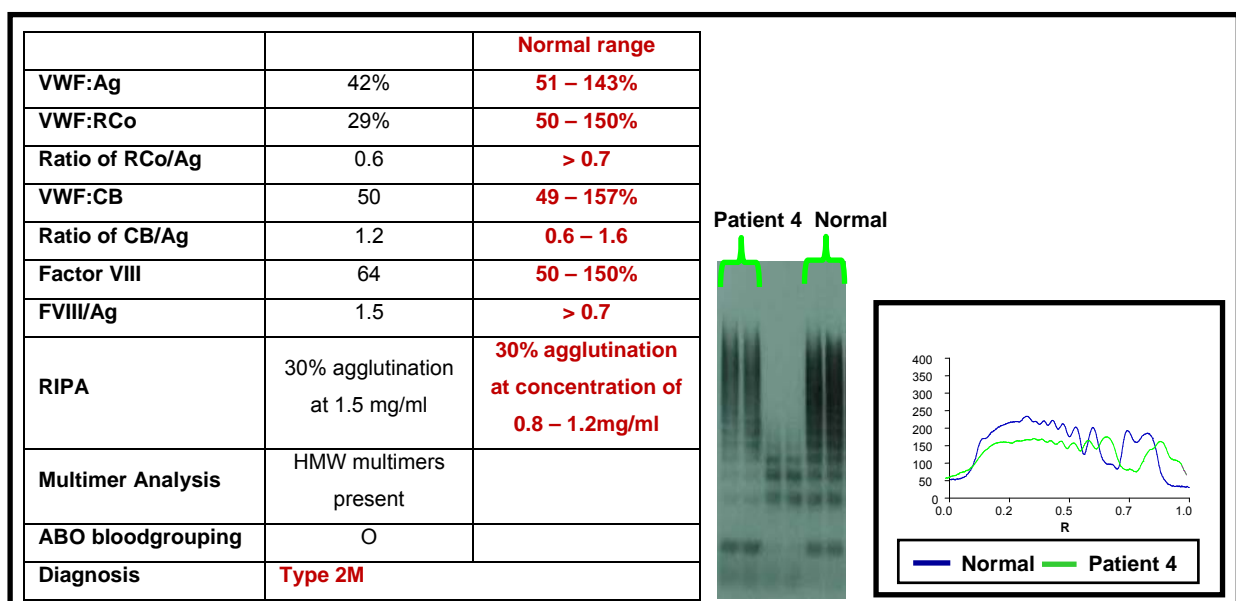
Three polymorphisms were detected. Table 12 is a report of the nucleotide alteration observed in patient 3. Figure 13 shows the sequencing data with the arrows illustrating the altered nucleotides. One homozygous polymorphism consists of a substitution of an adenine (A) with a guanine (G) in codon 4141 in both chromosomes resulting in amino acid alteration of the threonine (T) to alanine (A) at amino acid number 1381 of the protein. The second homozygous

polymorphism is an alteration of a guanine (G) to a cytosine (C) at codon 4414 in both chromosomes. This results in an amino acid change from a histidine (H) to aspartic acid (D) at amino acid number 1472 of the protein. The third homozygous polymorphism is a substitution of thymine (T) to a cytosine (C) at codon number 4641 in both chromosomes. These results in no amino acid substitution at amino acid number 1547 of the protein therefore threonine (T) remains unchanged. These polymorphisms have been previously described by in literature and are in the VWF database.

• **Patient 4 results**

Patient 4 is a Caucasian female who was a diagnosed with type 2M VWD The low VWF:Ag levels suggest either a type 1 or type 2 VWD. The laboratory results are shown in Table 13. The RCo/Ag ratio is less than 0.7 suggesting a type 2 VWD, and the CB/Ag ratio is normal. The decreased RIPA suggests either type 2A or 2M VWD. The presence of large multimer patterns as seen on the x-ray film and the presence of normal VWF:CB levels indicates the diagnosis of type 2M VWD. Comparing the normal and patient plasma multimers on a densitogram (see below) shows the presence of the HMW multimer and therefore confirms type 2M diagnosis.

Table 13: Laboratory analysis patient 4 including multimer patterns and densitogram



Three polymorphisms two homozygous and one heterozygous polymorphism were detected in this patient. One homozygous polymorphism is a substitution of an adenine (A) with a guanine (G) in codon 4141 in both chromosomes resulting in amino acid alteration of the threonine (T) to alanine (A) at amino acid number 1381 of the protein. The second homozygous polymorphism is a substitution of thymine (T) to a cytosine (C) at codon number 4641 in both chromosomes. This polymorphism was also found in patient 1 and 3. This results in no amino acid substitution at amino acid number 1547 of the protein therefore threonine (T) remains unchanged. The third heterozygous polymorphism is an alteration of a guanine (G) to a cytosine (C) at codon 4414 in one chromosome. This results in an amino acid change from a histidine (H) to aspartic acid (D) at amino acid number 1472 of the protein. These polymorphisms have been previously described by in literature and are also in the VWF database. Table 14 a summary report of the sequencing data.

Figure 14 shows the sequencing data from patient 4 with arrows showing the nucleotide change.

Table 14: Genetic analysis of patient 4

Patient No:	4						
	Exon studied	Exon 28					
		Diagnosis	Type 2M				
			VWF ref number	NM_00552.3			
				VWF protein ref	NP_000543.2		
					Polymorphisms	Nucleotide alteration	Amino acid change
4141G>A						A/A1381	Homozygous
4641C/T	T1547					Homozygous	
4414C>G	D/D1472	Heterozygous					
Mutations							

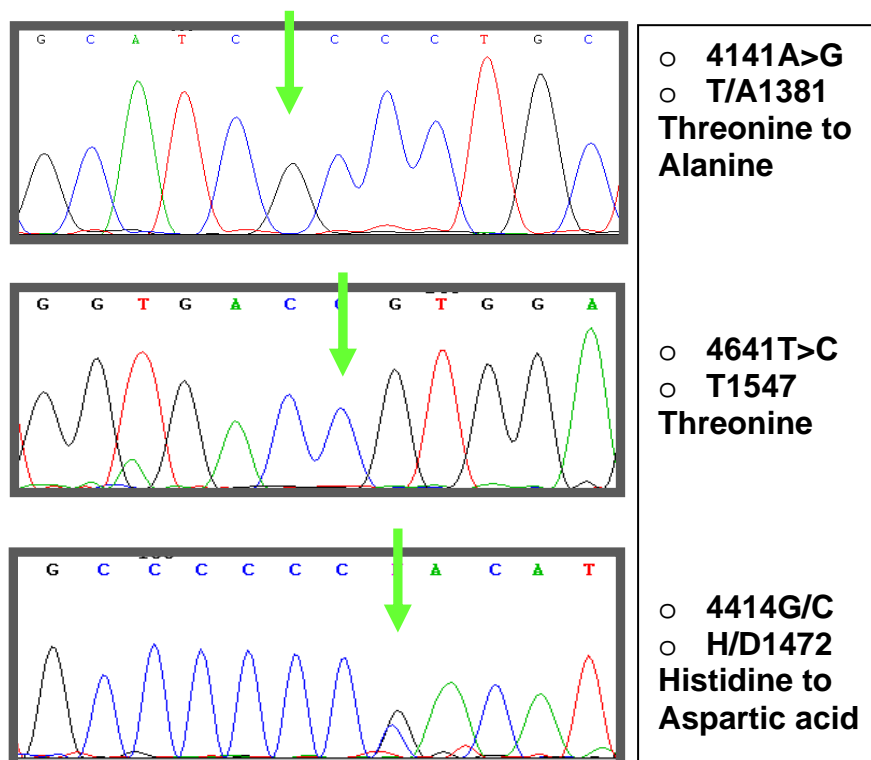


Figure 14: Sequencing analysis of patient 4

● **Patient 5 results**

Patient 5 is an African male and diagnosed with type 2B VWD. Table 15 shows the phenotype results from laboratory tests done in the diagnosis of patient 5. Attached are the multimer patterns and densitogram. The RCo/Ag and CB/Ag ratios were less than 0.7 that is indicative of type 2 VWD. The increased RIPA is suggestive of a type 2B VWD subtype. The absence of large multimer patterns validates the diagnosis to be type 2B VWD subtype. RIPA mixing studies using normal plasma can differentiate between PT-VWD and type 2B VWD. Diagnoses of PT-VWD can be confirmed by molecular studies of the GPIb gene. Diagnosis of type 2B can be confirmed by molecular analysis of VWF gene more especially exon 28. The absence of large multimer patterns on the X-ray film validates the diagnosis to be type 2B VWD. A comparison between the normal and patient multimers on a densitogram (see Table 15) shows the absence of the HMW multimers and thus confirms the type 2B diagnosis.

Table 15: Laboratory analysis of patient 5 including multimer patterns and densitogram

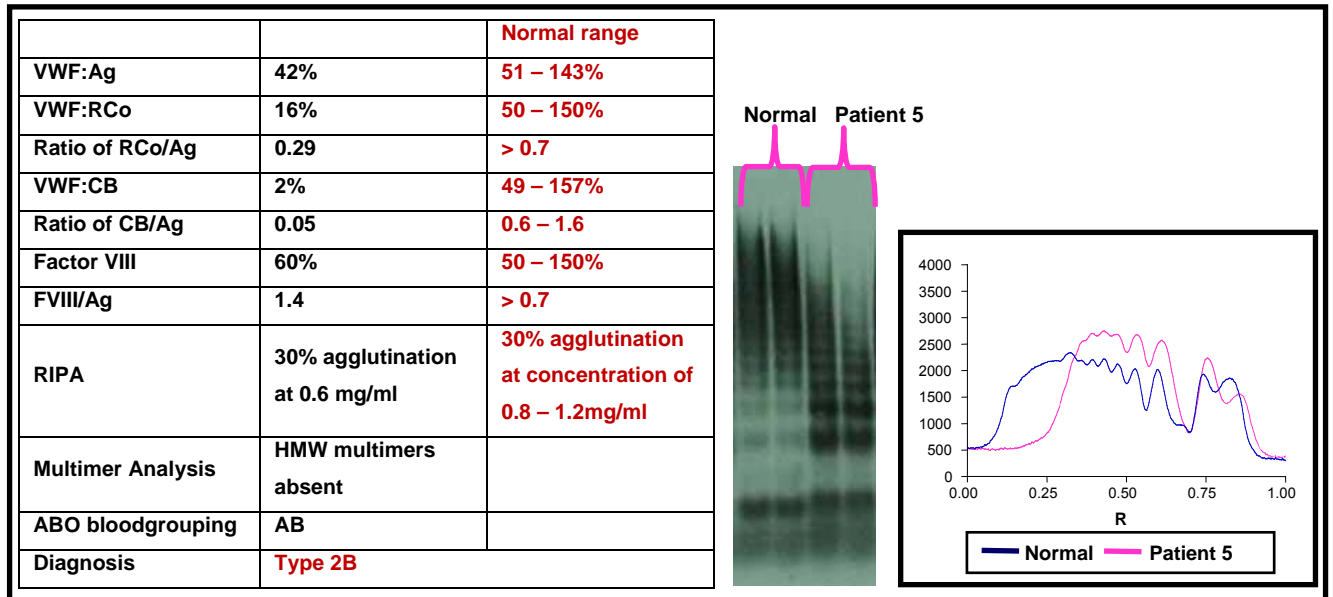


Table 16: Genetic analysis of patient 5

Patient No:	5		
Exon studied	Exon 28		
Diagnosis	Type 2B		
VWF ref number	NM_00552.3		
VWF protein ref	NP_000543.2		
	Nucleotide alteration	Amino acid change	Mode of inheritance
Polymorphisms	3795G/A	P1265	Heterozygous
	4641C/T	T1547	Homozygous
Mutations	4923G/A	R1641	Heterozygous

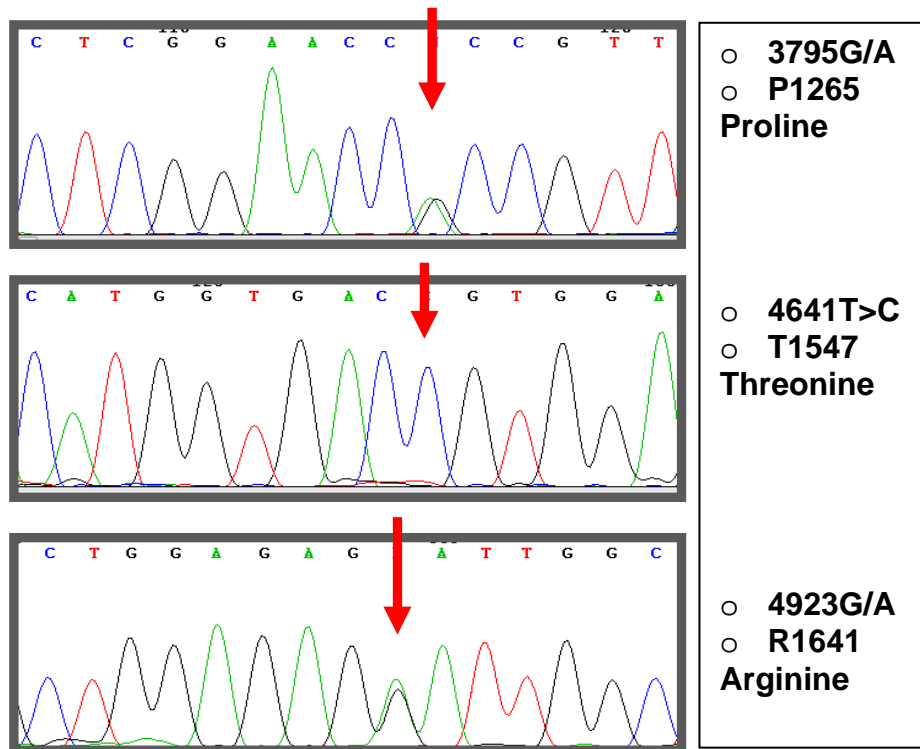


Figure 15: Sequencing analysis of patient 5

Three polymorphisms were detected two homozygous and one heterozygous. One heterozygous polymorphism is an alteration of a guanine (G) with an adenine (A) at codon number 3795 in one chromosome. This results in no amino acid substitution at codon 1265 of the protein where the proline (P) remains unchanged. The second homozygous polymorphism is an alteration of thymine (T) to a cytosine (C) at codon number 4641 in both chromosomes. This also results in no amino acid substitution at amino acid number 1547 of the protein where the threonine (T) remains unchanged. These polymorphisms have been previously described by in literature and are in the VWF database at <http://www.ragtimedesign.com/vwf/polymorphism/>. The third heterozygous polymorphism is an alteration of guanine (G) with an adenine (A) at codon number 4923 in one chromosome. This results in no amino acid substitution at amino acid number 1641 of the protein where the arginine (R) remains unchanged. This however is a novel SNP and has not been described in the literature. Table 16 shows the report of sequencing data results of patient 5.

Chapter 5: Discussion

In this study we search for mutations in exon 28 of the VWF gene in 5 patients with type 2 VWD. We had a limited sample size because patients with VWD only come to clinics when they have a bleeding episode. This is due to the fact that most type 2 VWD patients present with a mild bleeding phenotype and treatment is also given only on demand. This is the first study of this nature done in South Africa.

The VWF gene is highly polymorphic and many polymorphisms have been described (Sadler and Ginsburg 1993). We found a single nucleotide polymorphism (SNP), 4641C/T, at codon 4641 resulting from a substitution of thymine with a cytosine in one allele in all five patients (two with type 2B, two with type 2M and one with type 2A VWD). This polymorphism results in a silent amino acid change at position 1547. This SNP is inherited homozygous in three of the patients (patients 3, 4 and 5) and heterozygous in the other two patients (patients 1 and 2). According to the Ensemble VWF gene sequence (www.ensembl.org/Homo_sapiens/Gene/Summary?g=ENSG00000110799), a SNP occurs in this position, but the sequence that was published by Mancuso in 19989 shows this SNP as part of the wild type sequence. The frequency of this SNP, 4641C/T, was reported to be 0.64/0.36 (Sadler and Ginsburg 1993). In a Japanese population this SNP occurs with a frequency of 0.41/0.59 (Sadler and Ginsburg 1993) and in a British population at a frequency of 0.42/0.58 (Cumming *et al.* 2006). The fact that we found this SNP in all five of our patients could indicate a high frequency of this SNP in our population as well. This hypothesis can be proved using larger sample groups.

We found a SNP, 4141G/A, at codon 4141 caused by a substitution of adenine with a guanine in one of the alleles in three of the 5 patients (patient 1,3 and 4). This SNP results in an amino acid change from a threonine to alanine at position 1381 of the protein. However in patients 3 and 4 the SNP is inherited in a homozygous state, while heterozygous in patient 1. Both patient 3 and 4 have been diagnosed with type 2M VWD while patient 1 is a type 2B VWD subtype.

Again, there is a difference at this location between the Ensembl VWF gene sequence and the sequence that was published by Mancuso (1989) where they found that this SNP is part of the wild type VWF sequence. The frequency of this SNP, 4141G/A, was reported to be 0.35/0.65 in North American population, and 0.41/0.59 in Japanese population (Sadler and Ginsburg 1993). It was also observed in the British population at a frequency of 0.46/0.54 (Cumming *et al.* 2006). In the patients where this SNP is inherited in a homozygous state, the hydrophilic threonine is substituted with a hydrophobic alanine in both the alleles meaning that there is no wild type allele to compensate for the effect of the SNP. This SNP, T/A1381, is found in the A1 domain where VWF binds to the platelet GPIIb α complex. The T/T variant of this SNP increases interaction between VWF and platelet GPIIb under static conditions compared to the A/A and T/A variants. However, these variations are found to have no influence on normal haemostasis resulting in a null physiological effect (Szanto *et al.* 2007). No correlation between the A/A1381 SNP and an increase in proteolysis of VWF could be found (Bowen and Collins 2004).

In patient 1, we found a heterozygous mutation, 3922C>T at codon 3922 resulting in a cytosine to a thymine nucleotide alteration in one allele. This produces an amino acid change of a positively charged hydrophilic arginine to a polar hydrophilic cysteine at position 1308. Majority of mutations that are responsible for type 2B VWD are clustered in exon 28 with more than 20 different amino acid substitutions resulting in this gain in function phenotype (James and Lillicrap 2008). This mutation is the second most common mutation found in patients with type 2B see Table 4. It has been reported in family studies to induce a high affinity of VWF to GPIIb (Schmitt *et al.* 2006) (Baronciani *et al.* 2005). Interestingly Baronciani *et al.* (2005) showed that this mutation also reduces binding of VWF to collagen type I and type III. We also found a low VWF:CB activity of 27% in this patient.

Two heterozygous mutations were found in patient 2. Both mutations were previously reported to the VWF database. One heterozygous mutation, 4508T>C, results from a thymine (T) to cytosine (C) nucleotide alteration at codon 4508 in one allele. This produces an amino acid change from a non-

polar hydrophobic leucine to a non-polar hydrophobic proline at amino acid number 1503, L1503P. Expression studies done on similar mutations L1503R and L1503Q showed that the L1503Q mutation causes increased proteolysis by ADAMTS 13. The L1503R mutation causes protein instability whereby VWF is retained in the cells and not secreted (Kashiwagi *et al.* 2008). The substituted amino acid in this position, 1503, plays a significant role in the functionality of the protein. Thus the phenotypic variations observed in patients with the same subtype can clearly be distinguished by performing mutational analysis.

In the same patient a second heterozygous mutation was detected. This mutation 4517C>T results from a cytosine (C) to a thymine (T) at codon 4517 in one allele. This produces an amino acid change from a polar hydrophilic serine to a non-polar hydrophobic leucine at amino acid number 1506, S1506L. This mutation has been previously described in literature to cause type 2A VWD (Sugiura *et al.* 1992) in an unknown population. This mutation cause conformational changes in the A2 domain that (Sutherland *et al.* 2004) cause partial retention of the protein (Sutherland *et al.* 2004). However, the secreted protein is subjected to increased susceptibility to proteolysis by ADAMTS13 (Hassenpflug *et al.* 2006). This could be the result of the conformational change in the A2 domain exposing the cleavage site of ADAMTS13. This leads to the loss of large and intermediate multimers, typical of the type 2A subtype. The increased clearance can be seen with the triplet structure that is visible on a 3% agarose gel. This is due to the dimers that are formed by an increase in susceptibility to ADAMTS13. These dimers migrate faster than the normal multimer sizes and are clearly visible in between the normal bands on the gel, thus creating the triplet band structure (Furlan *et al.* 1993). The inheritance of these two mutations is reported in the VWF database and also in the same patient. This patient is an African female and therefore this report is the first on these mutations in an African patient.

In patients 3 and 4 with type 2M VWD, we found a homozygous SNP, 4414C/G, at codon 4414 resulting from guanine to a cytosine in both alleles. This results in an amino acid change from a positively charged hydrophilic histidine to a negatively charged hydrophobic aspartic acid at position 1472 of the VWF

protein. There is also a difference at this location between the Ensembl VWF gene sequence and the sequence that was published by Mancuso (1989), since the latter group classified it as part of the wild type sequence. The frequency of this SNP, 4414G/C has been reported in a population in North America at a frequency of 0.89/0.11 (Sadler and Ginsburg 1993). The amino acid alterations of this SNP do not seem to affect the proteolysis of VWF Bowen *et al.* (2008).

Type 2M VWD is classified by a decreased affinity of VWF for platelets in the presence of large multimers. We did not detect any mutations in the two patients with type 2M VWD although the laboratory analysis indicates VWD. We could only detect SNP's of which the majority is inherited homozygous. Previously, type 2M VWD was incorrectly diagnosed as type 1 (Budde and Schneppenheim 2001) because of the presence of the large multimers. This is one of the most elusive type 2 subtypes, with only two mutations reported more than once in the VWF database. Mutations that cause type 2M are found in exon 17, 27, 52 and mostly in exon 28. It is possible that our patients possess mutations elsewhere on the VWF gene as we only did mutational analysis on exon 28. Mutations in the A1 domain are mostly detected in patients with RCo/Ag ratio's < 0.4. Both the patients had RCo/Ag ratio's > 0.56. Type 2M and type 2A can easily be confused (Batlle *et al.* 2008), according to the revised classification (Sadler *et al.* 2006). Type 2A VWD is classified with decreased platelet affinity as a result of selective deficiency of HMW VWF multimers. Type 2M VWD is classified to have markedly defective platelet binding affinity with the presence of large multimers. Problems arise when identifying certain mutations in the A3 domain of VWF that cause type 2M VWD with low collagen binding activity. It was then suggested by Batlle *et al.* (2008) that the category of type 2A should include both variants that are type 2A and type 2M. This would eliminate the confusion in trying to distinguish between the two variants.

But the possibility also exists that these two patients might be type 1 VWD since only the RCo/Ag ratio and the RIPA are decreased. The presence of all multimer sizes in both types 1 and 2M makes distinguishing between the two tricky. Repeating the laboratory diagnosis of the patients would confirm or dispute the results. Furthermore DDAVP trials are an alternative, where the biological response of patients with VWD to Desmopressin is assessed.

Patients with type 1 VWD respond better to DDAVP as compared to patients with type 2M VWD (Federici *et al.* 2004).

In patient 5 with type 2B VWD we found a heterozygous SNP, 3795G/A resulting in an alteration of a guanine with an adenine in one allele. This results in no amino acid substitution at position 1265 (P1265). The frequency of this SNP, 3795G/A, was reported in to be 0.99/0.01 in French populations.

We also found a novel heterozygous SNP, 4923G/A, resulting in an alteration of guanine with an adenine at codon number 4923 in one allele in the same patient. This also results in no amino acid substitution at position 1641 (R1641) of the protein where the arginine remains unchanged. This is the first report on this SNP, 4923G/A. We are still unclear as to the frequency of this SNP in our population. Since this patient is an African male, we also would like to investigate if this SNP might be ethnic specific. Furthermore this patient was diagnosed with type 2B VWD using laboratory analysis but has no mutations in exon 28. All the mutations that have previously been reported in the VWF mutation database that cause type 2B VWD are found in exon 28. We hypothesize that this patient might have platelet type VWD instead of type 2B VWD, since this was not excluded in the initial diagnosis. This patient has very low VWF:CB levels indicating a loss of HMW multimers as seen in patients with type 2B VWD (Baroncini *et al.* 2005). The increased RIPA suggests type 2B VWD or PT-VWD (Othman and Favalaro 2008). RIPA mixing studies as well as molecular analysis of the GPIb gene can be used to confirm or dispute the PT-VWD. The influential role of other domains in the inhibition of the GPIb binding site (A1 domain) is also a possibility. It has been reported that mutations in the D'D3 domain regions can have a negative effect on the GPIb platelet binding site, thus inhibiting VWF platelet interaction (Ulrichs *et al.* 2006).

There has been reports of mutational analysis done in patients with VWD but no mutations were detected in half the patients (Melo-Nava *et al.* 2007). The effect of the collaboration of the SNPs in affecting the function and folding of VWF is another school of thought. If inheritance of certain SNP's may contribute negatively to the structural integrity of VWF, this will lead to impaired function.

Othman *et al.* (2008) highlighted that the GPIb binding site of VWF might be influenced by mutations outside the A1 loop of VWF.

Inheritance of the same SNP's or mutations in different patients does not result in the same VWF phenotype in patients (Cooney and Ginsburg 1996). Thus, mutations do not necessarily predict clinical phenotypic variations in patients but can however link specific subtypes. Mutations found in the same exon (i.e. exon 28) are specifically grouped according to the different subtypes on the VWF gene. For example, mutations that cause type 2B VWD are clustered at a different location in the A1 domain than those that cause type 2M VWD (Hillery *et al.* 1998). Molecular diagnosis in especially type 2 VWD is thus important (Song *et al.* 1999).

It is of significance to search for SNP's in our variety of populations in South Africa. No VWD mutational analysis studies have been done on African populations thus far. Furthermore, demographics in South Africa make it an interesting gene pool because of the diversity of ethnic groups and mixture between the groups. This is the first study to undertake the sequencing exon 28 in patient with type 2 VWD in South African. The fact that we found a novel SNP within a small sample size means that there might be more variations in exon 28 and that the variations might be specific for populations in the country or on the African continent. With this study, we have successfully implemented a method to detect mutations and polymorphisms in exon 28 of the VWF gene. We are still unclear as to the frequency of this SNP in our population. Since this patient is an African male, we also would like to investigate if this SNP might be ethnic specific. Furthermore this patient was diagnosed with type 2B VWD using laboratory analysis but has no mutations in exon 28. All the mutations that have previously been reported in the VWF mutation database that cause type 2B VWD are found in exon 28. We hypothesised that this patient might have platelet type VWD instead of type 2B VWD, since this was not excluded in the initial diagnosis.

Several studies have proven that mutational analysis might solve the laboratory diagnosis paradox (Roland *et al.* 2006), (Melo-Nava *et al.* 2007), (Favaloro

2008). Newer methods and techniques are being reported on making full length VWF mutational analysis faster and more efficient (Corrales *et al.* 2009). It is thus important to include molecular analysis in our laboratory to better understand our patient phenotype, to improve our VWD diagnosis and to setup a database of VWD mutations in South Africa. The enhanced understanding of VWD diagnosis can help the haematologist making an informed decision in VWD patient management. South Africa, as other developing countries, faces many challenges regarding health care and patient management. There are no statistical data on the prevalence of VWD in South Africa. There are however 8 haemophilia treatment centres throughout the country for the management of patients with bleeding disorders. The lack of knowledge and understanding about VWD remains a barrier. More importantly the undeveloped testing facilities where only certain tests are available also has an impact on the proper diagnosis of VWD in South Africa. The scarce resources allow that only outspoken diseases like HIV/AIDS are given prevalence over the less prominent diseases like VWD. The increase of awareness about VWD is highlighted in an article by Pasi (2005) which stressed the fact that an increase in awareness about VWD will lead to an increase in newly diagnosed patients.

Chapter 6: Conclusion

Genetic demographics in South Africa provide an interesting gene pool to investigate the genetics of Von Willebrand disease because of the diversity of ethnic groups and mixture between the groups.

We found the heterozygous single nucleotide polymorphism (SNP), 4641C/T in all five our patients which could indicate a high frequency of this SNP in our population as well. We found another heterozygous SNP, 4141G/A, in three of the 5 patients (patient 1, 3 and 4). However the SNP is inherited in a homozygous state in the type 2M VWD patients, while heterozygous in the first patient with type 2B VWD. In this patient with type 2B VWD, we also found a heterozygous mutation, 3922C>T which is the second most common mutation found in patients with type 2B VWD. Interestingly, this mutation also reduces the binding of VWF to collagen type I and type III and we also found a low VWF:CB activity of 27% in this patient. Two heterozygous mutations 4508T>C and 4517C>T were found in the type 2A VWD patient. Both mutations might cause either increased proteolysis by ADAMTS 13 or protein instability whereby VWF is retained in the cells and not secreted. The inheritance of these two mutations is reported in the VWF database and also in the same patient. We found a homozygous SNP, 4414C/G, in the 2 patients with type 2M VWD that do not seem to affect the proteolysis of VWF Bowen *et al.* (2008). We did not detect any mutations in the two patients with type 2M VWD. In the second patient with type 2B VWD, we found a heterozygous SNP, 3795G/A, that has been described on the VWF database. We also found a novel heterozygous SNP, 4923G/A, in the same patient that results in no amino acid substitution (R1641) of the protein. This is the first report on this SNP.

With the results obtained in this study we have proven the importance of mutational analysis to confirm laboratory diagnosis. We will set up mutational analysis as part of our Von Willebrand disease diagnostic tools in future. It could be worthwhile to investigate the other exons of VWF as well, especially exons encoding the A3 domain. We also recommend using a larger sample

size of patients with VWD. This study only focused on patients with type 2 VWD. By including genetic analysis in our Von Willebrand disease testing facility, will help us to compile a register of patients with VWD in South Africa and to report the statistics of patients with VWD in South Africa.

Chapter 7: Abstract

Keywords: Von Willebrand disease, genetics, polymorphisms, mutations, Von Willebrand factor, Inherited bleeding disorders, molecular pathogenesis.

Von Willebrand disease (VWD) is a common bleeding disorder caused by either quantitative (type 1 and 3) or qualitative (type 2) defects of von Willebrand factor (VWF). The diagnosis of VWD usually requires a panel of tests. Several analyses therefore are required to diagnose VWD. These tests are also subjected to pitfalls and it is important to take the pitfalls in to consideration when diagnosing VWD. Despite all these tests, the diagnosis and classification of VWD often remains a challenge. Identification of mutations that cause functional defects of VWF (type 2 VWD) is needed to improve the diagnosis of the disease. Mutations that cause functional abnormalities of VWF occur mostly in exon 28 of the VWF gene. Exon 28 primarily encodes the platelet GPIb and collagen binding domains of VWF (A1 domains) and the ADAMTS13 cleavage domain (A2 domains). Recently, studies in industrialised countries have been conducted on finding mutations on exon 28 but none have been done on South African populations. In this study we searched for mutations in exon 28 of the VWF gene in 5 patients with functional defects of VWF in order 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 analysed in four specific fragments, using PCR with primers that mismatch the pseudogene. The mutations were identified by automatic sequencing of the different fragments. The following polymorphisms were detected. A silent SNP 4641T/C in all five patients, the SNP 4141A/G in three patients, a silent SNP 3795G/A in one patient and a novel silent SNP 4923G/A in another patient. It is important to note that we found a novel SNP in an African patient with type 2B VWD, since no polymorphisms reported in exon 28 were from African populations. Several studies have proven the importance of mutational analysis in solving laboratory diagnosis paradox. The mutations found in the patients with type 2 VWD confirm the diagnosis and validates the importance of molecular diagnosis in VWD. With this study, we have successfully implemented a method to detect mutations in exon 28 of the VWF gene.

Hoofstuk 7: Abstrak

Von Willebrand siekte (VWS) is die mees algemeenste bloedingsiekte wat veroorsaak word deur kwalitatiewe (tipe 1 en 3) and kwantitatiewe (tipe 2) afwykings van Von Willebrand factor (VWF). Die diagnose van VWS benodig 'n paneel van toetse en dus is verskeie toetse nodig om die siekte te diagnoseer. Hierdie toetse het ook tekortkominge en dit is nodig om hierdie tekortkominge in aanmerking te neem wanneer VWS gediagnoseer word. Die diagnose en klassifikasie van VWS by steeds 'n uitdaging, nieteenstaande dat al hierdie toetse beskikbaar is. Dit is nodig om mutasies wat funksionele defekte van VWF veroorsaak, te identifiseer om sodoende die diagnose van VWS te verbeter. Sulke mutasies kom meestal in ekson 28 van die VWF geen voor. Ekson 28 kodeer vir die plaatjie- en kollageen bindingsdomeine van VWF, asook die ADAMTS13 snydingsdomein. Studies waar daar na mutasies in ekson 28 van die VWF geen gesoek is, is slegs in ontwikkelende lande gedoen en geen so 'n studie is nog op Suid Afrikaanse of Afrika populasies gedoen nie. Ons het in hierdie studie na mutasies in ekson 28 van die VWF geen gesoek in 5 pasiënte met funksionele defekte van VWF. Dit is uitgevoer om die metode daar te stel wat gebruik kan word om genetiese analise in VWS te bepaal. Ons het twee pasiënte met tipe 2M, twee met tipe 2B an een met tipe 2A VWS in die studie ingesluit. Die hele ekson 28 is in 4 spesifieke fragmente ge-analiseer deur polimerase-kettingreaksies te doen met pylstukke wat van die pseudo-geen verskil. Ons het die volgende enkel-nukleotied-polimorfismes (ENP) gevind. Die 4641T/C ENP in al vyf pasiënte, die 4141A/G ENP in 3 pasiënte, die 3795G/A ENP in een pasiënt en 'n nuwe ENP 4923G/A in 'n ander pasiënt. Dis is van belang dat hierdie nuwe ENP in 'n swart pasiënt met tipe 2B VWS gevind is, aangesien nog geen ENP's in VWF ekson 28 van swart pasiënte beskryf is nie. Verskeie studies het al die belang van mutasie-analise aangetoon in die oorkoming van die diagnostiese uitdagings van VWS. Die mutasies wat in ons tipe 2 VWS pasiënte gevind is, bevestig die belang van molekulere diagnose in VWS. Ons het die metode om mutasies in ekson28 van die VWF geen aan te toon suksesvol met hierdie studie geïmplementeer.

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