VALIDATION OF A VON WILLEBRAND FACTOR PROPEPTIDE ASSAY

By

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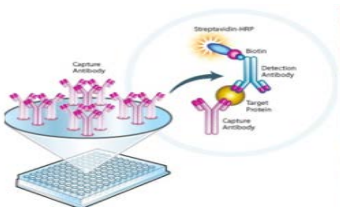

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Rethabile Brigette Maleka

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I would like to thank God; this would have never been possible without Him.

I would also like to thank the following people:

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I dedicate this dissertation to my mother, Winnie Mahlape Maleka, thank you so much for your love and support during this time. You have motivated me to be where I am and I will forever be grateful for that.

"Even though I walk through the valley of the shadow of death,
I will fear no evil,
for you are with me;
your rod and your staff,
they comfort me".

Psalm 23

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List of abbreviations

1C	1 clearance
ADAMTS13	A Disintegrin and Metalloproteinase with ThromboSpondin type 1 motifs, member 13
APTT	Activated partial thromboplastin time
bps	Base pairs
CVD	Cardiovascular disease
DDAVP	1-deamino-8-D-arginine vasopressin
E. coli	*Escherichia coli*
EACA	Epsilon-aminocaproic acid
ELISA	Enzyme-linked Immunosorbent Assay
FVIII	Factor VIII
FVIII:C	FVIII coagulant
GP	Platelet glycoprotein
gRAD	generic Rapid Assay Device
HMW	High molecular weight
HTCs	Haemophilia Treatment Centers
IL	Interleukin
IMAC	Immobilized metal ion affinity chromatography
IS	International Standard
kb	Kilobases
kDa	Kilo daltons
LFA(s)	Lateral flow assay(s)
LRP	Lipoprotein receptor
NO	Nitric oxide
OPD	o-phelylenediamine dihydrochloride
PBS	Phosphate buffered saline
PT	Prothrombin time
PT-VWD	Platelet type-VWD
RIPA	Ristocetin-induced platelet agglutination
S. cerevisiae	*Saccharomyces cerevisiae*
ScFv(s)	Single chain variable fragment(s)

TA	Tranexamic acid
TSP	Thrombospondin
TTP	Thrombotic thrombocytopenic purpura
tVWFpp	Truncated form of the VWFpp
VWD	von Willebrand disease
VWF	von Willebrand factor
VWF:Ag	VWF antigen
VWF:CB	VWF Collagen binding
VWF:RCo	VWF Ristocetin co-factor
VWFpp	von Willebrand factor propeptide
Y. lipotytica	*Yarrowia lipolytica*

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SUMMARY

Von Willebrand disease is the most common inherited bleeding disorder caused by a deficiency or defect in von Willebrand factor. Quantitative defects of von Willebrand factor include, type 1 von Willebrand disease (partial deficiency of von Willebrand factor) and type 3 von Willebrand disease (complete deficiency of von Willebrand factor). Type 2 von Willebrand disease includes all qualitative defects of von Willebrand factor. Type 1 von Willebrand disease is either due to decreased synthesis and secretion or increased clearance of von Willebrand factor from plasma. It is essential to diagnose individuals with an increased clearance rate of von Willebrand factor, as the treatment of these patients with 1-deamino-8-D-arginine vasopressin is not effective. The ratio between the von Willebrand factor propeptide and the von Willebrand factor antigen is used to identify conditions of reduced half-life, such as type 1 von Willebrand disease with increased clearance. Currently, there is only one commercial assay available to measure von Willebrand factor propeptide levels. This assay is not only too expensive to be used in developing countries but is also very time consuming. The von Willebrand factor propeptide protein assay is an expensive test as it uses monoclonal antibodies. Mammalian cells are commonly used for the expression of monoclonal antibodies. The production of monoclonal antibodies is expensive. With this research an effort was made to develop more cost-effective and more rapid assays to determine the von Willebrand factor propeptide levels in patient's plasma. The aim of this study was to therefore validate a von Willebrand factor propeptide assay. First, two single chain variable fragments that bind to the von Willebrand factor propeptide were expressed by yeast. The von Willebrand factor propeptide protein was also expressed, as it is not commercially available. However, the expression of the propeptide and the two single chain variable fragments were not successful. The von Willebrand factor propeptide protein is firstly too large and it also consists of 2 homologous cysteine-rich D domains. A primary bottleneck in recombinant protein production is the presence of the disulfide bond structure. We then produced two polyclonal antibodies against a truncated form of the von Willebrand factor propeptide. The two polyclonal antibodies could however only detect the truncated von Willebrand factor propeptide, but not the von Willebrand factor propeptide in

plasma. The reduced antigenicity of the truncation affected the epitope construction. We then developed a lateral flow assay using commercial antibodies to the von Willebrand factor propeptide. Lateral flow assays are low cost detection devices that are simple to use, rapid and portable. In the rapid von Willebrand factor propeptide lateral flow assay, a monoclonal and polyclonal clonal antibody was used. The polyclonal antibody did not bind specific to the von Willebrand factor propeptide as it can bind to both the full-length von Willebrand factor protein and to the von Willebrand factor propeptide. Polyclonal antibodies show higher cross reactivity. This assay could therefore not be validated, as it was not specific for the VWF propeptide. Lastly, a rapid enzyme-linked immunosorbent assay using the commercial antibody pair clone CLB-Pro 35 and CLB-Pro 14.3 was developed and validated. This rapid assay has equal sensitivity and precision as the commercial method and can be used to diagnose patients with increased von Willebrand factor clearance.

Key words: von Willebrand factor Propolypeptide, von Willebrand disease, Enzyme-linked Immunosorbent Assay, Lateral flow assay (LFA).

Chapter 1: Introduction

von Willebrand disease (VWD) is the most common inherited bleeding disorder and is caused by a deficiency or defect in von Willebrand factor (VWF) (Sanders et al., 2015). VWF plays an essential role in primary haemostasis, where it regulates platelet adhesion to damaged vascular subendothelium and subsequently platelet aggregation (Peyvandi et al., 2011). It also carries the blood clotting factor VIII (FVIII) and circulates together with factor VIII in plasma as a non-covalent complex (De Wit and Van Mourik, 2001). VWD is classified into three types, type 1, 2 and 3 (Sadler and Gralnick, 1994).

The quantitative deficiencies include, type 1 VWD (partial deficiency of VWF) and type 3 VWD (complete deficiency of VWF). Type 2 VWD includes all qualitative defects of VWF (Sharma and Flood, 2017). Type 1 VWD is either due to decreased synthesis and secretion of VWF, or increased clearance of VWF from plasma (Meiring *et al.*, 2009). Increased VWF clearance was seen in 45% of the type 1 VWD patients in South Africa (Meiring *et al.*, 2011). The glycosylation of the VWF protein has a significant impact on its clearance. Thus, the ABO antigens that are found on the N-linked sugars on VWF affect the clearance of VWF (Casari *et al.*, 2013; Van Schooten *et al.*, 2007). The blood group O antigens on VWF are associated with the increased clearance of this protein (Lenting *et al.*, 2007). The average VWF levels are about 25 % lower in individuals with the O blood type than in non-O blood type (Van Schooten *et al.*, 2007). VWF levels are also much lower in individuals with the Bombay phenotype, who do not express any of the ABO antigens (Lenting *et al.*, 2007). Interesting, the O blood group is more common in type 1 VWD than in the general population or in type 2 VWD patients (National Institutes of Health, 2011).

It is essential to diagnose individuals with an increased clearance rate of VWF, because the treatment of these patients using 1-deamino-8-D-arginine vasopressin (DDAVP) is not effective since the VWF in the plasma of these patients is cleared too rapidly from the circulation (Meiring *et al.*, 2009). The ratio between the von Willebrand factor propeptide (VWFpp) and the VWF antigen (VWF:Ag) is used to identify conditions of reduced VWF half-life, such as type 1 VWD with increased

clearance (Hubbard *et al.*, 2012). The VWFpp/VWF:Ag ratio varies amongst the blood groups, with increased ratios in individuals with blood group O compared to the non-O blood group individuals (Casari *et al.*, 2013).

The VWFpp assay is not only included in the diagnostic panel of VWD, it can also be used instead of the VWF:Ag assay in the assessment of acute or chronic endothelial activation (Haberichter, 2015a). The reason is because the VWFpp does not (like the mature VWF protein) bind or get trapped by the subendothelial connective tissues or platelets after its release. It also reflects endothelial secretion more accurately (Health and Human Services, 2014). Systemic VWF concentrations are therefore not an accurate measurement of endothelial cell secretory function during activation, perturbation or damage of the endothelium. Endothelial cell activation due to increased VWFpp level has been implicated in conditions, such as chronic renal failure, aortic stenosis, ischaemic stroke, sepsis, meningococcal disease, dengue, systemic sclerosis, sickle cell disease, HELLP syndrome and asthma (Haberichter, 2015a).

Currently, there is only one commercial assay available for the measurement of VWFpp levels. This assay is not only too expensive to be used in developing countries but is also very time consuming. With this research an effort was made to develop more cost-effective and more rapid assays to determine VWFpp levels in patient's plasma. Previously, a cost-effective VWFpp ELISA assay was developed, using antibodies that were produced by phage display technology. This study aimed to express these antibodies and validate the assay. This project also aimed to develop and validate a rapid lateral flow assay (LFA). Lastly, a rapid ELISA assay for the measurement of the VWFpp in plasma was developed and validated.

Chapter 2: Literature review

2.1 von Willebrand factor

2.1.1 Genetics of von Willebrand factor

The von Willebrand factor (VWF) gene is located on chromosome 12p13.2 (figure 2.1) (Federici, 2006). The gene is 178 kilobases (kb) long and contains 52 exons (Mancuso *et al.*, 1989). The exons of this gene range in size from 40 to 1379 base pairs (bps) in length and the introns range from 97 bps to 19.9 kb. The signal peptide and the von Willebrand factor propeptide (VWFpp) are encoded by the first 17 exons of the gene, and the mature VWF and the 3' untranslated region are encoded by exons 18 to 52 (Lillicrap, 2005).

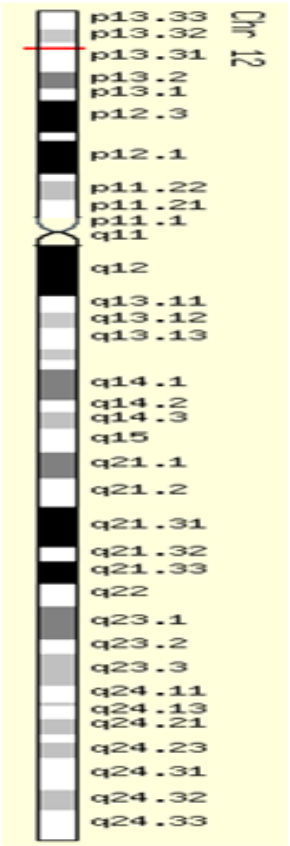

Figure 2.1 The locus of the VWF gene on chromosome 12 *(http://www.genecards.org/cgi-bin/carddisp.pl?gene=VWF).*

2.1.2 Structure of von Willebrand factor

VWF was first identified by Zimmerman and collaborators in 1971 (Ruggeri, 1999). The primary translation product of VWF is a 2813-residue precursor polypeptide known as pre-pro-VWF. It contains a signal peptide of 22 amino acids, an unusually large VWFpp of 741 amino acids and a mature subunit of 2050 amino acids (Ruggeri and Ware, 1993). The mature VWF has about 22 carbohydrate side chains, ten of which are O-linked to serine or threonine and 12 N-linked to asparagine (Preston *et al.*, 2013). The estimated carbohydrate content of VWF ranges from 10 to 19 % of the total mass of the mature VWF protein calculated at approximately 278 kilo daltons (kDa). A typical structural feature of VWF is the high cysteine content, which makes 169 of the total 2050 amino acids. The cysteine residues join the VWF subunits into a higher ordered structure (Ruggeri and Ware, 1993).

The VWFpp and the mature VWF, which together form the pro-VWF, consist of four types of repeating domains arranged from the amino to carboxyl terminal end (Ruggeri, 2003). These domains appear in the following order (figure 2.2): D1-D2-D'-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK, and have a number of specific ligand-binding sites. The D1 and D2 domains form the VWFpp. The D' to D3 domains form the factor VIII (FVIII) binding site. The A1 domain contains the binding site for platelet glycoprotein (GP) Ib. The A2 domain contains the cleaving site for the VWF cleaving protease, A Disintegrin and Metalloproteinase with ThromboSpondin type 1 motifs, member 13 (ADAMTS13). The A3 domain binds to collagen and the C4 domain binds to GPIIb/IIIa on the platelet membrane (Crawley and Scully, 2013).

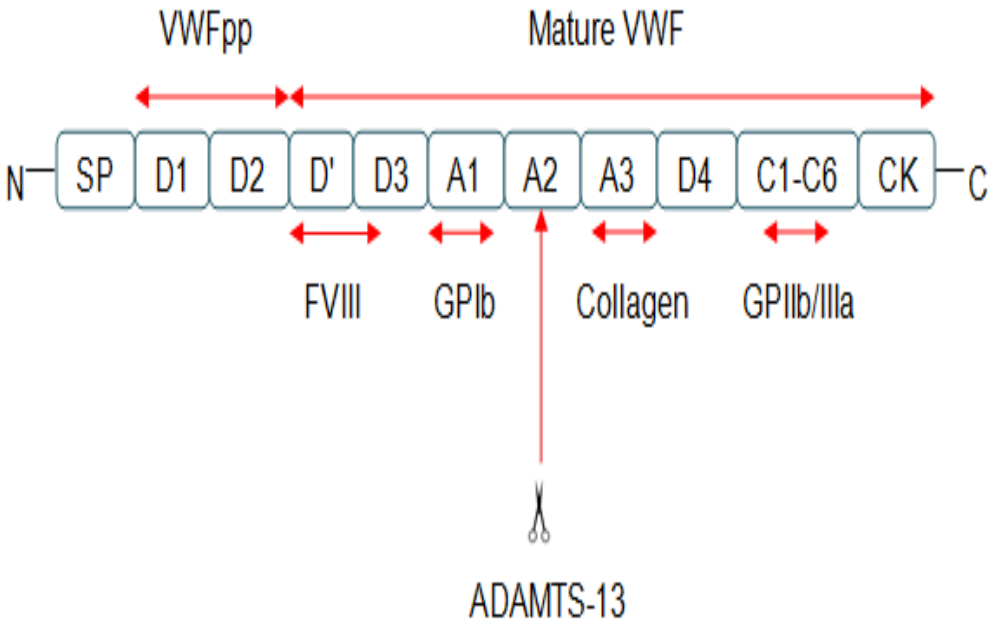

Figure 2.2 Domain structure of VWF.

Pro-VWF has four types of repeating domains arranged from the amino to carboxyl terminal end. N= N-terminal domain, CK= cysteine knot, C= C-terminal domain (Crawley and Scully, 2013).

2.1.3 Functions of von Willebrand factor

VWF is a multifunctional plasma protein that plays an essential role in primary haemostasis (Favaloro, 2016). It regulates platelet adhesion to the damaged vascular subendothelium and subsequently platelet aggregation (figure 2.3) (De Wit and Van Mourik, 2001; Peyvandi *et al.*, 2011). This protein also carries and protects FVIII from proteolytic degradation in the circulation (Davies et al., 2008). Both these functions of VWF are important for the normal arrest of bleeding. However, VWF can be involved in situations that can lead to arterial occlusion (Ruggeri and Ware, 1993).

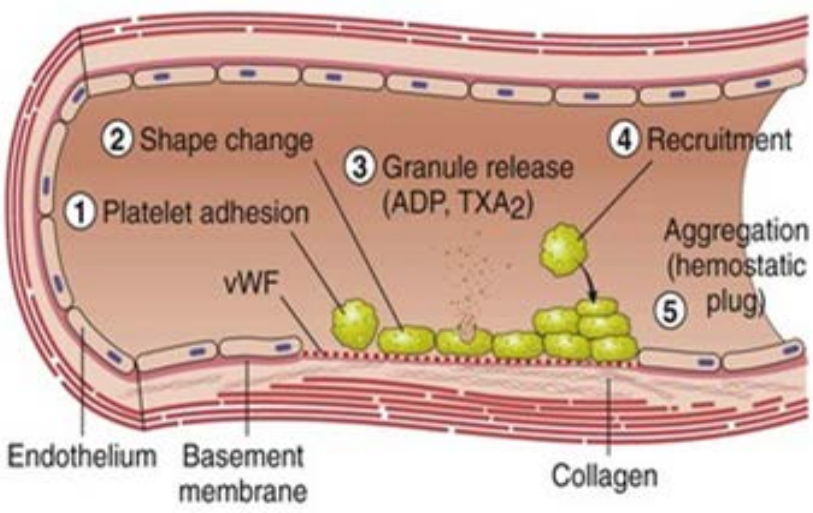

Figure 2.3 The role of VWF in primary haemostasis.

Platelets adhere to VWF on the endothelium, that leads to activation of the plateles and causes them to change their shape. Granule contents (ADP, TXA_2) of the platelets are then released. More platelets are recruited to the VWF on the endothelium, and a haemostatic plug is formed. ADP= adenosine diphosphate, TXA_2= thromboxane A_2 (https://veteriankey.com/bleeding-and-hemostasis/).

VWF in the sub-endothelial matrix and plasma interacts with the platelet receptor GP Ib-IX-V. This binding activates the GPIIb-IIIa receptor complex on the platelet membrane through which platelets adhere to each other. Platelet adhesion then becomes irreversible across the whole injured area and extra platelets are recruited to the growing thrombus (platetelet aggregation) (Ruggeri and Ware, 1993). For efficient platelet adhesion and aggregation to take place, appropriate haemodynamic situations, such as a high shear rate and highly polymerized molecules of VWF are needed (De Wit and Van Mourik, 2001).

The factor VIII binding function of VWF has a significant effect on the half-life of FVIII in the circulation (Leyte *et al.*, 1991). An alteration in plasma level of VWF also leads to a concordant change in the plasma concentration of FVIII for example, thus low levels of VWF are associated with decreased FVIII levels. In situations associated with an increased level of VWF, such as malignancy, sepsis or liver disease, the FVIII level is also elevated in a similar manner. Physiological stimuli such as exercise or pregnancy also increase the level of VWF and this can also result in a rise in FVIII

levels. All these examples indicate the importance of VWF in the stabilization of FVIII in the circulation. However, the level of VWF in plasma is not affected by the FVIII level (De Wit and Van Mourik, 2001).

2.1.4 Synthesis of von Willebrand factor

VWF is a large multimeric glycoprotein which is produced in the megakaryocytes and endothelial cells, and it is found in the subendothelial matrix, plasma and platelets (Dayananda *et al.*, 2011; Ruggeri, 2007). VWF is produced as a pre-pro protein. After the signal peptide has been cleaved off, a pro-protein is formed (figure 2.4) (Valentijn and Eikenboom, 2013).

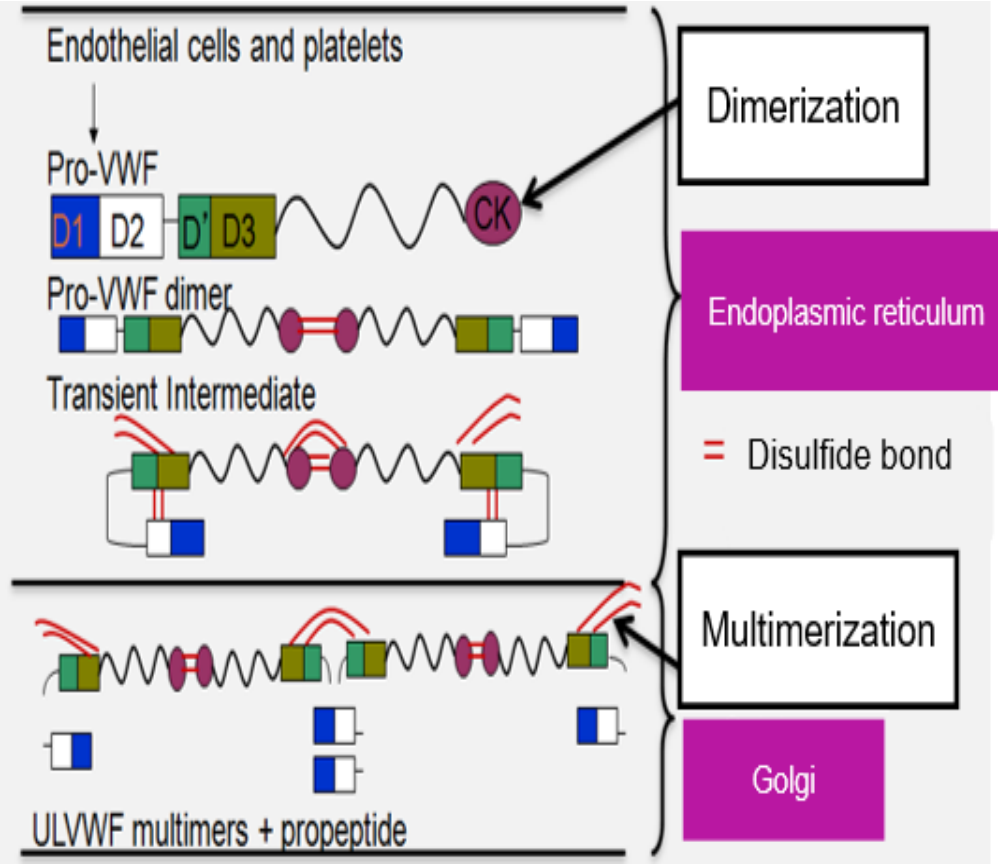

Figure 2.4 Synthesis of VWF.

The VWF molecule is synthesized in endothelial cells and megakaryocytes. Pro-VWF monomers undergo dimerization at the C-terminal ends in the endoplasmic reticulum. The pro-VWF dimers are then transported to the golgi apparatus where they undergo glycosylation and sulfation, and multimerization at the N-terminal ends.

Two pro-VWF molecules are joined in a tail-to-tail configuration through disulfide bonds at C-terminal ends to form pro-VWF dimers in the endoplasmic reticulum. The tail-to-tail jointed pro-VWF dimers are then transported to the Golgi apparatus, where they undergo modification through glycosylation and sulfation. They also multimerize in a head-to-head configuration by forming additional disulfide bonds at the N-terminal ends (Pimanda and Hogg, 2002). In the trans-Golgi network, furin cleaves the VWFpp, which stays non-covalently bound to the mature VWF until the VWF is released. If cleavage of the VWFpp is prevented by introducing a mutation at the cleavage site, VWF multimerization still takes place, however targeting of VWF to storage granules is inhibited. The non-covalent link between the VWFpp and the mature VWF is thus important for the arrangement of VWF into tubules (Valentijn and Eikenboom, 2013).

After synthesis, VWF is secreted through one of two pathways. The constitutive pathway is linked directly to synthesis (where molecules are released immediately after production), while the regulated pathway involves the storage of mature molecules which are secreted after stimulation by secretagogues (Ruggeri, 1999). When endothelial cells are stimulated by agonists that increase cytosolic free calcium ions, such as thrombin, histamine or calcium ionophore A23187; or to factors that raise the level of cyclic adenosine monophosphate, like epinephrine or forskolin, they release VWF rapidly from the cell. The rate of this type of secretion is higher when compared to the biosynthetic rate of VWF (De Wit and Van Mourik, 2001). VWF is stored in two organelles, namely the Weibel-Palade bodies in the endothelial cells and the α-granules in the megakaryocytes and platelets. The genesis of the Weibel-Palade bodies solely depends on VWF. Thus, endothelial cells that lack VWF cannot store any other proteins, such as P-selectin, interleukin (IL)-8 and endothelin which are normally also found in the Weibel-Palade bodies (Ruggeri, 2003). In megakaryocytes, only the regulated pathway of VWF secretion exists. Therefore, circulating plasma VWF all originates from endothelial cells, as platelets only secrete their α-granule content upon stimulation. VWF released from endothelial cells, either through the constitutive or regulated pathway, is directed towards both the lumen and subendothelial matrix (Bowie *et al.*, 1986).

2.1.5 Regulation of von Willebrand factor size

The size of VWF is controlled by the metalloprotease, ADAMTS13 (Dayananda *et al.*, 2011). This enzyme cleaves VWF at the peptide bond located between tyrosine at position1605 and methionine at position 1606 within the A2 domain (figure 2.5) (Kobayashi *et al.*, 2008). ADAMTS13 binds to VWF under static conditions and in conditions of venous and arterial shear stress. This interaction between ADAMTS13 and VWF is not effective unless shear stress is highly sufficient to stretch VWF and expose the hidden A2 domain for cleavage. In static conditions, ADAMTS13 only cuts VWF under denaturing conditions, but in high shear stress conditions as in the microvasculature, VWF cleavage occurs very quickly. The binding of VWF to the platelet GPIb receptor causes conformational changes in the A1 and A2 VWF domains that are needed for the cleavage by ADAMTS13. On the other hand, binding of chloride ions to the A1 VWF domain prevents this cleavage by ADAMTS13, and this leads to other conformational changes in the A1 and A2 VWF domains that make the cleavable peptide bond in VWF unavailable for proteolysis (Di Stasio *et al.*, 2008).

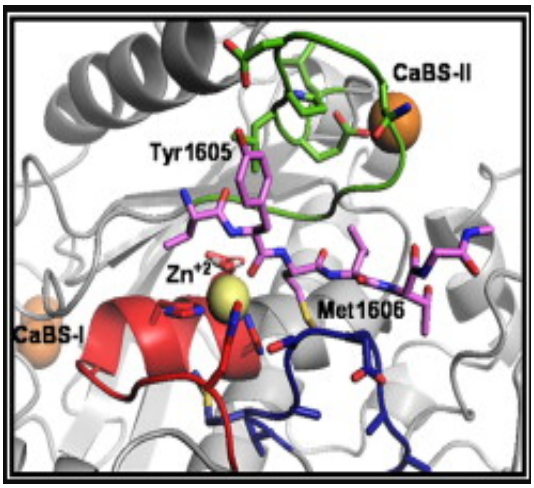

Figure 2.5 Interaction between VWF and ADAMTS13.

Interaction at the peptide bond located between tyrosine at position1605 and methionine at position 1606 within the A2 domain. When the ADAMTS13 protease cleaves VWF at this peptide bond, a N-terminal and a C-terminal domain is created from the VWF monomer. Tyr= tyrosine, Met= methionine, Zn^{2+}= zinc, CaBS-I= calcium binding site 1, CaBS-II= calcium binding site 2 (Pozzi et al., 2012).

The ADAMTS13 gene is located on chromosome 9 (Zheng *et al.*,2001). A characteristic feature of ADAMTS13 is a Zn^{2+} binding motif (HEXXHXXGXXHD) that involves three histidine amino acid residues and a glutamic acid residue in the active site of this proteolytic enzyme (Crawley and Scully, 2013). In addition to the active site, Zn^{2+} and Ca^{2+} ions are also required for ADAMTS13 functionality (Gardner *et al.*, 2008). ADAMTS13 weighs 180 kDa, and consists of a metalloprotease, disintegrin-like, thrombospondin (TSP) type 1 repeat, cysteine rich domain, a spacer domains, additional seven TSP repeats and two C-terminal CUB domains (figure 2.6). The C-terminal domains of ADAMTS13 that include the TSP repeats 2 to 8 and the CUB domains are essential for the binding of ADAMTS13 to the globular form of VWF. In this form of VWF, the A2 domain within VWF is folded and this cleavage side is hidden (Crawley and Scully, 2013).

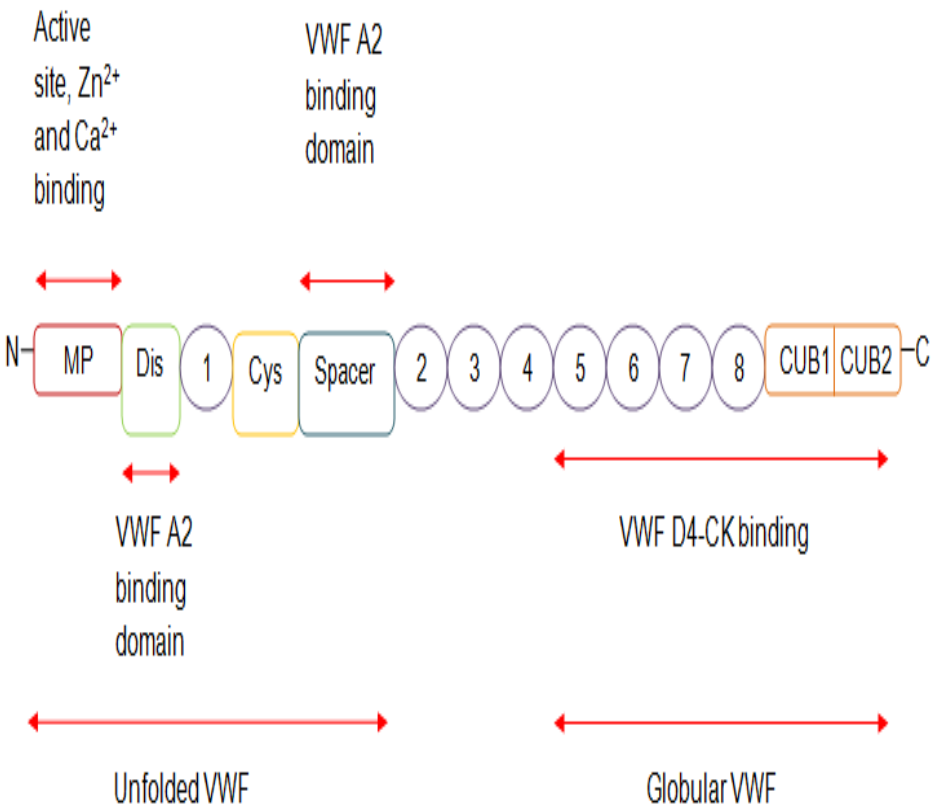

Figure 2.6 Structure of ADAMTS13.

During shear stress, VWF unfolds from a globular form to an elongated form. In this form, ADAMTS13 cleaves VWF into smaller fragments, thereby controlling the size of VWF. MP= metalloprotease domain, Dis= disintegrin domain, Cys= cysteine domain, 1-8= TSP repeats (Crawley and Scully, 2013).

Shear stress unfolds VWF from a globular form to an elongated form (Casa *et al.*, 2015). This conformational change forms the basis of how shear stress increases the susceptibility of VWF to proteolytic cleavage. Shear stress also enhances the adhesive ability of VWF. The response of the conformation and function of VWF to shear stress explains why this protein has the ability to support platelet adhesion and aggregation under high shear stress situations. When elongated forms of VWF accumulate in the blood circulation, platelet aggregation and intravascular thrombosis might occur (Tsai *et al.*, 2003). Thrombotic cytopenic purpura (TTP) occurs due to a deficiency of ADAMTS13. In TTP, the microvascular platelet aggregation and thrombus formation leads to thrombocytopenia, microangiopathic haemolytic anaemia, variable renal and neurological dysfunction, and fever (Meiring et al., 2012). Lack of ADAMTS13 is also seen in metastasizing malignancies, liver disease, connective tissue disorders and the post-surgical state (Matsukawa et al., 2007). A severe deficiency of this metalloprotease activity of less than 5 % of that in normal plasma, results from either a mutation in the ADAMTS13 gene or by autoantibodies to ADAMTS13 (Kobayashi *et al.*, 2008).

2.1.6 Clearance of von Willebrand factor

There are numerous factors which have an effect on the clearance of VWF, such as glycosylation and missense mutations. In addition to this, cells that lead to the catabolism of VWF have been identified. This has led to the identification of receptors that regulate the cellular up take of the VWF protein (Denis *et al.*, 2008).

The glycosylation of the VWF protein has a significant impact on its plasma levels. The primary VWF sequence has 10 O-linked and 12-N-linked glycosylation sites with carbohydrate residues. The first N-linked carbohydrates are attached to VWF during the early stages of synthesis. Further processing into complex N-linked site chains continues in the Golgi apparatus, where O-linked glycosylation also takes place, as well as the addition of sialyl to both the O- and N-linked sugars. VWF is one of the rare plasma proteins with N-linked sugars that contain the ABO blood group. However, the ABO blood group antigens are not found on O-linked sugars (Van Schooten *et al.*, 2007). The ABO antigens affect the clearance rate of VWF (Casari *et al.*, 2013). The average VWF levels are about 25 % lower in individuals with the O

blood type than in non-O blood type (Van Schooten *et al.*, 2007). VWF levels are also much lower in individuals with the Bombay phenotype, who do not express the ABO antigens (Lenting *et al.*, 2007). Individuals with the AB blood type have the highest VWF levels. Blood group O is more common in type 1 von Willebrand disease (VWD) than in the general population (National Institutes of Health, 2011).

The ratio of the VWFpp to the VWF antigen (VWF:Ag) varies amongst the blood groups, with increased ratios for the O blood group individuals compared to the non-O blood group individuals (Casari *et al.*, 2013). The blood group O antigens on VWF are thus associated with increased clearance of this protein (Lenting *et al.*, 2007). Glycosylation has an effect on VWF plasma levels as it has been shown that the half-life of endogenous VWF is reduced more in mice that are genetically deficient of the sialyl-transferase ST3Gal-IV. In addition, in patients with real or suspected bleeding disorder, reduced sialyl-transferase ST3Gal-IV-mediated sialylation was associated with decreased plasma levels of VWF (Van Schooten *et al.*, 2007). To further support the relationship between the ABO blood group and the clearance of VWF, a significantly decreased half-life of VWF has been reported after the administration of 1-deamino-8-D-arginine vasopressin (DDAVP) in individuals with the O blood group compared to the non-O blood group individuals (Casari *et al.*, 2013).

Mutations also have a significant effect on the clearance of VWF (Denis *et al.*, 2008). More than 20 different mutations have been identified that have an effect on the clearance of VWF (figure 2.7) (Casari *et al.*, 2013). Missense mutations mostly in the D3 domain of the VWF gene lower the half-life of VWF (National Institutes of Health, 2011). These mutations can have an effect on the levels of VWF by affecting any part of the biosynthetic pathway, such as trafficking, storage, secretion, and/ or clearance of VWF (Hospital Physician Hematology Board Review Manual, 2014). The p.Arg1205His variant, also known as the Vicenza variant is the best characterized and the most common of these missense mutations. These mutations are referred to as type 1 clearance (1C), even though this has not been recognized by the the Scientific and Standardization Committee on the classification of VWF of the International Society on Thrombosis and Haemostasis (National Institutes of Health, 2011). In type 1C VWD, the patients usually have very low levels of VWF, an increased VWFpp/VWF:Ag ratio and a reduced response to DDAVP. On the other

hand, the half-life of VWF/FVIII concentrates is normal in these patients (Hospital Physician Hematology Board Review Manual, 2014).

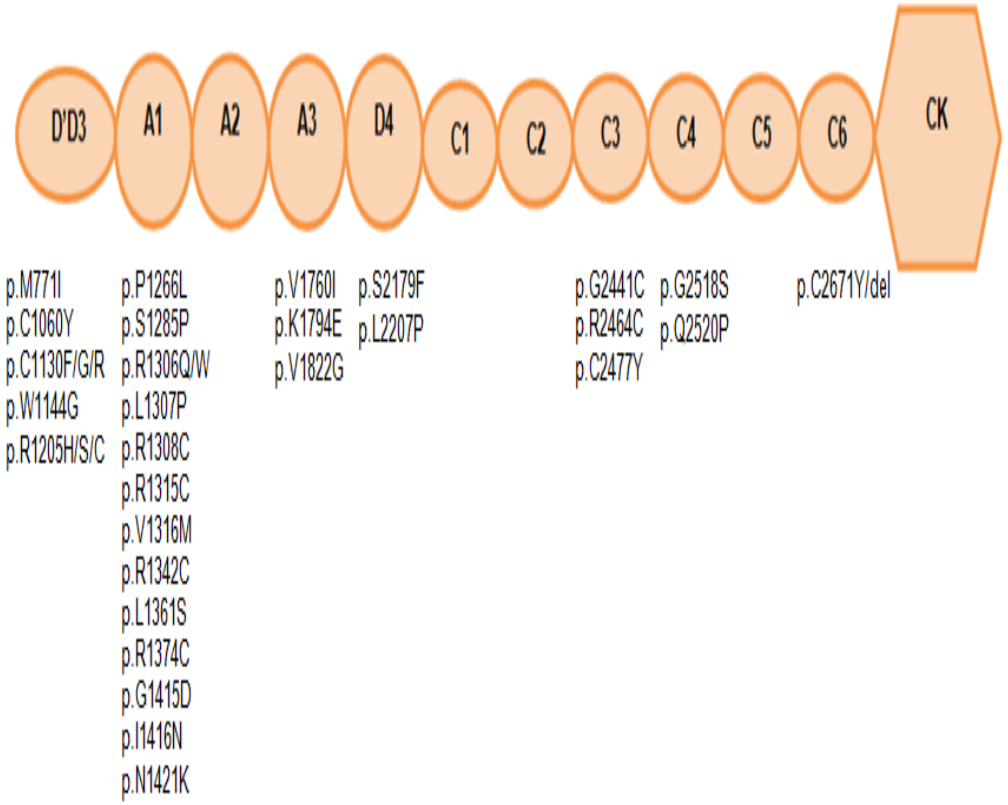

Figure 2.7 VWF clearance mutations.

The VWFpp/VWF:Ag ratio has been used to identify more than 20 different mutations that are potentially associated with increased VWF clearance. These mutations occur throughout the mature VWF molecule. Most of these mutations occur in the D'D3 and A1 domains. Additionally, 30 to 40 % of these mutations lead to the appearance or disappearance of a cysteine amino acid (Casari, et al., 2013).

Macrophages in both the liver and spleen are the dominant cell type that is involved in the uptake of VWF (Van Schooten *et al.*, 2008). Chemical inactivation of macrophages results in a prolonged VWF half-life. VWF is also bound and internalized by macrophages in *in vitro* experimentations (Casari *et al.*, 2013).

VWF also acts as an adhesive surface for leukocytes through an interaction with β2 integrins (Gragnano *et al.*, 2017). In particular, αMβ2 integrin (also known as MAC-1 or CR3) which is involved in the uptake of microbes and proteins like fibrinogen by

macrophages, and it therefore serves as an endocytic receptor for VWF (Lenting *et al.*, 2007). Siglec-5 has also been identified as a potential receptor for VWF as it recognizes the sialic acid structures on VWF. It is expressed on a number of cell types, such as macrophages, and therefore can contribute to the removal of VWF from the circulation (Casari *et al.*, 2013).

Other receptors that determine plasma VWF levels include stabilin-2, CLEC4M and lipoprotein receptor (LRP)1. Stabilin-2 is expressed in liver sinusoidal endothelial cells and it is commonly known for its role in the clearance of apoptotic bodies and heparins. The interaction of stabilin-2 to VWF is unknown, and requires further investigation. CLEC4M is a C-type lectin receptor that is expressed on endothelial cells. It is involved in the interaction with pathogenic viruses and has the intrinsic capacity to recognize glycan structures that are present on VWF. The LRP1, which is also known as CD91, was initially identified as a scavenger receptor for lipoproteins and serine protease/inhibitor complexes. Recently, more than 30 functionally and structurally different LRP1 ligands have been discovered, including FVIII. The binding of VWF to LRP1 is regulated by shear stress. Furthermore, a prolonged VWF half-life has been observed when there is a macrophage-specific deficiency of LRP1. These observations show that LRP1 functions as a clearance receptor for VWF and the VWF-FVIII complex (Casari *et al.*, 2013).

2.2 von Willebrand factor propeptide

In 1978, Montgomery and Zimmerman were the first to identify the VWFpp protein which is also known as VWD antigen II. This protein represents 26.3 % of the primary translation pro-VWF and it is composed of 2 homologous cysteine rich D domains (D1 and D2), with 32 cysteines in each of these domains (Rosenberg et al., 2002). The VWFpp and mature VWF multimers are produced through proteolytic processing in the acidic compartment of the trans Golgi, and both proteins are stored in the α-granules or Weibel-Palade bodies (Haberichter et al., 2006). Stimulation of exocytosis leads to equimolar amounts of these two proteins. The VWFpp has a circulating half-life of 2 to 3 hrs, and the half-life of mature VWF is more than 12 hrs (Scheja *et al.*, 2001). After secretion into plasma, the VWFpp protein dissociates from VWF and circulates as a homodimer at a concentration of approximately 1 µg/ml,

whereas the mature VWF circulates at approximately 10 µg/ml (Haberichter *et al.*, 2006).

2.2.1 Functions of von Willebrand factor propeptide

VWF multimers are linked through disulfide bonds. The formation of disulfide bonds is normally limited to the endoplasmic reticulum, where neutral pH and necessary oxidoreductase enzymes promote this process. The Golgi is a hostile environment for disulfide bond formation or rearrangement as a result of its acidic nature and lack of oxidoreductase. To overcome this, VWF uses the VWFpp as its own oxidoreductase to promote the rearrangement or disulfide bond formation (Purvis and Sadler, 2004). The Cys-X-X-Cys sequences within each of the two D domains of the VWFpp have intrinsic disulfide isomerase activity and catalyze multimerization of VWF (Haberichter *et al.*, 2003). These sequences are similar to the active sites of the protein disulfide isomerase family of enzymes that catalyze disulfide bond formation during the synthesis of secretory proteins in the endoplasmic reticulum (Allen *et al.*, 2000).

The VWFpp also promotes vesicular segregation of VWF into storage granules (Rosenberg *et al.*, 2002). Even though the VWFpp is required for both VWF multimerization and regulated storage, these two events are independent of each other. Disruption of either of the vicinal cysteine motifs in the D1 domain leads to defective multimerization, but has no effect on the regulated storage of VWF (Haberichter, 2015a).

Furthermore the VWFpp also has other functions, it acts as an antagonist of platelet function and mediates inflammation (De Wit and Van Mourik, 2001).

2.2.2 The role of VWF and its propeptide in endothelial cell diseases

There is increasing interest in the evaluation of VWF and the VWFpp when endothelial cell perturbation, activation and/or vascular damage are suspected since both these proteins are processed and secreted by the endothelium through the constitutive or regulated secretion pathways (Health and Human Services, 2014).

During perturbation of the endothelium, both VWF and VWFpp concentrations quickly increase. The VWFpp concentration returns to its baseline value much faster due to its rapid turnover after termination of the vascular challenge compared to the levels of VWF. Based on these observations, measuring both the VWFpp and VWF levels can provide a means to examine the extent and time course of endothelial cell activation under clinical conditions. High VWF and VWFpp levels are indicative of acute vascular perturbation, whereas situations in which only the VWF is increased, indicates chronic endothelial cell activation (Van Mourik *et al.*, 1999).

Furthermore, the measurement of the VWFpp levels has many advantages over that of the VWF levels. The plasma levels of VWF are determined by many factors, such as age, pregnancy, and diseases associated to endothelial dysfunction. Genetic variations like the ABO blood group also have an effect on VWF levels as mentioned previously. On the other hand, the VWFpp plasma levels are not affected by the ABO blood group (Marianor *et al.*, 2015). In addition, a part of VWF can be trapped in the sub-endothelium at the site of release and fail to reach the circulation. Furthermore, VWF can also be rapidly consumed by platelet aggregation (Vischer *et al.*, 1997). Consequently, systemic VWF concentrations are therefore not an accurate measurement of endothelial cell secretory function during activation, perturbation, or damage of the endothelium. Therefore, the systemic VWFpp level is more accurate in reflecting endothelial secretion of VWF (Health and Human Services, 2014).

Increased VWFpp level has been found in clinical conditions, such as chronic renal failure, aortic stenosis, ischaemic stroke, sepsis, meningococcal disease, dengue, systemic sclerosis, sickle cell disease, HELLP syndrome and asthma. VWFpp and VWF levels have also been used to identify endothelial cell activation in diabetes. Significant increases in both VWF and VWFpp have been seen in insulin-dependent diabetic individuals that have microalbuminuria or overt diabetic nephropathy (Haberichter, 2015a). In children with malaria, acute endothelial cell activation is also associated with increased VWF and VWFpp levels (Hollestelle *et al.*, 2006). Increased VWF and VWFpp plasma levels have also been noticed in thrombotic microangiopathy (Ito-Habe *et al.*, 2011). In addition, high plasma VWF concentrations have also been described in other vasculopathies, such as hypertension and diabetes (Scheja *et al.*, 2001).

Vascular injury leads to increased secretion of VWF and its propeptide. The endothelium lines the entire vascular system and consists of a single layer of endothelial cells. The vascular endothelium regulates thrombosis and thrombolysis, platelet adhesion, modulates vascular tone and blood flow, and modulates immune and inflammatory responses by monitoring leukocyte, monocyte and lymphocyte interactions to the blood vessel wall (Sumpio *et al.*, 2002). The endothelium also produces nitric oxide (NO) that regulates vascular dilator tone, regulates local cell growth and protects the blood vessel from injurious consequences of platelets and circulating cells in the blood (Cannon III, 1998). Free radicals can however disrupt the NO balance, and damage the endothelium and make it permeable to toxins. The human body has sufficient antioxidants that are obtained from a variety of foods to neutralize these free radicals; but if these antioxidants are depleted, damage to the endothelium and a change in NO balance can occur. There are several factors that may increase the number of free radicals, such as obesity, smoking, sleep deprivation, acute microbial infections, high glucose intake, and exposure to metals and air pollutants (Rajendran *et al.*, 2013).

Dysfunction of the endothelium is characterized by a shift in its functions towards decreased vasodilation, a proinflammatory state and prothrombotic properties. Endothelial dysfunction is associated with most types of cardiovascular disease (CVD), such as hypertension, coronary artery disease, chronic heart failure, peripheral vascular disease, diabetes, chronic kidney failure and severe viral infections (Rajendran *et al.*, 2013). CVD is main cause of death in the world, (World Health Organization, 2017). Endothelial dysfunction also contributes to the pathogenesis of CVD through dysregulation of vascular tone, growth, thrombogenicity and inflammation. There are a number of inflammatory and haemostatic biomarkers of endothelial dysfunction that have been associated with CVD, such as C-reactive protein, IL-6, fibrinogen, fibrin D-dimer, plasminogen activator inhibitor-1, cell adhesion molecules, VWF and the VWFpp (Frankel *et al.*, 2008).

2.3 von Willebrand disease

In 1926, Erik von Willebrand (1870-1949) a Finnish clinician, discovered VWD in families in the Åland Islands (Berntorp, 2007). He did not find the cause of this disease but he managed to differentiate it from haemophilia and other bleeding disorders (Bharati and Prashanth, 2011). One of his patients, a girl called Hjördis, died at the age of 13 from her fourth menstruation, and both her parents suffered from nose bleeds. von Willebrand concluded that this bleeding disorder occurs in both females and males and must be an unknown form of haemophilia. He decided to name it hereditary pseudohaemophilia (Nilsson, 1999). Today, this disorder is known as von Willebrand disease.

VWD is the most common inherited bleeding disorder, with a prevalence of 1 % in the general population. This disease is more common in women, as a result of physiological haemostatic challenges, such as menstruation and pregnancy (Payandeh *et al.*, 2013; Rodeghiero, 2013). Type 1 VWD is the most common form of VWD, with a prevalence of 85 % (Sharma and Flood, 2017). VWD patients can have a mild, moderate or severe bleeding tendency from childhood (Meiring *et al.*, 2009). The symptoms of VWD are due to either a deficiency or defect of VWF, and include nose bleeds, bleeding from small lesions in the skin, mucosa or gastrointestinal tract, menorrhagia, and excessive bleeding from trauma, surgery or childbirth (Lillicrap, 2007). Patients with severe forms of VWD can also bleed into their joints and muscles, as with haemophilia patients (Schneppenheim, 2011).

2.3.1 Classification of von Willebrand disease

VWD is classified into three types, type 1, 2 and 3 (figure 2.8; Sadler and Gralnick, 1994). Type 1 VWD is a partial quantitative deficiency of VWF (Sharma and Flood, 2017). It is either due to decreased synthesis and secretion of VWF, or increased clearance of VWF from plasma (Meiring *et al.*, 2009). Increased clearance of VWF was found in 45 % of the type 1 VWD patients in South Africa (Meiring *et al.*, 2011). Type 1 VWD has an autosomal dominant inheritance and variable penetrance (Haberichter *et al.*, 2006). It is characterized by a decrease of VWF:Ag and the VWF multimer distribution is normal (Bharati and Prashanth, 2011). This is the most

common form of VWD, but it is also the most problematic to diagnose, particularly its milder form. Diagnosis of type 1 VWD can be complicated by factors, such as the ABO blood type (Lillicrap, 2005). The diagnosis of this type of VWD is complicated even more by incomplete penetrance of the disease and the effect of several genetic and environmental factors on the levels of VWF. There is no single test that can be used to diagnose type 1 VWD. Mutation studies on the VWF gene can potentially contribute to the diagnosis, but due to the size and complexity of this gene, complete analysis of the gene has only been done in a small number of patients. A more reliable diagnosis of type 1 VWD is needed, to prevent false positive diagnoses that can lead to stigmatization and unnecessary treatment, and also to prevent false negative diagnosis with the risk of unnecessary complications of bleeding (Goodeve *et al.*, 2007). Type 1 VWD is treated with DDAVP. However, it is not effective in patients with increased VWF clearance since the VWF in plasma of these patients is cleared rapidly from the circulation. It is therefore essential to diagnose individuals with an increased clearance rate of VWF (Meiring *et al.*, 2009). The VWFpp/VWF:Ag ratio is important for the diagnosis of patients with increased VWF clearance. VWFpp levels are only measured in type 1 VWD patients (Meiring *et al.*, 2011).

Type 2 VWD is characterised by a heterogeneous group of qualitative defects of VWF (Favaloro and Mohammed, 2014). It is further subdivided into four groups (type 2A, 2B, 2M and 2N) (Favaloro, 2011). Type 2A is due to mutations that lower the proportion of large functional VWF multimers which result in decreased VWF-dependent platelet adhesion. In type 2B, mutations increase VWF-dependent platelet binding and this leads to the reduction of large functional multimers. Type 2M, is also due to mutations that decrease VWF-dependent adhesion, but do not lower the large multimers. Then type 2N, is caused by mutations that impair the binding of VWF to FVIII and lower FVIII levels are found in these patients (Yawn *et al.*, 2009).

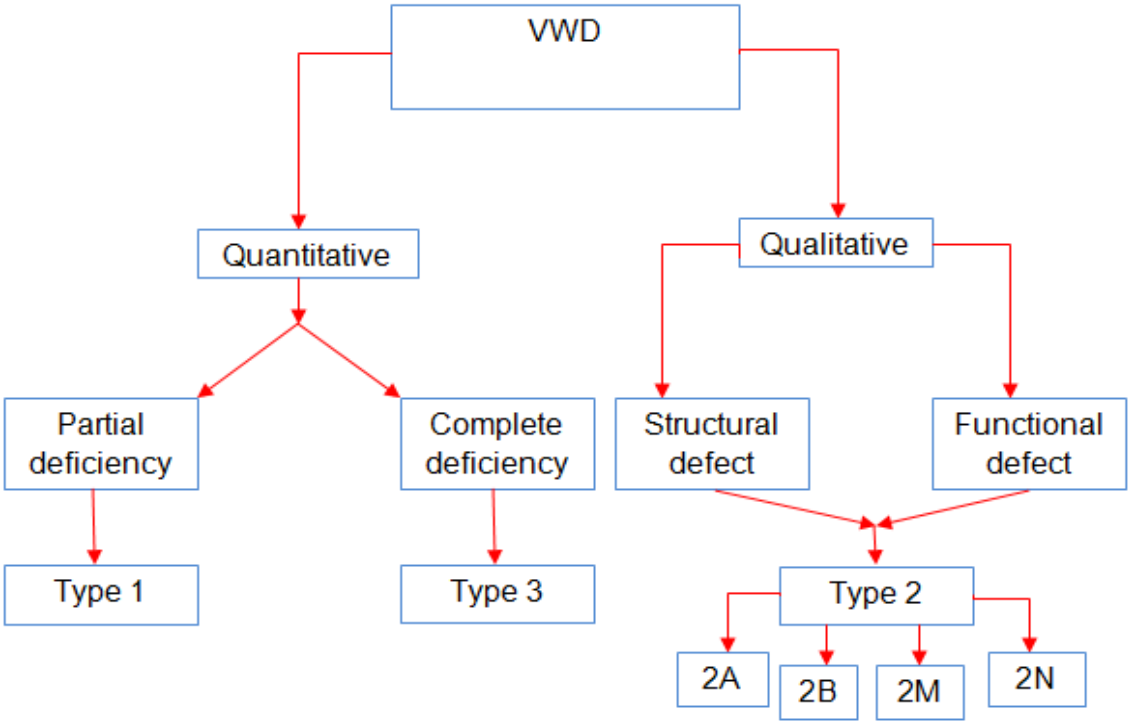

Figure 2.8 Classification of VWD.

Type 1 and 3 VWD are quantitative deficiencies of VWF, and type 2 VWD is a qualitative defect of VWF (Schneppenheim, 2011).

In type 3 VWD, there is complete quantitative deficiency of VWF, and the FVIII levels are very low (Favaloro and Mohammed, 2014; Yawn *et al.*, 2009). Type 3 VWD is rare but it is the most severe form of VWD. It is characterized by severe bleeding, such as bleeding in the soft tissue and joints, which rarely occurs in the other forms of VWD. The very low levels of FVIII in type 3 VWD can result in the development of haemarthroses and haematomas which are also rarely observed in the other types of VWD (Federici, 2009).

Most (about 70 %) of the mutations that occur in type 1 VWD are missense mutations located in the coding sequence of VWF, with 10 % of the mutations being splice site, transcription, small deletions, small duplications and nonsense mutations. Defects have also been identified in the VWF promoter that are associated with type 1 VWD. Type 2A, 2B and 2M VWD all have an autosomal dominant inheritance pattern, whereas type 2N has an autosomal recessive inheritance pattern (Flood, 2014). In type 2A VWD, missense mutations have been identified in the D2, A1 and A2

domains and in the C-terminal domain of VWF. Type 2B is caused by missense mutations in the GPIb binding region of the A1 VWF domain, which is encoded by exon 28 of the VWF gene. In Type 2M VWD missense mutations have been identified in the A1 VWF domain, and type 2N VWD is a result of mutations in the D' to D3 VWF domains (Lillicrap, 2007). Type 3 VWD has an autosomal dominant inheritance pattern. This type of VWD is caused by disruption of the expression of both VWF alleles through point mutations, such as missense or null mutations, or deletions. Some of the deletions can be small and only affect 1 or 2 exons, whereas others affect larger segments of the VWF gene. The most commonly found deletion in type 3 VWD is in exons 4 to 5 (Flood, 2014).

2.3.2 Statistics of von Willebrand disease in South Africa

Patients with bleeding disorders in South Africa are cared for in 20 Haemophilia Treatment Centers (HTCs) which are distributed throughout the country. The HTCs work in collaboration with the South African National Department of Health, the South African Haemophilia Foundation, the Medical and Scientific Council of South Africa and the National Haemophilia Nurses Committee to ensure the best management of individuals with bleeding diatheses, which include VWD (Meiring *et al.*, 2017).

The precise prevalence of VWD in South Africa is unknown. The central plateau of this country has a dry climate. Therefore, epistaxis in the general population is quite common, and there is not a high index of suspicion of bleeding diseases (Meiring *et al.*, 2011). The 2016 Global Survey of the World Federation of Haemophilia predicted 632 patients to be diagnosed with VWD in South Africa; 375 female and 257 male. From these patients, 431 have mild, 61 moderate and 42 severe VWD. The majority of these patients are diagnosed between the ages of 14 and 44 years. Patients with VWD are mostly diagnosed in five academic centres (Meiring *et al.*, 2017).

The classification of the VWD subtypes in South Africa is only done by the VWD testing facility situated in Bloemfontein. The academic complex in Bloemfontein serves patients from the Free State and the Northern Cape provinces. However, the VWD testing facility receives samples from across the whole of South Africa.

2.3.3 Treatment

Correct classification of VWD is important as treatment options are based on the type of VWD (Federici, 2009). Treatment of VWD includes the following; replacement of VWF-containing concentrates or the release of VWF from endogenous storage organelles using DDAVP. Antifibrinolytic agents are also used in patients with mucosal tissue bleeding (Keesler and Flood, 2018; Schneppenheim, 2011,).

VWF/FVIII concentrates are used for individuals who do not respond to DDAVP, patients with major bleedings, short VWF half-life or type 3 VWD patients (Schneppenheim, 2011). Although cryoprecipitate was used in the past, the treatment of choice is viral inactivated VWF/FVIII concentrates (Strong, 2006).

DDAVP is a synthetic derivative of the antidiuretic hormone, and it is used for the treatment of type 1 VWD and in some of the type 2 VWD patients (Yawn *et al.*, 2009). DDAVP causes the release of endogenous VWF from the Weibel-Palade bodies in the endothelial cells. It binds to receptors on the endothelial cells and stimulates the release of VWF. This rapidly increases the levels of VWF with a high proportion of large VWF multimers. However, DDAVP is not effective in individuals with type 3 VWD, since they cannot produce VWF. Similarly, it is not used in patients who have type 2B VWD, because this can aggravate the associated thrombocytopenia in these patients (Schneppenheim, 2011).

In type 1 VWD patients, testing for DDAVP response is an essential step in determining optimal treatment. The response to DDAVP differs amongst type 1 VWD patients, depending on the of VWF mutation (Schneppenheim, 2011). In patients who are responsive, DDAVP should be the preferred choice of treatment when there is a need for an efficient haemostasis for no longer than 2 to 3 days, thus not exceeding 3 to 4 infusions after which tachyphylaxis usually ensues (Rodeghiero, 2013). If a longer duration or shorter intervals for the use of DDAVP are necessary, the patient must be monitored for fluid and electrolyte problems as DDAVP treatment can result in hyponatraemia (Yawn, 2009). This is the most common disorder of decreased electrolytes that appears in 15 to 30 % of acutely or chronically hospitalized patients (Verbalis *et al.*, 2013). Hyponatraemia occurs when the serum sodium concentration

is less than 135 mmol/l. This condition is associated with increased mortality, morbidity and duration of hospital stay in patients with a range of conditions (Spasovski et al., 2014).

Antifibrinolytic agents are useful in many clinical conditions. The two most commonly used antifibrinolytic agents include tranexamic acid (TA) and epsilon-aminocaproic acid (EACA), which are both derivatives of lysine. These drugs bind to the lysine-binding site of plasminogen which results in the inhibition of fibrinolysis. TA is more potent than EACA and has a longer half-life as well. Both these drugs can be taken either orally or intravenously (Villar *et al.*, 2002). Antifibrinolytic agents have a risk of thrombosis (Strong, 2006). They can also cause gastrointestinal complains like nausea, vomiting, dyspepsia or diarrhoea. These symptoms normally disappear with dose reduction. Hypersensitivity such as rush occurs occasionally and anaphylaxis to TA has also been reported (Mikhail and Kouides, 2010).

Menorrhagia is the first sign of VWD in women, although other causes of menorrhagia should be ruled out (Yawn *et al.*, 2009). It is the most common complaint in women of reproductive ages (Hassan *et al.*, 2012). Menorrhagia is seen in about 5 % of women between the ages of 30 and 49. The aetiology of this disorder can be local or systemic, but a specific cause is only noticed in less than 50 % of the affected women. It has been suggested that bleeding disorders like VWD and platelet function disorders are more common in women with menorrhagia. VWD has a prevalence of 1 % in the general population. However, there is a higher prevalence of 10 to 20 % in women with menorrhagia (Payandeh *et al.*, 2013). The treatment of VWD in women with menorrhagia includes combined oral contraceptives, DDAVP, antifibrinolytic agents or VWF concentrates (Yawn *et al.*, 2009).

2.3.4 Diagnosis

Laboratory diagnosis of VWD is challenging. First, samples should be stored immediately after centrifugation in polypropylene tubes at -70 °C until analysis. Cryoprecipitate can form if plasma samples are stored at temperatures that are warmer than -70 °C. Cryoprecipitate contains large numbers of VWF and particularly high molecular weight (HMW) multimers. Therefore, all tests should be performed

using aliquots that were not previously thawed. Plasma samples must be thawed at 37 °C before performing diagnostic tests. Special care should be taken to make sure that no cryoprecipitate forms in samples (Meiring *et al.*, 2011; Meiring *et al.*, 2017). Cold storage of whole blood can result in artificially low levels of VWF, and in such cases patients are then wrongly diagnosed with VWD (Mikhail and Kouides, 2010). It is important to note that the level of VWF changes during surgery, collagen vascular disease, general inflammatory diseases or infection, pregnancy and increased endothelial activation (for example, diffuse intravascular coagulation, liver disease, TTP and haemolytic uraemic syndrome) (Branchford and Di Paola, 2012).

The three main criteria that are needed for the diagnosis of VWD include a positive history of bleeding from childhood, a family history of bleeding with a dominant or recessive inheritance pattern and decreased activity of VWF in plasma (Federici, 2009). There is no single test that can be used for the diagnosis of VWD. The diagnosis of this disorder is based on a combination of a patient's medical history and the results of the many tests that are included in its diagnosis (Schneppenheim, 2011).

Genetic testing for VWD is not indicated except for particular situations where the test results would make a difference in a patient's therapeutic management or counselling. There are a number of complicating factors that can make genetic testing problematic for VWD. Firstly, the VWF gene is very large as it spans 178 kb and has 52 exons. VWF has a highly homologous partial pseudogene in chromosome 22 and this makes the sequencing and its interpretation very difficult. In addition, the VWF gene is also very polymorphic with more than 300 single nucleotide polymorphisms that have been reported (Ng *et al.*, 2015).

Screening tests for bleeding disorders usually include; a platelet count, bleeding time, prothrombin time (PT), activated partial thromboplastin time (APTT), plasma FVIII levels and the blood group of the patient. These tests are normally performed by a routine coagulation laboratory (Meiring *et al.*, 2009). In individuals with type 2B VWD, the platelet count is usually normal but mild thrombocytopenia may appear (Bharati and Prashanth, 2011; Strong, 2006). A platelet count must always be performed when investigating patients with a possible bleeding condition (Laffan *et al.*, 2004).

The platelet function analyzer (PFA-100) and the bleeding time are normally prolonged but can also be normal in mild forms of VWD (Strong, 2006). Its clinical utility is limited, because of insensitivity and lack of reproducibility (Laffan *et al.*, 2004). The PT is normal but the APTT can be prolonged depending on FVIII levels in plasma. In pregnancy, the physiological increase in FVIII levels can make diagnosis of VWD challenging (Strong, 2006). Although the half-life of FVIII is controlled by VWF and is normally reduced in VWD, FVIII coagulant (FVIII:C) levels are not always similar to those of VWF and can be normal in the presence of VWD. However, a normal FVIII:C does not exclude VWD (Laffan *et al.*, 2004). The ABO blood group has an influence on VWF concentrations as previously mentioned and can complicate the diagnosis of type 1 VWD (Lillicrap, 2005; Vischer *et al.*; 1998).

The first line of laboratory tests for the diagnoses of VWD includes, the VWF concentration in plasma (VWF:Ag), VWF ristocetin co-factor (VWF:RCo) assay, VWF collagen binding (VWF:CB) assay and recent VWF-GPIb binding activity assays. Confirmatory tests include, the ristocetin-induced platelet agglutination (RIPA), VWF multimer analysis, FVIII binding assay and the VWFpp assay (Meiring *et al.*, 2009). The VWF:Ag assay is frequently performed using the Enzyme-linked Immunosorbent Assay (ELISA) or using new technologies like the Latex immunoassay. The VWF:Ag can be used to detect all type 3 VWD, most type 1 VWD and only some of the type 2 VWD patients since most of these individuals will have a normal VWF:Ag result (Favaloro, 2001).

The VWF:RCo assay is the most commonly performed VWF activity based test (Favaloro and Mohammed, 2014). However, this assay has low sensitivity and poor reproducibility (Federici, 2009). This assay measures the ability of VWF to bind to GPIb in the presence of ristocetin. The VWF:RCo assay is performed by measuring the agglutination of normal fixed platelets in dilutions of test plasma that contains excess ristocetin. Dimers of ristocetin both bind to VWF and GPIb leading to crosslinking of the platelets. The patient's VWF:RCo is evaluated by using a plasma standard as a reference (Laffan *et al.*, 2004). This assay is performed using formalin-fixed platelets in an aggregometer (Meiring *et al.*, 2009).

The other VWF activity assay, the VWF:CB is used by a small number of laboratories (Favaloro and Mohammed, 2014). However, the VWF:CB assay is very sensitive for the presence of the HMW multimers (Federici, 2009). The assay is based on the ability of HMW VWF multimers that preferably bind to collagen. This is an ELISA based technique where the patient's plasma is added to a collagen-coated ELISA plate. The type of collagen seems to be important, but discordance exists about the type of collagen (type 1, type 3 or a combination of both these collagens) that can be used. The VWF:CB assay has been shown to be sensitive in the identification of type 1, 2A and 2B VWD. However, the VWF:CB assay is normal in type 2M VWD individuals (Meiring *et al.*, 2009). The VWF:RCo and VWF:CB assays measure the two important functions of VWF namely, platelet GPIb and collagen binding respectively. Both these assays also have a similar preference for HMW VWF multimers (Favaloro and Mohammed, 2014).

The RIPA and the VWF multimer analysis are confirmatory tests used to diagnose the type 2 VWD subtypes (Meiring *et al.*, 2009). The RIPA test measures platelet agglutination at different ristocetin concentrations. The sensitivity for ristocetin in RIPA depends on both the level and the activity of VWF. Individuals with type 3 VWD do not show platelet agglutination at any concentration of ristocetin (Favaloro, 2001). RIPA is measured by mixing various ristocetin concentrations that range from 0,2 to 2 mg/ml together with the patient's PRP in an aggregometer. The results of this test are given as the concentration of ristocetin (in mg/ml) that is able to induce 30 % platelet agglutination. RIPA-mixing studies are performed to differentiate between type 2B VWD and platelet type-VWD (PT-VWD). The PT-VWD is identified when RIPA-mixing studies have confirmed a platelet origin (Meiring *et al.*, 2009).

The VWF multimer analysis detects VWF of various molecular weights (the high-, intermediate- and low-molecular weight multimers), and also identifies specific structural abnormalities of VWF. This test is performed by a small number of laboratories due to its complexity and time or cost. It is used to differentiate type 2M VWD from type 2A and type 2B VWD (Favaloro, 2001). The assay is also used to distinguish type 1 VWD from type 2A and 2B VWD (Meiring *et al.*, 2009). The steps performed in the analysis of VWF mutimerization include, electrophoresis of plasma proteins in agarose gel, either fixation of the gel or transfer of the electrophoretic

protein product to a membrane, immunodetection of the protein, and evaluation of the protein in the gel or membrane (Ledford-Kraemer, 2010).

The FVIII binding assay is used to measure the ability of VWF to bind to exogenously added FVIII (Bharati and Prashanth, 2011). It can be used as confirmation for the diagnosis of type 2N VWD in patients with a low FVIII/VWF:Ag ratio and can also be used to rule out mild to moderate forms of haemophilia A (Federici, 2009). This assay is an ELISA (Favaloro, 2001). A microplate is coated with a rabbit polyclonal anti-human VWF antibody. After adding the patient plasma, factor VIII is removed from the plasma using 350 mmol/l Ca_2Cl_2 and recombinant FVIII is added. The bound FVIII is quantified with a peroxidase conjugated sheep polyclonal antihuman FVIII antibody (Meiring *et al.*, 2009).

The VWFpp assay is normally used in the diagnosis of type 1 VWD patients with increased clearance of VWF. The steady state levels of the VWF:Ag and VWFpp are an equilibrium between secretion and clearance of VWF (Davies *et al.*, 2008). The ratio between the VWFpp to VWF:Ag can be used in the assessment of the rate of synthesis, secretion and clearance of VWF (Haberichter, 2015b). This ratio has been used to identify conditions in which there is reduced VWF half-life, such as type 1 VWD with increased clearance (Hubbard *et al.*, 2012).

A proportionate reduction of VWF:RCo and VWF:Ag with a RCo:Ag ratio that is > 0.7 together with a proportionate reduction of VWF:CB and VWF:Ag with a CB:Ag ratio > 0.7 is indicative of type 1 VWD (figure 2.9). If type 1 VWD is suspected, it is important to determine the VWF clearance rate; and in such a case the VWFpp assay is used. If the ratio between the VWFpp and the VWF:Ag is > 2, this means that the patient has an increased clearance rate of VWF. The VWFpp assay is also used in the assessment of acute or chronic endothelial activation (Haberichter, 2015a).

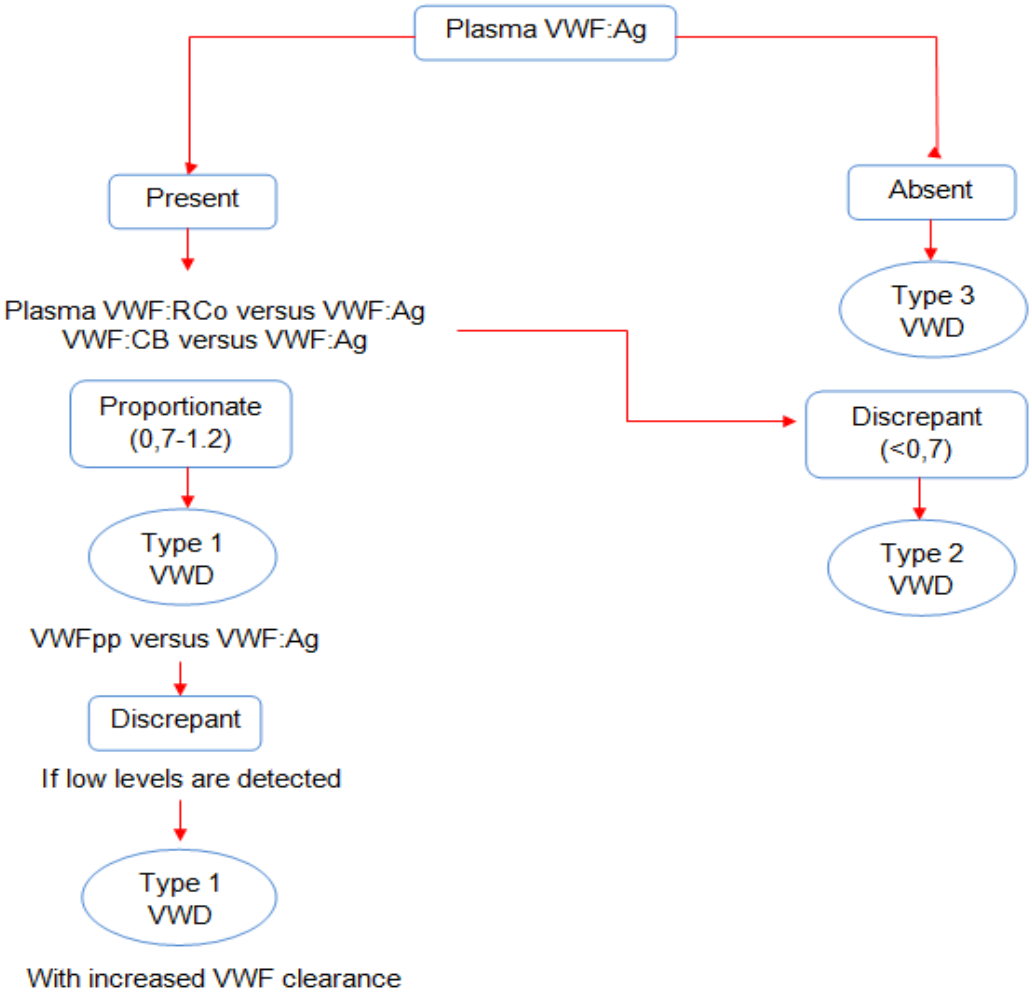

Figure 2.9 Algorithm for diagnosis of VWD.

The VWFpp assay is used in the algorithm for the diagnosis of VWD. A VWFpp/VWF:Ag ratio of > 2 indicates increased VWF clearance (Meiring et al., 2009).

Currently, there is only one commercial assay (CLB-Pro 35 and CLB-Pro 14.3 mAb, Cellsciences) to measure VWFpp levels. This assay is not only too expensive to be used in developing countries but is also very time consuming. The VWFpp assay is expensive as it uses monoclonal antibodies. Mammalian cells are commonly used for the expression of monoclonal antibodies because of their capability to perform post-translational modifications (Spadiut *et al.*, 2014). However, mammalian cells have disadvantages, such as slow growth rate and low expression yield. These factors make mammalian cell cultivation expensive and the cost of manufacturing is further increased by expensive media and the difficult culturing techniques (Cha *et al.*,

2005). Furthermore, the VWFpp is not commercially available. The VWFpp is too large for easy expression as it has a molecular weight of 95 kDa (Sadler, 1991) In addition, the VWFpp is composed of 2 homologous cysteine rich D domains as previously mentioned (Rosenberg *et al.*, 2002). Intra-chain disulfide bonds form between cysteines in a protein (Shewry and Tatham, 1997). As a result, it is very challenging to express this protein. Therefore, more research must be done on finding the optimal expression method that will enable the expression of such a protein. Once this is achieved, it will also be possible to produce appropriate antibodies to the VWFpp.

2.4 Phage display technology

In 1985, George Smith developed phage display technology where he inserted foreign fragments of deoxyribonucleic acid into filamentous phage gene III that encodes for the phage coat protein III. Thereby, he created a fusion protein that contains a foreign sequence (Burton, 1995). Phage display is currently used to identify peptides, proteins or antibodies with a high affinity for a particular target. With many rounds of affinity selection or biopanning, a phage library is enriched with high affinity binders (‘t Hoen *et al.*, 2012). The applications for this technique include production of potent and novel antibodies, *in vitro* improvement of protein affinity and function, epitope discovery, development for vaccine research and the identification of interacting proteins (Burton, 1995).

The linkage of genotype to phenotype is the essential aspect of phage display technology. In this technique, the starting point is normally an antibody library that comprises a population of about 10^9 to 10^{11} clones. After two or a maximum of three rounds of selection, the population is increased for a high percentage of antibody fragments that are specific for the target antigen (Carmen and Jermutus, 2002).

One of the main advantages of phage display is the production of single chain variable fragments (ScFvs) that bind to a specific antigen, which can be performed within a couple of weeks. ScFvs have been successfully produced using phage display libraries. The expression of ScFvs has a less toxic effect on the *Escherichia coli (E. coli)* cell compared to larger antibody fragments. This leads to a higher yield

and variation in single chain variable fragment (ScFv) libraries (Carmen and Jermutus, 2002).

Figure 2.10 illustrates the process of phage display.

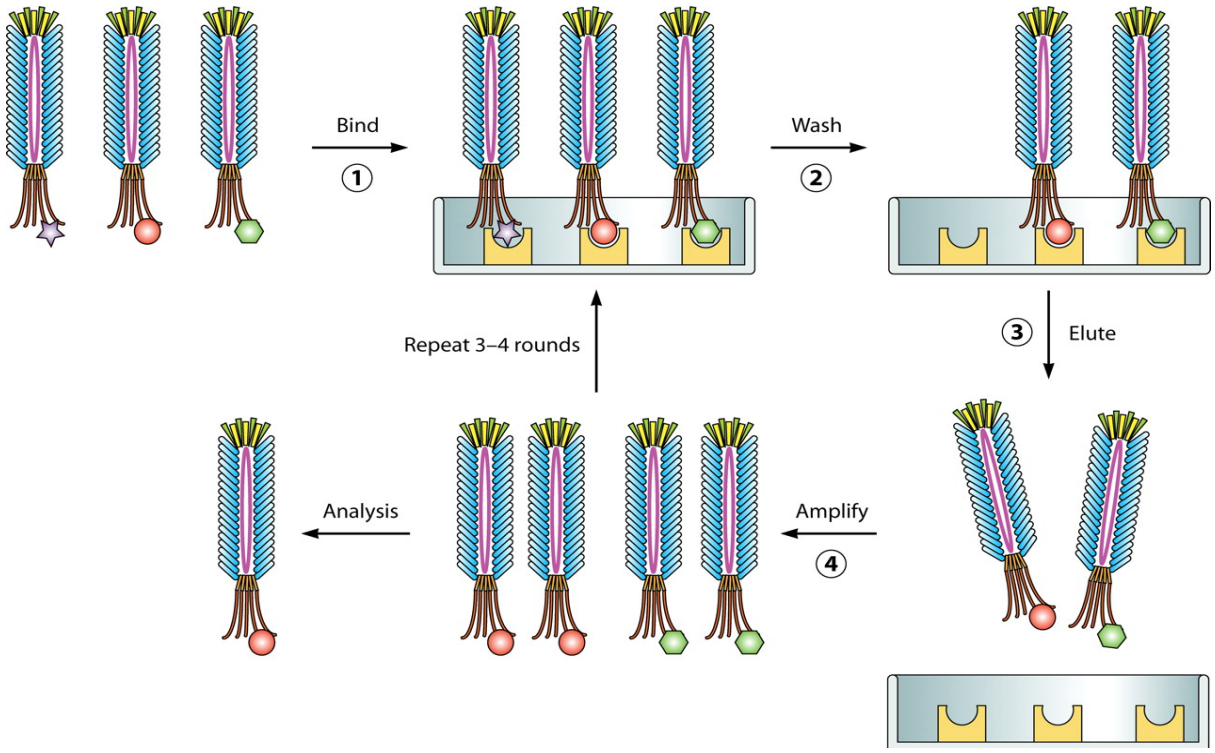

Figure 2.10 Phage display technology.

1. An antibody library is added to the target protein. 2. The unbound phages are washed off. 3. The specific binding phages are eluted. 4. The phages are amplified by infection of E. coli. Some of these phages are then used in the next round of selection and others are analysed for binding (http://aac.asm.org/content/56/9/4569/F3.expansion.html).

2.5 Yeast display

Yeast display is mostly used for the generation of eukaryotic recombinant proteins. Yeast, as an expression host offers many advantages of which the most important is increased level of protein production (Kumari *et al.*, 2015). Yeast expression combines the ease of manipulation and growth of unicellular organisms with eukaryotic-post translational processing and modifications (Nicaud *et al.*, 2002). *Saccharomyces cerevisiae* (*S. cerevisiae*) was the first yeast used for heterologous

protein production. Many proteins have since been successfully cloned and expressed using this yeast. However, *S. cerevisiae* is prone to hyperglycosylation, which alters the protein structure and causes the protein to be more allergenic (Kumari *et al.*, 2015). The *S. cerevisiae*-Aga2p system is the most widely used system. In this display system, target proteins are expressed as cell surface fusions to the Aga2p subunit of the protein a-agglutinin in *S. cerevisiae* (Gera *et al.*, 2013).

Amongst the yeast host systems that are used for the generation of heterologous proteins, the *Yarrowia lipolytica* (*Y. lipotytica*) is the most attractive microorganism since it has been used in several industrial processes. In addition, the entire genome of the yeast is available (Yue *et al.*, 2008). The *Y. lipotytica* genome can be analysed efficiently using bioinformatical software which makes it possible to identify and subsequently clone required genes (Yuzbasheva *et al.*, 2012). Bragg *et al.*, (2017) patented a *Y. lipolytica* expression system that is used for the secretion of expressed polypeptides into either the extracellular space or onto the surface of this host cell wall. These polypeptides can be used for human and animal health applications.

2.6 *Escherichia coli* expression

E. coli is the most commonly utilised expression host for the production of recombinant proteins (Robichon *et al.*, 2011). *E. coli* is simple to manipulate, inexpensive to culture and can readily produce recombinant proteins (Khow and Suntrarachun, 2012). However, when proteins are expressed in large numbers and contain too many disulfide bonds, they might precipitate and form inclusion bodies (Khow and Suntrarachun, 2012). These insoluble aggregates are generally misfolded and are biologically inactive (Villaverde and Carrio, 2003). Therefore, extra *in vitro* refolding is needed to obtain biologically functional proteins. However, *in vitro* refolding of proteins in inclusion bodies is unpredictable and is challenging, and it is time consuming and this requires a large number of reagents. As a result, the expression of a fully functional target protein, without the need for additional *in vitro* refolding is preferred. *E. coli* strains that do not contain thioredoxin reductase, glutathione reductase and/ or glutathione biosynthesis have been successfully used to improve the soluble expression of proteins that carry disulfide bonds. Another commonly adapted solution is to co-express chaperones or oxidase to promote the

formation of disulfide bonds in order to stabilize the recombinant proteins in the cytoplasm and also to improve protein expression (Kong and Guo, 2014).

2.7 Monoclonal and Polyclonal antibody production

When producing antibodies there are many factors that one should consider, such as whether the antibody of interest should be monoclonal or polyclonal depending on the use of it.

2.7.1 Antibodies

Antibodies that are secreted by a single clone of B lymphocytes are referred to as monoclonal antibodies. When they are produced by a mixture of different B lymphocyte clones they are known as polyclonal antibodies (Leenaars and Hendriksen, 2005). A schematic diagram of (A) monoclonal antibodies and (B) polyclonal antibodies binding to epitopes on an antigen is shown in figure 2.11.

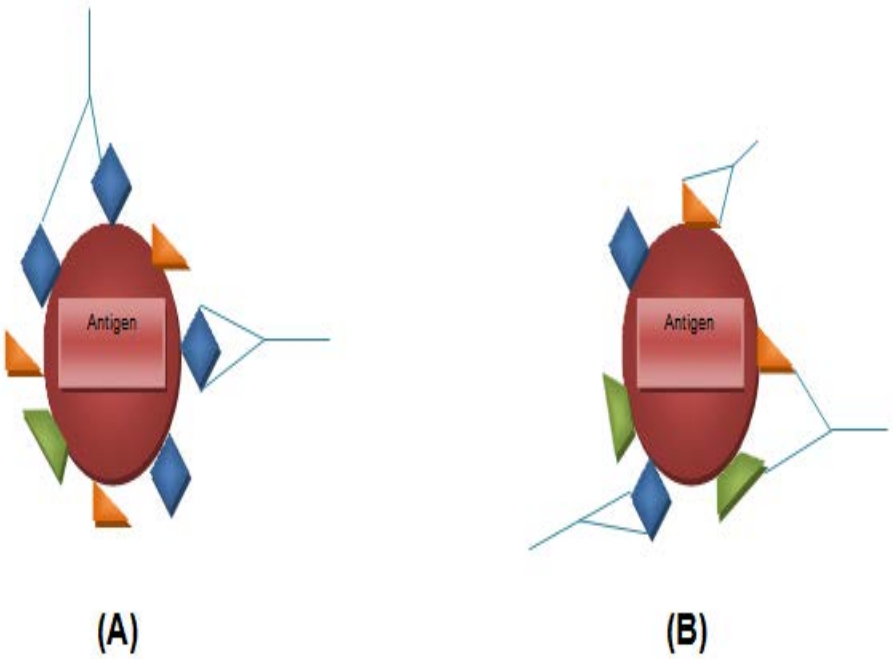

Figure 2.11 Monoclonal and polyclonal antibodies.

(A) Monoclonal antibodies bind to a specific epitope on an antigen, and (B) polyclonal antibodies bind to different epitopes on an antigen.

The decision whether to use polyclonal or monoclonal antibodies depends on the time and cost of production of the antibody. Numerous research questions can be answered using polyclonal antibodies (Leenaars and Hendriksen, 2005). Polyclonal antibodies can be produced more quickly, at low cost and with less technical skill when compared monoclonal antibodies. Polyclonal antibodies can be generated within several months after starting immunizations, whereas the production of hybridomas and the subsequent production of monoclonal antibodies may take up to a year or longer in some situations, as this requires more expenses and time. In addition, polyclonal antibodies are more stable over a wide pH- and salinity range, while monoclonal antibodies may be highly susceptible to minor alterations in both (Lipman *et al.*, 2005).

Polyclonal antibody production includes the preparation of the antigen, selection of the animal for inoculation, selection and preparation of the adjuvant, injection protocol, post injection observation, and collection of the antibodies. The specificity of the immune response that will be obtained relies on the purity of the antigen that is used. The toxicity of the antigen preparation must also be considered. These factors are important as they may have a negative effect on the well-being of the animal and also on the immunological results (Leenaars and Hendriksen, 2005). When choosing an animal for producing polyclonal antibodies, it is essential to consider the amount of antibody or antiserum that is required. Another important factor to consider is the phylogenetic relationship between the recipient and the donor of the antigen. For generating polyclonal antiserum to an antigen, a donor/recipient pair whose members are not closely related on phylogenetic basis is usually chosen (Hanly *et al.*, 1995). The procedure for polyclonal antibody production is shown in figure 2.12.

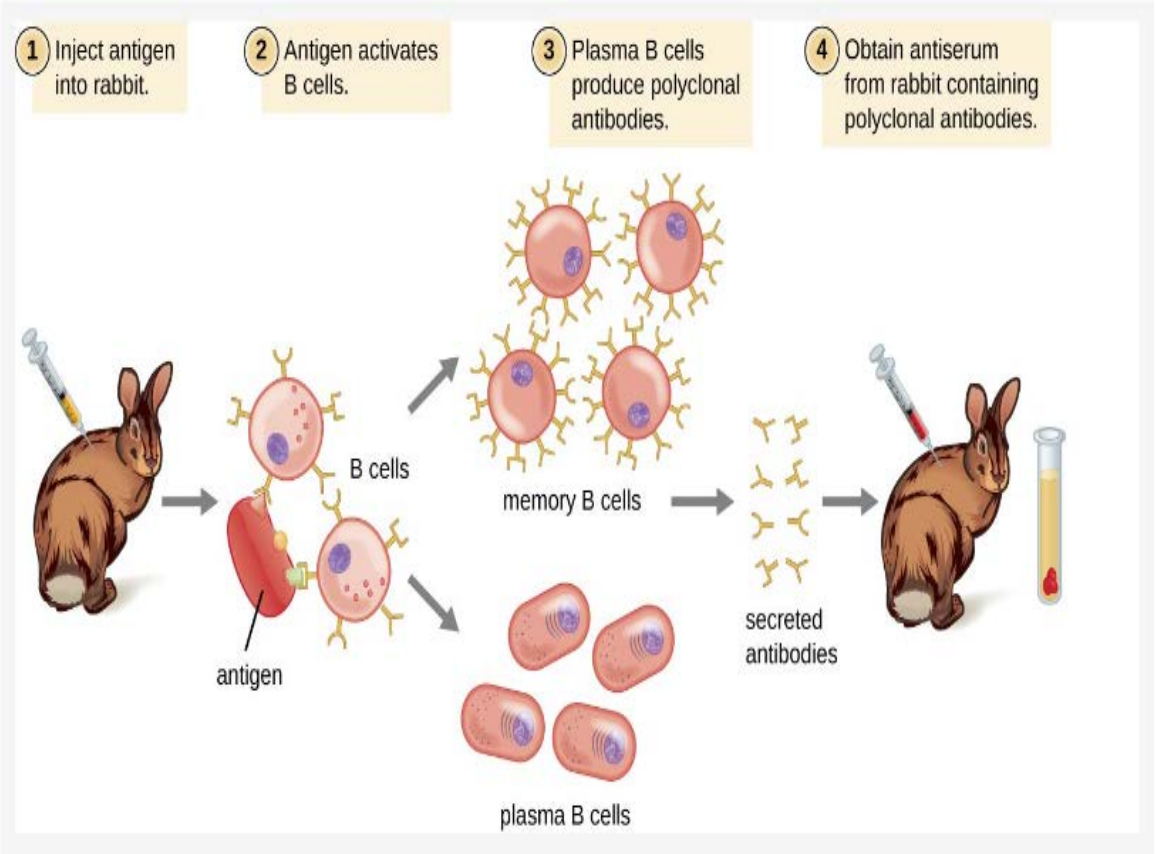

Figure 2.12 Polyclonal antibody production.

A rabbit is injected with an antigen against the polyclonal antibodies of interest. This leads to activation of B cells, which produces the antibodies of interest. Antiserum containing the polyclonal antibodies is then obtained from the rabbit. These antibodies can then be purified and tested in various applications (https://courses.lumenlearning.com/microbiology/chapter/polyclonal-and-monoclonal-antibody-production/).

The main advantages of monoclonal antibodies are their homogeneity and consistency. However, the monospecificity of monoclonal antibodies can also have a limitation to their use. Minor changes in the structure of an epitope (for example due to genetic polymorphism, glycosylation and denaturation) may greatly have an effect on the function of the monoclonal antibody. For this reason, monoclonal antibodies should be produced based on the antigen to which it will bind. Another important advantage of monoclonal antibodies is that once the antibody of interest has been produced, the monoclonal antibodies can be produced as a constant and renewable resource (Lipman *et al.*, 2005).

The procedure of monoclonal antibody development includes the production of antigen specific B cells, fusion of these cells with myeloma cells, cloning and selection of the specific hybridoma clone using limiting dilution, and up-scaling the monoclonal antibody production. For immunization, the BALB/c mice are normally used since many of the myeloma cells available for fusion have a BALB/c origin (Leenaar and Hendriksen, 2005). Figure 2.13 shows a basic illustration of monoclonal antibody production.

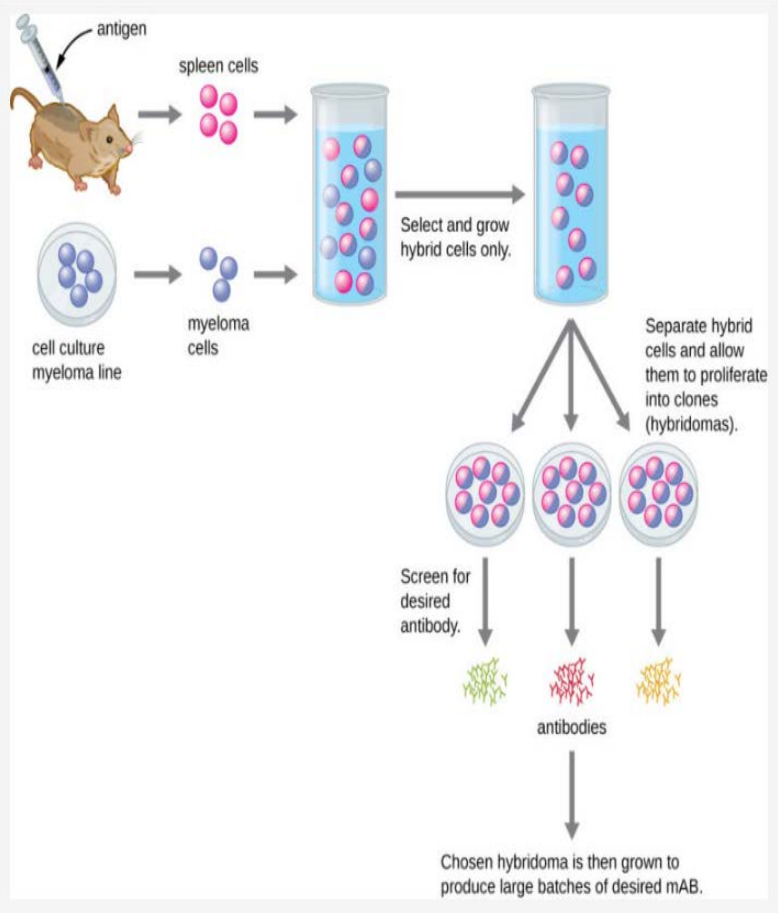

Figure 2.13 Monoclonal antibody production.

A mouse is injected with the antigen against the monoclonal antibodies of interest. Spleen cells are then removed from the mouse and are fused with myeloma cells, to form a hybrid. Only the hybrid cells are selected and grown. The hybrid cells are then separated so that they proliferate into hybridomas. The hybridomas are screened for the target antibody. Finally, the chosen hybridoma is grown to produce many batches of the desired antibody (https://courses.lumenlearning.com/microbiology/chapter/polyclonal-and-monoclonal-antibody-production/).

2.8 Protein purification

2.8.1 Protein purification systems

The most widely used methods for preparative protein purification all involve chromatography. It separates proteins based on differences between the properties of the target protein that will be purified and the properties of other substances in the sample. Chromatographic methods includes affinity chromatography, immobilized metal ion affinity chromatography (IMAC), ion exchange chromatography, size exclusion chromatography, hydrophobic interaction chromatography and chromatofocusing (GE Healthcare handbook, 2015).

IMAC purification involves the interaction between proteins with 6x histidine-tags with divalent metal ions (for example, Ni^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}) that are immobilized on the column matrix. The principle of the IMAC is that, the histidine-tagged proteins will bind to the column matrix via interaction with the divalent metal ions, while the untagged proteins will pass through the column. This allows for the isolation of the target protein from the crude extract. The histidine-tagged proteins will bind to the column while displacing the imidazole counter ligands. The column is then washed with the binding buffer to remove unbound proteins, which is followed by elution of the target protein using an increasing imidazole gradient (20mM to 300mM) (GE Healthcare handbook, 2010).

2.8.2 Protein Characterization

Once protein purification has been performed, samples must be resolved using sodium dodecyl sulphate- polyacrylamide gel electrophoresis. When stained using a dye such as Coomassie brilliant blue, the intensity of the bands are proportional to the amount of protein. This allows for estimation of purity of the sample and also whether the purified protein is of expected size. The Bradford assay is the simplest method for quantifying the purified protein. This method spectroscopically measures the binding of Coomassie brilliant blue to the protein (National Institutes of health, 2008).

Once the protein has been isolated, it should be stored correctly to ensure accurate and reliable analytical results during future testing. It is recommended to evaluate the stability of the protein to concentration and freeze-thaw cycles before processing the whole protein batch. The frozen and thawed protein should be compared with a protein that was not frozen for biochemical activity, noticeable precipitation or alterations in physical properties. A relatively small number of proteins are irreversibly inactivated by a single freeze-thaw cycle. In such rare situations, the protein may be stored at 4 °C for short periods of time, at -20 °C in high concentrations of glycerol, or as ammonium sulphate suspension (National Institutes of health, 2008). For long term storage, single-use aliquots of the protein in liquid-nitrogen may be made. The common form of frozen protein storage is at -20 °C or -80 °C. Repeated freeze-thaw cycles should be avoided at these storage temperature conditions, as they decrease the stability of the protein. Many compounds can be added to the protein to increase its shelf-life. These include cryoprotectants, such as gylcerol or ethylene gylcol to help stabilize the protein by preventing ice crystal formation at -20 °C. Protease inhibitors to prevent proteolytic cleavage of the protein can also be added to the protein sample. Anti-microbial agents, such as sodium azide or thimerosal may also be required. Metal chelators, such as EDTA to prevent metal-induced oxidation of -SH groups to help maintain the protein in a reduced state. Reducing agents, such as dithiothreitol and 2-mercaptoethanol also help maintaining the protein in a reduced state by preventing the oxidation of cysteines (Pierce, 2003).

2.9 Enzyme-linked immunosorbent assay

The ELISA detects and quantifies peptides, proteins, antibodies and hormones. First, an antigen is immobilized to a solid surface. The antigen then binds to an antibody that is linked to a detection enzyme that cleaves a substrate. Detection is accomplished by incubation of the enzyme complex with a substrate that generates a detectable product. The most essential component of the detection strategy is a highly specific antibody-antigen interaction (Thermo Scientific handbook, 2011).

Two types of ELISA exist. They are the direct ELISA and the indirect ELISA (figure 2.14).

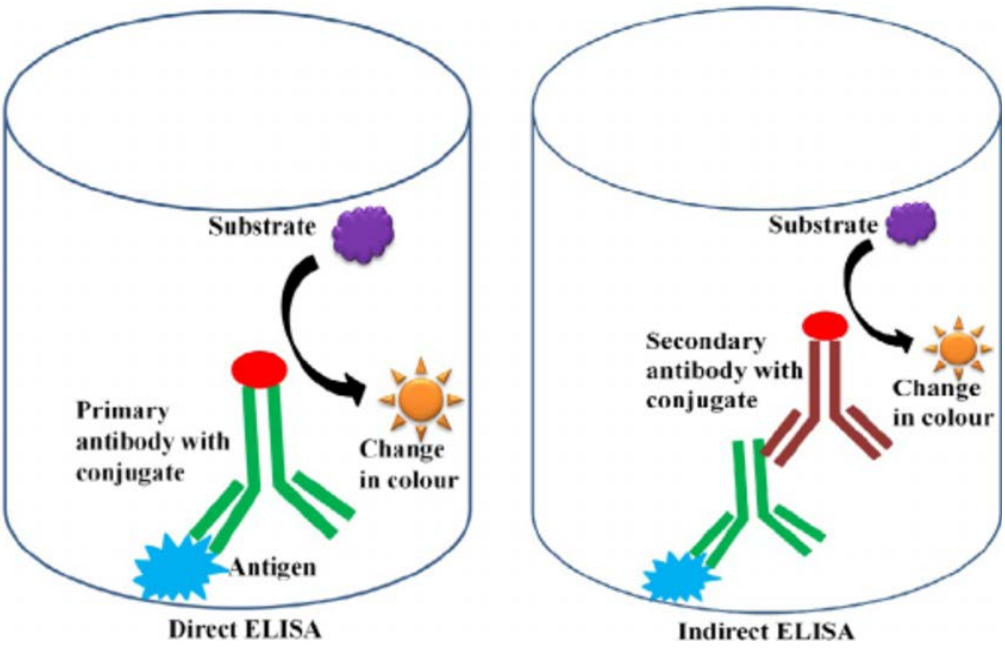

Figure 2.14 Direct and indirect ELISA methods.

In both the methods, between each step there is washing with a buffer such as PBS/0.1% Tween-20 to remove unbound proteins (http://canacopegdl.com/synonym/ELISA-test.html).

The direct ELISA technique detects an antigen coated to a multi-well plate using an antibody that is directly conjugated to a detection enzyme that cleaves a colorimetric substrate. This technique is a good choice when there are no commercially available ELISA kits for a target protein. It is fast since only one antibody is used, and the cross-reactivity of the secondary antibody is eliminated. The disadvantages include, labelling primary antibodies for each specific ELISA system, which is time-consuming and is also expensive. Furthermore, there is no flexibility in the choice of primary antibody labelling from one experiment to another, and minimal signal amplification occurs (Boster Biological Technology handbook).

The competitive and sandwich ELISA are two forms of the indirect ELISA. The fundamental part of the competitive ELISA is the competitive reaction between the sample antigen and the antigen that is bound to the wells of a microtiter plate. In the competitive ELISA, a primary antibody is incubated with the sample antigen, and the antibody-antigen complexes are then added to wells that have been already coated with the same antigen. After an incubation period, any antibody that is unbound

becomes washed off. The more antigen in the sample, the more primary antibody will be bound to the sample antigen. Therefore, there will be a small amount of the primary antibody that is available to bind to the antigen that is coated on the wells and this leads to a signal reduction (Boster Biological Technology handbook).

The sandwich ELISA quantifies the amount of antigen between two layers of antibodies, namely the capture and detection antibody. The antigen that is to be quantified should contain at least two antigenic sites that are capable of binding to an antibody, as at least two antibodies are used in this method. Either monoclonal or polyclonal antibodies may be used as the capture and detection antibodies in the sandwich ELISA (Abcam). An essential consideration in designing the sandwich ELISA technique is that the capture and detection antibodies should recognise two non-overlapping epitopes. The capture and detection antibodies that do not interfere with each other and bind simultaneously are taken as a matched pair and they are suitable for developing a sandwich ELISA (Thermo Scientific handbook, 2011). The advantage of the sandwich ELISA method is that the sample does not have to be purified before it is analysed and the assay is very sensitive (Abcam).

2.10 Lateral flow assays

Lateral flow assays (LFAs) are low cost detection devices that are simple to use, rapid and portable. This assay uses a paper-based platform, where a sample is placed on a test device and results are obtained within 5 to 30 minutes. The low developmental cost and simplicity of producing LFAs have led to the expansion of its application to many fields in which rapid assays are needed. LFAs are commonly used in hospitals, physicians' offices and clinical laboratories for the qualitative and quantitative detection of particular antigens and antibodies, as well as gene amplification products. Many different kinds of biological samples can be analysed using LFAs. These include urine, saliva, sweat, serum, plasma, whole blood, and other fluids. LFAs have a long shelf life and do not need to be refrigerated. These assays are therefore suitable for use in developing countries, small ambulatory care settings, remote areas and battlefields (Koczula and Gallotta, 2016). The advantages and limitations of LFAs are summarised in table 2.1.

Table 2.1 The advantages and limitations of LFAs.

Advantages:	Disadvantages:
One-step assay, no washing steps are required	One-step assay, washing steps cannot be included
Rapid assay	Qualitative or semi-quantitative results
Cost-effective	Imprecise sample volume decreases assay precision
Small sample volume required	In one- step assay it is not possible to increase the response by enzymatic reaction
Easy test procedure	Good antibody preparation or hybridisation nucleic acid sequence is required
Applications at point of care	Pretreatment of sample is required if sample is not a fluid
Sensitive for analytes, such as proteins, haptens and nucleic acid amplicons	
Longer shelf half-life without the requirement for refrigerating and larger batches can be prepare in time	
Qualitative or semi-quantitative results	
Pretreatment of sample is normally not required when sample is a fluid	

(Posthuma-Trumpie et al., 2009).

The lateral flow assay (LFA) is performed on a test strip that consists of different parts that are held together by a plastic backing (figure 2.15). The parts include a sample application pad, a conjugate pad, a nitrocellulose membrane and an adsorption pad. The strip also contains pre-immobilized reagents that become active upon contact with a liquid sample (Bahadir and Sezgintürk, 2016; Sajid *et al.*, 2015).

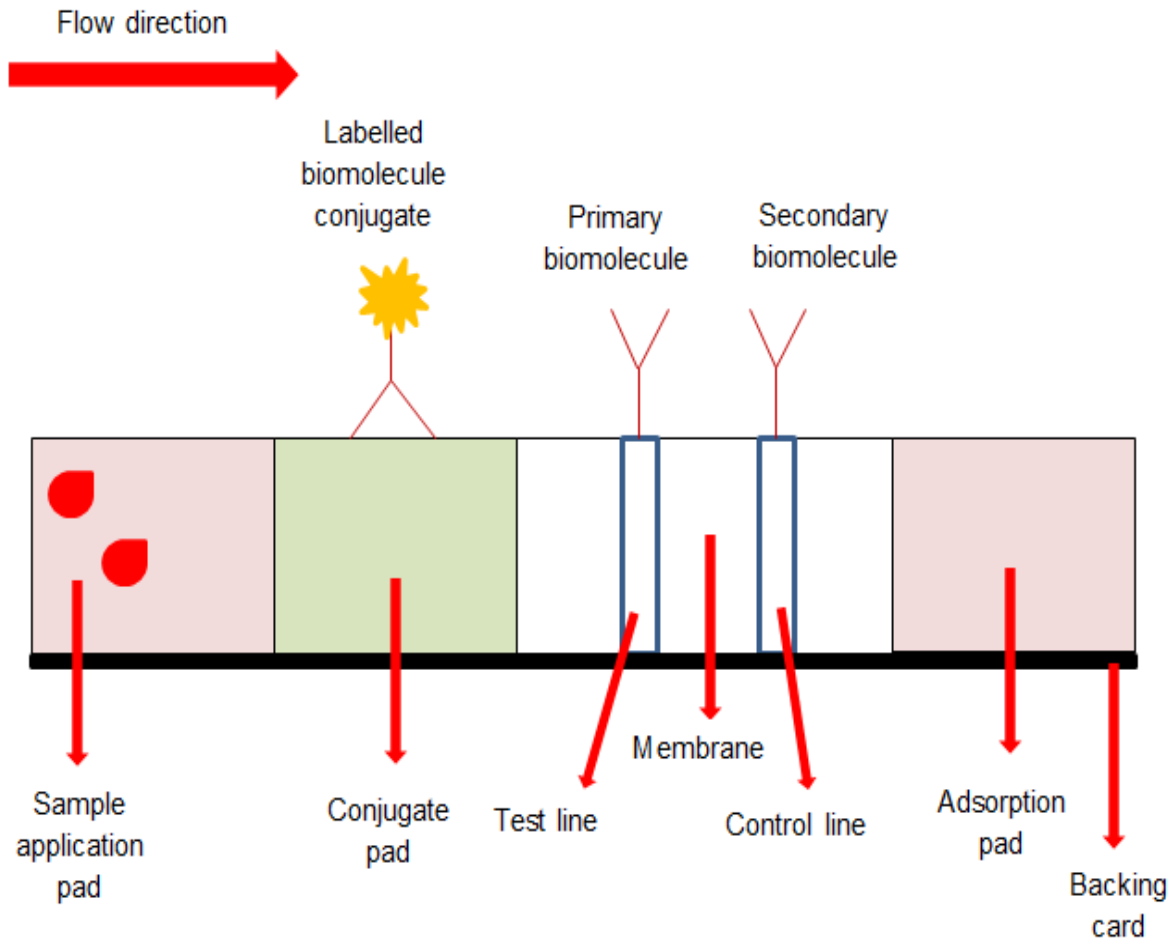

Figure 2.15 Basic design of a LFA.

In the LFA, a sample is applied to the sample application pad. The sample moves by capillary action through the strip. The sample then moves through to the conjugate pad which contains antibodies that are specific to the analyte. The sample, along with the conjugated antibody bound to the analyte then migrates to the detection area. The detection of the analyte results in an appropriate response on the test line, and a response on the control line shows proper liquid flow through the strip (Koczula and Gallotta, 2016; Sajid et al., 2015).

The sample application pad is composed of cellulose and/ or glass fibre, and the sample is applied to this pad to start the test. The function of the sample application pad is to transport the sample to other parts of the test strip device. Sample pads are designed to pretreat the sample before it is transported to the other parts of the strip. The pretreatment can include the separation of components, the removal of interferences and pH adjustment (Sajid *et al.*, 2015).

The conjugate pad contains antibodies specific to the analyte, and are conjugated to coloured or fluorescent particles, usually colloidal gold or latex microspheres, quantum dots, carbon nanotubes or colloidal carbon (Bahadir and Sezgintürk, 2016; Koczula and Gallotta, 2016) .The material of the conjugate pad immediately releases the labelled conjugate when in contact with a sample. A poorly labelled conjugate can negatively affect the sensitivity of the test. The conjugate pad is made of glass fibre, cellulose or polyesters. The nature of the conjugate material has an effect on the release of the labelled conjugate and also on the sensitivity of the test (Sajid *et al.*,2015).

The test strip consists of a membrane that is made of nitrocellulose, nylon, polyethersulfone, polyethylene or fused silica (Posthuma-Trumpie *et al.*, 2009). The membrane is highly important in determining the sensitivity of the strip test. An ideal membrane should provide support binding to the antibodies. Non-specific adsorption on the test and control lines can affect the results enormously (Sajid *et al.*, 2015).

The adsorption pad controls the flow rate of the liquid through the membrane and prevents back flow of the sample (Bahadir and Sezgintürk, 2016). It also allows the use of bigger sample volumes, which leads to increased test sensitivity. Commonly used adsorption pads are made of cellulose filters (Koczula and Gallotta, 2016).

There are two formats of LFAs, namely the sandwich and competitive LFAs. The sandwich format is usually used for detecting large analytes that have at least two binding sites. In this assay, the antibody that binds to one binding site of the analyte is conjugated to a nanoparticle and the another antibody that binds to the second binding site is used for the test line (Bahadir and Sezgintürk, 2016; NanoComposix handbook, 2016). In the sandwich format, the intensity of colour at the test line correlates to the amount of the analyte of interest (Sajid *et al.*, 2015).

The competitive format is used for the detection of small analytes that are not able to bind to two antibodies simultaneously (NanoComposix handbook, 2016). In this assay, the absence of colour at the test line indicates the presence of the analyte, while the appearance of colour at both the test and control line indicates the absences of the analyte (Sajid *et al.*, 2015).

2.11 Assay validation

Assay validation evaluates the fitness of a method and provides evidence that a method is suitable for its intended purpose (OIE Terrestrial Manual, 2013; Trombetta *et al.*, 2015). A full validation is essential for the development and implementation of a novel bio-analytical method (Food and Drug Administration, 2013). It includes inter-assay precision, intra-assay precision and comparison studies.

Precision of an analytical process reflects the closeness of agreement between a series of measurements that are obtained from multiple sampling of the same homogeneous sample under prescribed conditions (Lavanya *et al.*, 2013). Inter-assay precision reflects within laboratory variations (for example different days, different analysts, different equipment) (European Medicines Agency, 2006). Intra-assay precision is the smallest measure of precision, and it involves measurements that are carried out under the same conditions (for example same operator, reagent lots, instrument, laboratory, time) (Burd, 2010).

Accurate measurement of clinical values (for example, measuring of VWFpp levels) is important, thus inaccurate measurement of these values results in inappropriate management of a patient. New instruments and tests are constantly developed, with the goal of providing cost-effective, non-invasive, more convenient and safe methods. When a new method of measurement or instrument is invented, the quality of the instrument must be evaluated. A method comparison or validation study is then needed (Rafdzah *et al.*, 2013). Method comparison studies assess the relative agreement between two analytical methods that measure the same analyte, mainly to assess the performance of a newly developed method. The current method of analysis is called the reference method, and the new method is called the test (Magari, 2002).

The inter-laboratory variability of the VWFpp and VWF:Ag assays have been estimated in literature and an assigned value for the VWFpp in plasma was provided to the WHO 6th International Standard (IS) for FVIII/VWF in plasma (Hubbard *et al.*, 2012). The importance of establishing laboratory quality standards ensures the accuracy of test results, increases the confidence of patients, clinicians and

communities in the value of laboratory testing, and informs patient management (World Health Organization, 2011).

Chapter 3: Aim

The aim of this study was to validate a VWFpp ELISA assay.

Objectives:

- Yeast expression of two single chain variable fragments to the VWFpp that were previously selected by phage display technology. The two single chain variable fragments were to be used to validate the VWFpp assay.
- *E. coli* expression of a truncated VWFpp and production of two polyclonal antibodies to it. The truncated VWFpp was expressed in order to produce polyclonal antibodies to epitopes on it. The two polyclonal antibodies were to be used instead of the two yeast display single chain variable fragments, to validate the VWFpp ELISA.
- Development of a lateral flow assay for the measurement of VWFpp in plasma.
- Development and validation of a rapid ELISA to measure the VWFpp level in plasma, using the commercial antibody pair CLB-Pro 35 and CLB-Pro 14.3.

With this study we aim to validate a rapid assay that can be used for the diagnosis of type 1 VWD with an increased clearance. Furthermore, the patients will be diagnosed on time to receive the correct treatment.

Chapter 4: Methodology

4.1 Ethical considerations

Ethical approval for this study was obtained from the Health Sciences Research Ethics Committee of the University of the Free State (HSREC 124/2016).

4.2 Study design

This was an experimental study, performed at the Department of Haematology and Cell Biology, University of the Free State. The VWFpp protein and two single chain variable fragments (JA9 and JG7) (from our previous study by Setlai, 2014) were expressed on yeast, and this part of the study was performed by the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State. The purification of these two ScFvs and the VWFpp was performed at the Department of Haematology and Cell Biology, University of the Free State. A truncated form of the VWFpp (tVWFpp) was also expressed in *E. coli* and polyclonal antibodies were produced against the tVWFpp in rabbits by BiologicsCorp, Indianapolis, USA. Commercial antibodies to the VWFpp were used to develop a lateral flow assay and the commercial antibody pair clones (CLB-Pro 35 and CLB-Pro 14.3 mAb) from Cellsciences, Newburyport, MA, USA were used to develop and validate a rapid ELISA that measures VWFpp levels in plasma.

4.3 Study samples

Plasma from 20 type 1 VWD patients was used in this study. The WHO 6th IS for FVIII/VWF in plasma was also used. It is lyophilised plasma with known concentrations of the VWF:Ag (100 %) and VWFpp (103 %). Normal pooled plasma was prepared according to the Clinical and laboratory Standards Institute guideline (2003). Whole blood was collected from normal healthy individuals in sodium citrate tubes. The blood was checked for clots by gentle inversion and observation. Normal pooled plasma was obtained by centrifuging the blood at 1500 g for 15 minutes at room temperature and the plasma was pooled.

4.4 Study procedure

In a previous study (Setlai, 2014) by our laboratory, a cost-effective ELISA was developed that measures VWFpp levels. Recombinant VWFpp was first displayed on the surface of yeast cells. ScFvs that bound to the displayed VWFpp were screened from phage-display libraries. Two ScFvs (JA9 and JG7) were selected that bound specifically to the displayed VWFpp and not to the yeast cells alone (unpublished data).

The first aim of this study was to express these ScFvs in a yeast expression system (*Yarrowia lipolytica*). Recombinant VWFpp is not commercially available, therefore the VWFpp was first expressed in yeast before expressing the ScFvs.

4.4.1 Expression of the VWFpp, the ScFv JA9 and ScFv JG7 by yeast

4.4.1.1 Materials

Bacterial and yeast media components were obtained from Sigma-Aldrich, USA and Merck, Darmstadt, Germany. DNA modification enzymes were obtained from Thermo Fisher Scientific, USA and New England Biolabs, Massachusetts, USA. Sig 10 5α chemically competent *E. coli cells* were purchased from Sigma-Aldrich, USA, for use in plasmid modification and propagation. The *Y. lipolytica* strain P01h was obtained from the University of the Free State yeast culture collection (Bragg *et al.*, 2017). The parent *Y. lipolytica* expression vector pINA1317 for secretion of recombinant proteins (Nicaud *et al.*, 2002) was obtained from Dr Catherine Madzak (INRA, France). A modified version of this vector, containing the LIP2t terminator replacing the XPR2t terminator, and with the signal sequence removed, was also used for accumulation of recombinant proteins intracellularly. The nucleotide sequences encoding the VWFpp were synthesised by GeneArt, Thermo Fisher Scientific, Germany and supplied in a standard pKM-RQ plasmid. Figure 4.1 shows the protein sequence of the VWFpp.
The nucleotide sequences encoding the two ScFvs, JA9 and JG7 were synthesised by GenScript, USA and supplied in copies of the standard pUC57 plasmid. Protein sequences of the two ScFvs are supplied in the results section. All sequences were

designed to include flanking restriction sites of either *SfiI* or *PvuI* (5' terminus) and *HindIII* or *BamHI* (3' terminus) to allow incorporation of the polynucleotides into expression vectors of choice.

```
  1 MIPARFAGVL LALALILPGT LCAEGTRGRS STARCSLFGS DFVNTFDGSM YSFAGYCSYL
 61 LAGGCQKRSF SIIGDFQNGK RVSLSVYLGE FFDIHLFVNG TVTQGDQRVS MPYASKGLYL
121 ETEAGYYKLS GEAYGFVARI DGSGNFQVLL SDRYFNKTCG LCGNFNIFAE DDFMTQEGTL
181 TSDPYDFANS WALSSGEQWC ERASPPSSSC NISSGEMQKG LWEQCQLLKS TSVFARCHPL
241 VDPEPFVALC EKTLCECAGG LECACPALLE YARTCAQEGM VLYGWTDHSA CSPVCPAGME
301 YRQCVSPCAR TCQSLHINEM CQERCVDGCS CPEGQLLDEG LCVESTECPC VHSGKRYPPG
361 TSLSRDCNTC ICRNSQWICS NEECPGECLV TGQSHFKSFD NRYFTFSGIC QYLLARDCQD
421 HSFSIVIETV QCADDRDAVC TRSVTVRLPG LHNSLVKLKH GAGVAMDGQD VQLPLLKGDL
481 RIQHTVTASV RLSYGEDLQM DWDGRGRLLV KLSPVYAGKT CGLCGNYNGN QGDDFLTPSG
541 LAEPRVEDFG NAWKLHGDCQ DLQKQHSDPC ALNPRMTRFS EEACAVLTSP TFEACHRAVS
601 PLPYLRNCRY DVCSCSDGRE CLCGALASYA AACAGRGVRV AWREPGRCEL NCPKGQVYLQ
661 CGTPCNLTCR SLSYPDEECN EACLEGCFCP PGLYMDERGD CVPKAQCPCY YDGEIFQPED
721 IFSDHHTMCY CEDGFMHCTM SGVPGSLLPD AVLSSPLSHR SKRSLSCRPP MVKLVCPADN
```

Figure 4.1 Protein sequence of the VWFpp.

The VWFpp contains of 741 amino acids (highlighted in yellow colour), (http://www.ncbi.nlm.nih.gov/protein/NP_000543.2).

4.4.1.2 Preparation of expression vectors and recombinant yeast strains

The preparation of the expression vectors and recombinant yeast strains was performed at the Department of Microbial, Biochemical and Food Biotechnology of the University of the Free State. Standard molecular biology techniques were carried out as described by Sambrook and Russel (2001), and enzymes were applied according to the specifications of the manufacturers. The sequences encoding either target protein were removed from the supplied vectors using appropriate combinations of either *SfiI* or *PvuI* and *HindIII* or *BamHI*, and ligated into modified versions of pINA1317, which were digested with the same restriction enzyme combination. The modified vectors allowed the incorporation of sequences encoding six consecutive histidine residues as histidine-tags on either or both of the 5' and 3' termini, depending on the restriction enzymes used. The histidine-tags were designed to be removable by treatment with enterokinase. The authenticiy,

orientation and reading frame of the recombinant plasmids were verified by DNA sequencing.

The modified vectors were transformed into *Y. lipolytica* strain P01h according to the method described by Lin-Cereghino *et al.* (2005). A negative control strain was constructed by transformation of *Y. lipolytica* strain P01h with the original derivative of pINA1317 excluding an insert in the multiple cloning site.

Transformants were randomly selected from yeast nitrogen base selective plates [yeast nitrogen base without ammonium sulphate and amino acids 0.17 % (w/v), ammonium chloride 0.4 % (w/v), glucose 1 % (w/v), casamino acids 0.2% (w/v), agar 2 % (w/v); pH 6.5]. Chromosomal integration in transformants was confirmed by Polymerase chain reaction using genomic DNA as template.

4.4.1.3 Purification of recombinant VWFpp and ScFvs

Y. lipolytica transformants secreting proteins of interest were cultivated in yeast extract peptone dextrose broth [yeast extract 1 % (w/v), peptone 2 % (w/v), glucose 2 % (w/v)] on a rotary shaker at 30 °C. After 48 hours cultivation, cells were harvested by centrifugation at 5000 g for 5 minutes. The resultant supernatants were either applied directly to Talon metal affinity IMAC purification resin columns (Clonetech, USA), or concentrated using Amicon® centrifugal filter units (Merck, USA) prior to Talon metal affinity IMAC purification following the protocols from the manufacturers. Purified proteins were confirmed using spectrophotometric analysis with the Gene Quant pro (GE Health Sciences, USA) at 280 nm. Protein purification and spectrophotometric analysis were performed at the Department of Haematology and Cell Biology, University of the Free State.

In the case of intracellular accumulation of recombinant proteins, cells were cultured and harvested as previously described. Harvested cells were resuspended in 50 mM Phosphate buffer, pH 7, containing 0.25 mM PMSF and 40-50 % glycerol. Cells were lysed using the one-shot cell disruptor (Constant Systems, England, UK), at 30 kPsi. The lysate was centrifuged at 4000 g for 10 minutes, and the resulting supernatant was kept for protein purification as previously described. Purified proteins were

confirmed using spectrophotometric analysis and SDS-PAGE. The spectrophotometric analysis of the purified proteins was performed as previously described. The expression of the intracellular proteins of interest was showed on 10 % SDS-PAGE stained with 0.05 % Coomassie blue R-250 (Sigma-Aldrich, USA) based on the method described by Fairbanks *et al.* (1971). The Precision Plus Protein™ Prestained Protein Standard (BioRad, Rosebank, Johannesburg, South Africa) was used to aid in size estimation. Protein purification and spectrophotometric analysis were performed at the Department of Haematology and Cell Biology, and SDS-PAGE analysis was performed at the Department of Microbial, Biochemical and Food Biotechnology.

4.4.2 Expression of a truncated VWFpp in *E. coli* and production of two polyclonal antibodies in rabbits

The VWFpp and the two ScFvs (JA9 and JG7) could not be expressed by yeast. Two polyclonal antibodies (anti-tVWFpp and HRP-anti-tVWFpp) were then produced against a truncated form of the VWFpp in rabbits by BiologicsCorp, Indianapolis, USA. Recombinant VWFpp is not commercially available as previously mentioned, thus the VWFpp was first expressed in *E. coli* in a truncated form. The protein sequence of the tVWFpp is shown in figure 4.2. The tVWFpp gene was first codon-optimized for expression in *E. coli* (BL21(DE3)) and synthesized. It was then sub-cloned into the expression vector pET30a. An N-termial His_6-tag was added to the target tVWFpp protein. Finally pilot expression optimization and scale-up expression of the protein was performed. After Talon metal affinity purification, the tVWFpp was analysed on 12.5 % SDS-PAGE gel in Western-blot analysis. A 120 kDa molecular weight marker was used.

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  1 MHHHHHHRCS LFGSDFVNTF DGSMYSFAGY CSYLLAGGCQ KRSFSIIGDF QNGKRVSLSV
 61 YLGEFFDIHL FVNGTVTQGD QRVSMPYASK GLYLETEAGY YKLSGEAYGF VARIDGSGNF
121 QVLLSDRYFN KTCGLCGNFN IFAEDDFMTQ EGTLTSDPYD FANSWALSSG EQWCERASPP
181 SSSCNISSGE MQKGLWEQCQ LLKSTSVFAR CHPLCPAGME YRQCVSPCAR TCQSLHINEM
241 CQERCVDGCS CPEGQLLDEG LCVESTECEC LVTGQSHFKS FDNRYFTFSG ICQYLLARDC
301 QDHSFSIVIE TVQCADDRDA VCTRSVTVRL PGLHNSLVKL KHGAGVAMDG QDVQLPLLKG
361 DLRIQHTVTA SVRLSYGEDL QMDWDGRGRL LVKLSPVYAG KTCGLCGNYN GNQGDDFLTP
421 SGLAEPRVED FGNAWKLHGD CQDLQKQHSD PCALNPRMTR FSEEACAVLT SPTFEACHRA
481 CPKGQVYLQC GTPCNLTCRS LSYPDEECNE ACLEGCFCPP GLYMDERGDC VPKAQC
```

Figure 4.2 Protein sequence of the tVWFpp.

The tVWFpp consists of 536 amino acids.

The two polyclonal antibodies against the tVWFpp were purified from rabbit immune sera by ammonium sulfate precipitation followed by antigen-immunoaffinity chromatography (CNBr-activated sepharose 4B, GE Healthcare Life Sciences, USA) according to the manufacturer's protocol. One of the antibodies was HRP-labelled. The HRP-labelling process was as follows. First, the antibody and HRP label were dissolved in carbonate buffer. One µl of $NaIO_4$ was then added to each 10 µl of antibody and incubated for 3 hours at room temperature in the dark. The mixture was washed with carbonate buffer to elute the conjugate. One µl of $NaBH_4$ was added to the mixture, and incubated for 60 minutes in the dark. The antibody solution was finally dialysed against phosphate buffered saline (PBS) and stored at -20 ºC. The purity and reactivity of the two polyclonal antibodies against the tVWFpp was analysed on a 12.5 % gel in Western Blot analysis.

4.4.2.1 Binding of polyclonal antibodies to the VWFpp in plasma

The polyclonal antibodies from BiologicsCorp were tested for binding to the tVWFpp and to VWFpp in normal pooled plasma. First, an ELISA 96-well plate (Greiner-bio-one, Germany) was coated with 100 µl per well of the unlabelled polyclonal antibody (1:100 dilution in PBS containing 11.68 g NaCl; 9.44 g Na_2HPO_4; 5.28 g $NaH_2PO_4.2H_2O$, 0.1 % Tween 20, in 2 l (d) H_2O, pH 7.2) overnight at 4 °C. The next day, the plate was washed with PBS/0.1% Tween-20 (PBS with 0.1 % Tween 20, in 2 liter (d) H_2O) using a microplate washer. The tVWFpp and normal pooled plasma

(diluted in PBS/0.1% Tween-20) were each added to the ELISA plate in different concentrations ranging from 0 to 10 µg/ml (100 µl per well), and the blank contained only PBS/0.1% Tween-20. The plate was then incubated for 2 hours at 37 °C. One hundred µl of the HRP-labelled antibody (1:2000 dilution in PBS/0.1% Tween-20) was added to the plate after another washing cycle with PBS/0.1% Tween-20, and the plate incubated for 1 hour at room temperature. Following this incubation, the plate was washed with PBS/0.1% Tween-20, and the binding of the antibodies to the tVWFpp and the VWFpp in normal pooled plasma was detected with o-phelylenediamine dihydrochloride (OPD), a substrate for horseradish peroxidase. The substrate mixture contained 10 ml 0.1 M citric acid, 10 ml 0.2 M sodium phosphate, 200 µl of a 5 % OPD solution (0.25 g OPD (Sigma-Aldrich, USA) in 2.5 ml 0.2 M sodium phosphate, 2.5 ml 0.1 M citric acid,) and 8 µl of 30 % H_2O_2 (Merck, Darmstadt, Germany). Ninety µl of this mixture was added per well to the plate. The reaction was stopped after 10 minutes by adding 30 µl of 4M H_2SO_4 per well. The ELISA plate was read at OD 490-630 nm using an ELISA plate reader (Synergy HT, BioTek, Winooski, VT, USA).

The commercial antibody pair CLB-Pro 35 and CLB-Pro 14.3 (Cellsciences, Newburyport, MA, USA) was also tested for binding to the tVWFpp and to VWFpp in normal pooled plasma. This was done to determine if the two polyclonal antibodies could be used to measure VWFpp levels in plasma. An ELISA similar to the previous one, was used to perform this, and the CLB-Pro 35 and CLB-Pro 14.3 antibody pair kit was used according to manufacturer's instructions. In short, 100 µl of a 1:100 dilution in PBS of CLB-Pro 35 was used to coat a plate. Then the tVWFpp and normal pooled plasma (diluted in PBS/0.1%Tween-20/1% BSA) were each added in different concentrations ranging from 0 to 10 µg/ml (100 µl per well) to the ELISA plate.

The blank contained PBS/0.1%Tween-20/1% BSA. A hundred µl of a 1:100 dilution in PBS/0.1%Tween-20/1% BSA of CLB-Pro 14.3 detection antibody was used and the colour reaction was performed as previously described. This reaction was stopped after 15 minutes, and the plate was read as previously described.

It was realised that the truncated form of the VWFpp does not contain similar epitopes than VWFpp in plasma and therefore the polyclonal antibodies could not be used in the VWFpp assay. The next effort was to develop a rapid lateral flow assay by using the commercially available antibodies.

4.4.3 The development of a rapid VWFpp lateral flow assay (LFA)

4.4.3.1 Principle of the rapid VWFpp lateral flow assay

It is important to note that HRP-conjugated detection antibodies cannot be gold-labelled. Therefore, CLB-Pro 14.3 could not be used as the detection antibody. A VWF/VWFpp polyclonal antibody (Novopro, Shanghai, China) was then used as the detection antibody in the rapid VWFpp LFA. This antibody is able to detect both the VWF antigen as well as the VWFpp.

4.4.3.2 LFA with CLB-Pro 35 as capture antibody and VWF/VWFpp polyclonal antibody as detection antibody

A 1:100 dilution in PBS of the Mouse Anti-human VWFpp antibody CLB-Pro 35 (Cellsciences, Newburyport, MA, USA) was used as the capture antibody and 0.3 µg/µl of the VWF/VWFpp polyclonal antibody (Novopro, Shanghai, China) was used as the detection antibody. CLB-Pro 35 was first biotinylated using the ab201795-Biotin type A fast conjugation kit (Abcam, UK), according to the manufacturer's instructions. In short, one µl of biotin modifier was added to each 10 µl of capture antibody. This mixture was added to a lyophilized conjugation mix. Then this second mixture was incubated for 15 minutes at room temperature. One µl of biotin quencher was then added to the previous mixture, and this was incubated for 4 minutes at room temperature. After the 4 minutes, the biotinylated antibody was stored at 4 °C for later use.

The detection antibody (VWF/VWFpp polyclonal antibody) was gold conjugated using the Naked Gold Conjugation kit, NGIB18-1 (Bioporto diagnostics, Tuborg Havnevej, Denmark) as recommended by the manufacturer. Firstly, ten buffer solutions were prepared in Eppendorf tubes with pH ranging from 5.4 to 10.1 by

using different concentrations of buffers. Next, the detection antibody was added to each of the buffer solutions. This antibody-buffer mixture was then added to a second set of 10 tubes with the gold conjugate and mixed by vortexing for 10 seconds. The mixture was incubated for 30 minutes at room temperature. Following the 30 minutes, only the tubes that did not change to a dark colour were kept. The reason for this is that when the pH is too low, the gold solution aggregates and the conjugation mixture changes to a darker colour. The aggregated gold solutions are not suitable for immunoassays. The reactions in the tubes that did not change to a darker colour were stopped by adding 100 µl of stabilizing buffer and the mixture was incubated for a further 30 minutes. The effectiveness of the conjugation was tested using a salt according to the instructions from the Naked Gold Conjugation kit (NGIB18-1). The gold conjugated antibody was added to 1 mol/100 ml NaCl (5.844 g NaCl, in 100 ml dH_20). When the mixture turned black, it was discarded. When the mixture remained red it was kept at 4 ºC or it could immediately be used.

A standard curve was then set with the WHO 6th IS for FVIII/VWF in plasma using different dilutions (0 %, 12.5 %, 25 % and 50 %). The rapid VWFpp LFA was used for determining different colour intensities on the generic Rapid Assay Device (gRAD) dip strip (provided in the LFA kit from Bioporto diagnostics, Tuborg Havnevej, Denmark) based on the different dilutions made using the WHO 6th IS for FVIII/VWF in plasma.

The rapid VWFpp lateral flow assay was performed according the gRAD (1) 2015-09 instruction manual (Bioporto diagnostics, Tuborg Havnevej, Denmark) as follows. The capture antibody (bioitynilated CLB-Pro 35) and detection antibody (gold-labelled anti VWF/VWFpp polyclonal antibody) were added first to a test tube. The sample solution was then added to the antibody mixture and this was left to incubate for 5 minutes (at room temperature) to allow the VWFpp in plasma to form a complex with the two antibodies. Next, the gRAD strip was placed into the test tube for 15 minutes after which colour change could be observed on the strip and the colour intensity could be compared to a calibration card. Figure 4.3 is a (A) photo of the calibration card, and (B) the gRAD dip strip that is used in this assay.

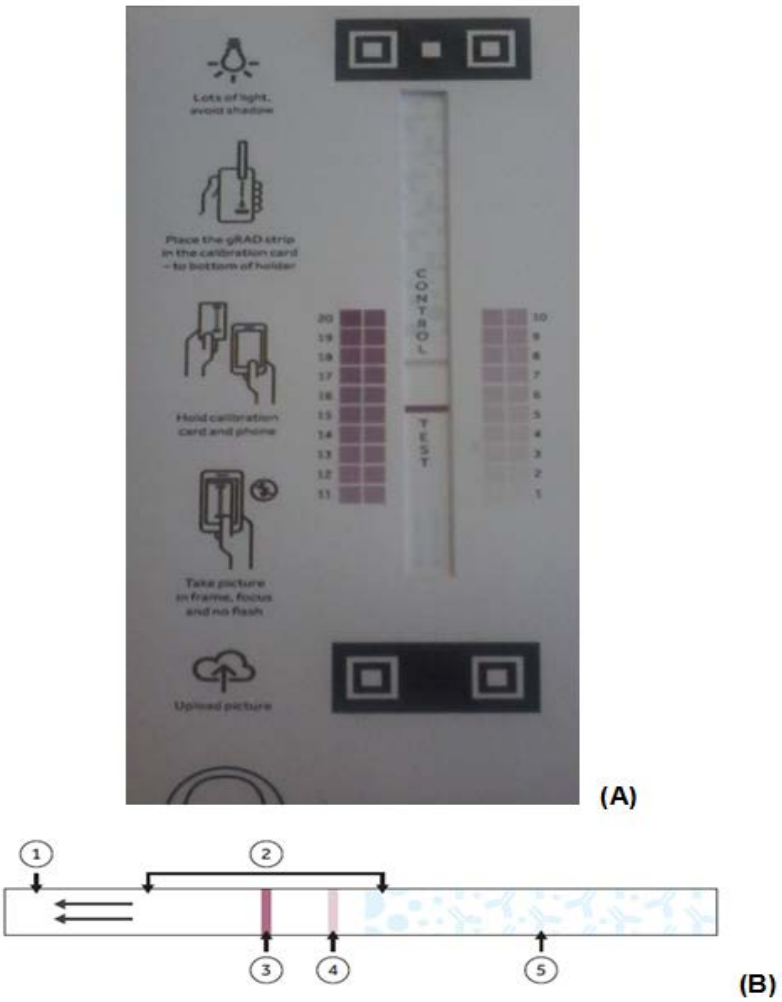


Figure 4.3 Calbration card and gRAD dip stip.

(A) The calibration card used for comparing colour intensity for the determination of VWFpp levels in plasma. The intensity of colour on the strip depends on the concentration of the VWFpp in plasma. (B) The design of the gRAD dip strip. 1= sample application pad, 2= analytical membrane, 3= test line, 4= control line, 5= adsorption pad (gRAD(1)-Kit, Bioporto diagnostics; http://www.bioporto.com/Files/Images/Fra%20Files/Marketing-material/IFU-gRAD-OneDection-Kit-Nov-2015_3.pdf).

4.4.3.3 LFA with VWF/VWFpp polyclonal antibody as capture antibody and CLB-Pro 35 as detection antibody

The antibodies were swopped, the VWF/VWFpp polyclonal antibody was used as the capture antibody and CLB-Pro35 as the detection antibody. The biotinylation of the capture was done using the ab201795-Biotin type A fast conjugation kit, according to the manufacturer's instructions as previously described. The detection

antibody was gold conjugated using the Naked Gold Conjugation kit (NGIB18-1) according to the manufacturer as previously described. The rapid VWFpp LFA was performed. Following this, biotinylation determination of the VWF/VWFpp polyclonal antibody was performed using an ELISA on a 96-well plate (Greiner-bio-one, Germany). The polyclonal antibody was added to the ELISA plate using different dilutions, and was detected with a 1:2000 dilution in PBS/0.1% Tween-20 streptavidin-HRP (Biolegend, San Diego, CA). The blank used contained PBS. The results were evaluated by observation of a concentration dependent colour change on the ELISA plate.

The VWF/VWFpp polyclonal antibody was then concentrated using the AbSelect antibody concentration & clean-up kit (Innova biosciences, Cambridge, UK) as recommended by the manufacturer. It was also biotinylated using a different kit (Lightning-link rapid biotin conjugation Kit type A (Innova biosciences, Cambridge, UK), as recommended by the manufacturer. In short, 1 µl of LL rapid modifier reagent was added to each 10 µl of concentrated antibody. This mixture was added to a lightning-link rapid mix (lyophilized material), and this was incubated for 15 minutes at room temperature. One µl of rapid quencher reagent was then added to the previous mixture. The biotin conjugate could be used after 5 minutes incubation at room temperature, or it could be stored at 4 °C.

After the biotinylation, the rapid VWFpp LFA assay was performed to determine if the biotinylated antibody could be used as the capture antibody. Next, an ELISA was performed in order to determine if the biotinylated VWF/VWFpp polyclonal antibody could detect VWFpp in plasma. In this ELISA, CLB-Pro 35 was used as the capture antibody, the biotnylated VWF/VWFpp polyclonal antibody was used as the primary (detection) antibody and the secondary antibody was streptavidin-HRP (1:1000 dilution in PBS/0.1% Tween-20). The WHO 6th IS for FVIII/VWF in plasma was used as a sample, and was added to the ELISA plate using different dilutions. A blank was also included which contained PBS/0.1%Tween-20/1% BSA. The biotinylation determination results were evaluated by observation of a concentration dependent colour change on the ELISA plate and by reading the plate at an OD between 490-630 nm using an ELISA plate reader (Synergy HT, BioTek, Winooski, VT, USA).

The last part of this study was to develop and validate a rapid VWFpp ELISA with the commercial monoclonal antibody pair.

4.4.4 The development and validation of a rapid VWFpp ELISA

4.4.4.1 The development of a rapid VWFpp ELISA

In this assay, the incubation times were reduced by 78%. Thus, the whole assay takes 1.30 hours instead of the 6 hours of the commercial assay. An ELISA 96-well microplate (Greiner-bio-one, Germany) was coated with 100 µl of a 1:100 dilution in PBS of CLB-Pro 35 antibody (Cellsciences, Newburyport, MA, USA) over night at 4°C. The next day, the plate was washed with PBS/0.1% Tween-20, and was then blocked with PBS/0.1%Tween-20/1% BSA for 30 minutes and not 2 hours as in commercial assay. After a washing step, the WHO 6th IS for FVIII/VWF in plasma was added to the plate with the following dilutions in PBS/0.1%Tween-20/1% BSA: 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, and 1:640. A blank was also included that only contained PBS/0.1%Tween-20/1% BSA. A 1:50 dilution was used for the samples. The standard and samples were then incubated at 37 °C for 30 minutes instead of the 2 hours used in the original assay. After washing the plate, a 100 µl of a 1:100 dilution in PBS/0.1%Tween-20/1% BSA of the detection antibody CLB-Pro 14.3 HRP (Cellsciences, Newburyport, MA, USA) was added, and the plate was incubated at 37 °C for another 30 min instead of the 2 hours according to the commercial assay kit. The plate was then washed. OPD was used as a substrate. The substrate mixture included 10 ml 0.1 M citric acid, 10 ml 0.2 M sodium phosphate, 200 µl of 5 % OPD and 8 µl of 30 % H_2O_2 (Merck, Darmstadt, Germany). The plate was coloured for 15 minutes by adding 90 µl of the substrate mixture, and the reaction was stopped by adding 30 µl of 4M H_2SO_4. The plate was then read at OD 490-630nm using an ELISA plate reader (Synergy HT, BioTek, Winooski, VT, USA).

4.4.4.2 The validation of a rapid VWFpp ELISA

The commercial assay was compared to the 30 minutes incubation assay in a validation study using plasma of 20 type 1 VWD patients. After the comparison of the two methods, the rapid 30 minute VWFpp ELISA was validated over five

consecutive days. Two samples were used which were named, sample (A) and (B). (A) was the WHO 6th IS for FVIII/VWF in plasma and (B) was a type 1 VWD patient with an increased VWFpp/VWF:Ag ratio. These samples were tested four times in duplicate each day for 5 days in order to determine the inter-assay and intra-assay precision.

4.4.5 Statistical data analysis

The commercial method and our rapid ELISA were compared to each other using linear regression, Deming regression and Bland-Altman analysis. The inter-assay precision was determined using the mean, SD and % CV. In addition, a margin of error, % bias and total allowable error % were also determined. The intra-assay precision was evaluated using precision plots of sample (A) and (B), and was also determined using the mean, SD and % CV.

Chapter 5: Results

The first part of this study was to express two ScFvs to the VWFpp. These ScFvs were previously selected from an antibody library using phage display technology.

5.1 Expression of the VWFpp, the ScFv JA9 and ScFv JG7 by yeast

The molecular structures of the ScFvs are shown in figure 5.1 and the protein sequences in table 5.1. Although the protein sequences of the two ScFvs shows more than 90% homology, the molecular structures of the two ScFvs differs substantially.

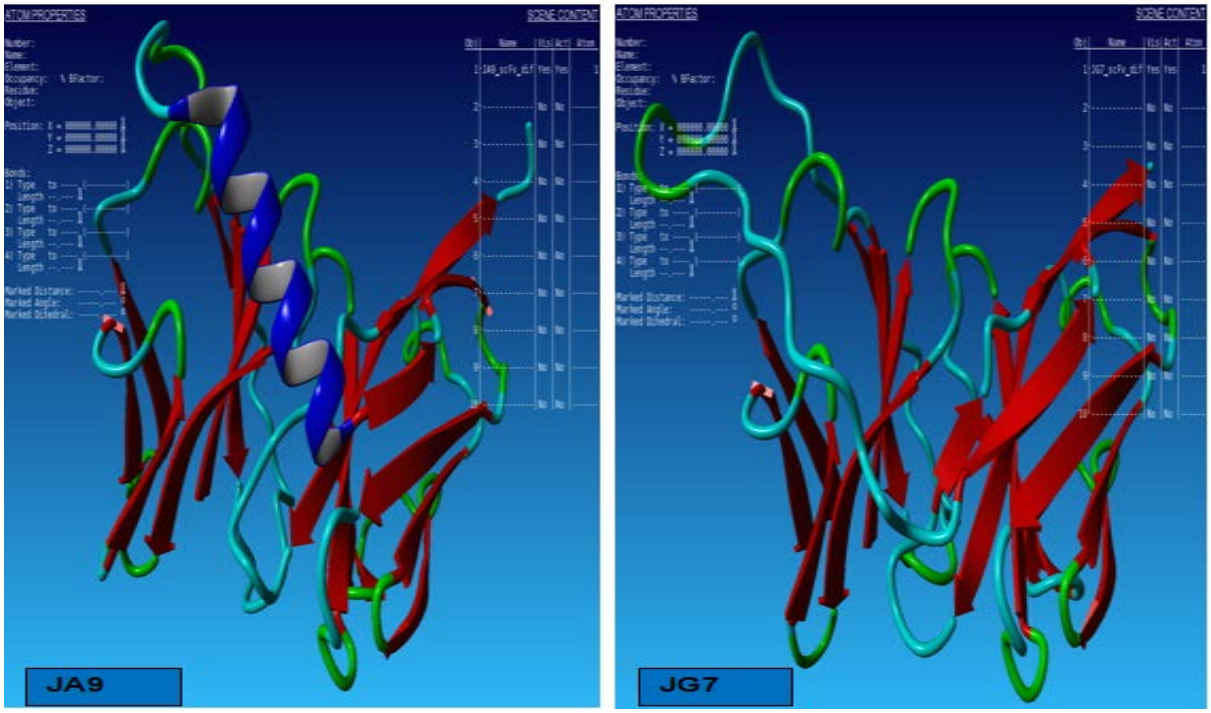


Figure 5.1 The molecular structures of JA9 and JG7.

Molecular structures were created using the YASARA software programme.

Table 5.1 Protein sequences of JA9 and JG7 ScFvs.

JA9	**VARIABLE LIGHT CHAIN:** QLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSSIYEEGVLTMYADSVKGRFTI SRDNSKNTLYLQMNSLRAEDTAVYYCAKRSVIR **LINKER SEQUENCE:** FDYWGQGTLVTVSSGGGGSGGGGSGGGGSTDIQMTQ **VARIABLE HEAVY CHAIN:** SPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASCKVGSPSRFSGSGSGTDFTLTI SSLQPEDFATYYCQQENCEYTFGQGTKVEIKR
JG7	**VARIABLE LIGHT CHAIN:** QLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSSIVETGPETSYADSVKGRFTI SRDNSKNTLYLQMNSLRAEDTAVYYCAKSPQL **LINKER SEQUENCE:** FDYWGQGTLVTVSSGGGGSGGGGSGGGGSTDIQMTQ **VARIABLE HEAVY CHAIN:** SPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYRASSLQSGVPSRFSGSGSGTDFTLT ISSLQPEDFATYYCQQGHPTPGTFGQGTKVEIKR

The differences between the two ScFvs are indicated in yellow.

After concentrating the proteins using Amicon® centrifugal filter units and purifying them on Talon metal affinity purification resin columns, the protein concentrations were extremely low as shown in table 5.2.

Table 5.2 Protein concentrations of the two ScFvs.

JA9	JG7
78 µg/ml	64 µg/ml

The 26 kDa ScFvs (JA9 and JG7) were expressed in very low quantities in the yeast cells. Figure 5.2 shows the SDS-PAGE of the yeast cell lysates.

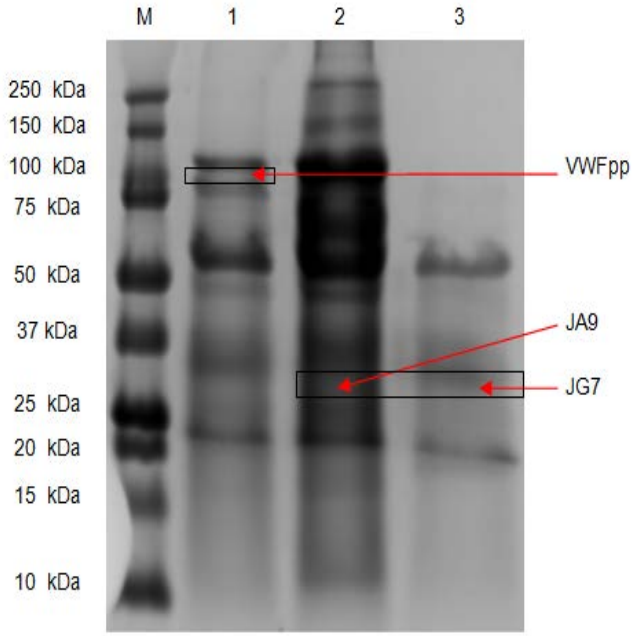


Figure 5.2 SDS-PAGE of yeast cell lysates.

A 10 % SDS-PAGE analysis of the yeast cell lysates. A 250 kDa molecular weight marker was used. Lane M= molecular weight marker, lane 1= VWFpp (95 kDa), lane 2= JA9 (26 kDa) and lane 3= JG7 (26 kDa). No VWFpp or antibody fragments JA9 and JG7 were expressed.

Due to the low protein yield in yeast, polyclonal antibodies were then produced to the VWFpp. Since no commercial VWFpp is available, a truncated form of the VWFpp was first expressed in *E.coli* (BL21(DE3)).

5.2 Expression of a truncated VWFpp in *E. coli* and production of two polyclonal antibodies in rabbits

A total amount of 0.473 mg tVWFpp was expressed. The purity of the tVWFpp was confirmed using SDS-PAGE analysis and Western-blot as shown in figure 5.3.

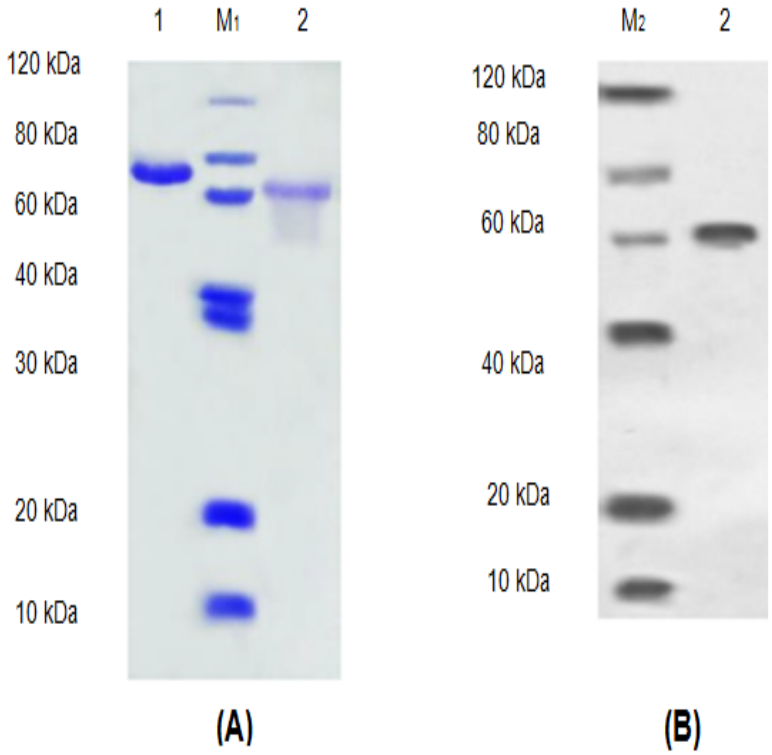


Figure 5.3 SDS- PAGE and Western-blot of the tVWFpp.

(A) 12.5 % SDS-PAGE analysis and (B) 12.5 % gel for Western-blot analysis of the tVWFpp. Lane 1= bovine serum albumin (66 kDa; used as molecular weight standard), Lanes M_1 and M_2 = molecular weight marker and Lane 2= tVWFpp (60kDa).

The purity of the two polyclonal antibodies (anti-tVWFpp and HRP-anti-tVWFpp) against the tVWFpp after antigen-immunoaffinity chromatography was confirmed using Western-blot as shown in figure 5.4. The final amount of purified anti-tVWFpp was 2.73 mg and the HRP-anti-tVWFpp was 2.35 mg.

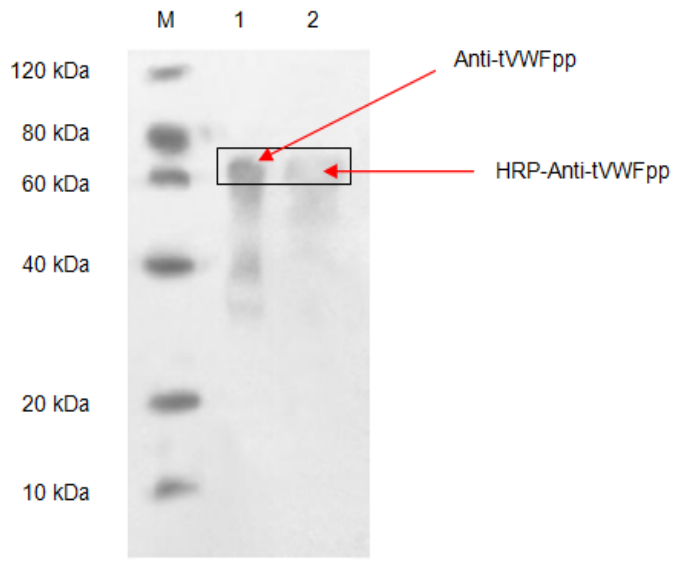


Figure 5.4 Western-blot of anti-tVWFpp and HRP-anti-tVWFpp.

A 12.5 % gel for Western-blot analysis of the anti-tVWFpp and HRP-anti-tVWFpp polyclonal antibodies. Lane M= molecular weight marker, Lane 1= anti-tVWFpp (60 kDa) and lane 2= HRP-anti-tVWFpp (60 kDa).

5.2.1 Binding of polyclonal antibodies to the VWFpp in plasma

The two polyclonal antibodies were tested for binding to the tVWFpp and to VWFpp in normal pooled plasma. Figure 5.5 shows that the anti-tVWFpp and HRP-anti-tVWFpp antibodies could only detect the tVWFpp in a concentration dependent manner. They however could not detect VWFpp in normal pooled plasma. Thus, the tVWFpp expressed different binding sites than VWFpp in normal plasma. Therefore, these polyclonal antibodies could not be used for the measurement of VWFpp levels in plasma.

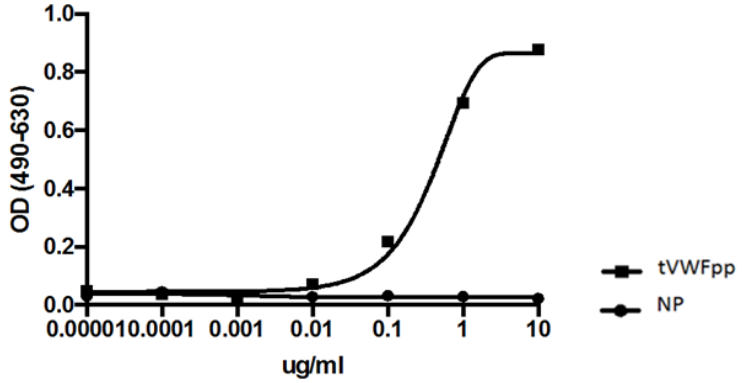


Figure 5.5 Binding of anti-tVWFpp and HRP-anti-tVWFpp to tVWFpp and to VWFpp.

The binding of the polyclonal antibodies to the tVWFpp is indicated by squares, and the binding to the VWFpp in normal pooled plasma (NP) is indicated by circles.

To confirm this assumption the commercial antibody pair (CLB-Pro 35 and CLB-Pro 14.3) was tested for binding to the tVWFpp and to VWFpp in normal pooled plasma (figure 5.6). Interesting to note, the commercial antibodies could only bind to the VWFpp in plasma and not to the tVWFpp.

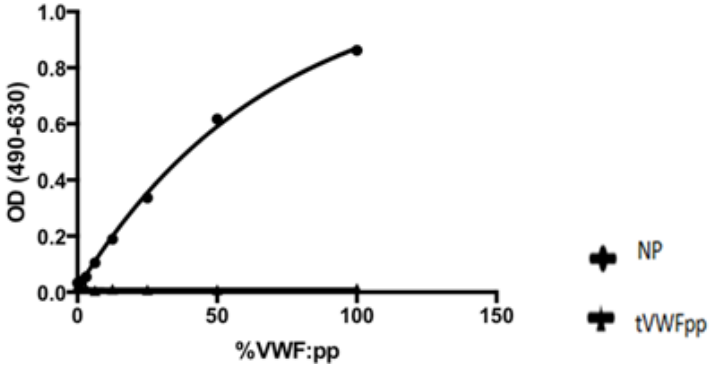


Figure 5.6 Binding of the commercial antibody pair to tVWFpp and to VWFpp.

The binding of the commercial antibodies to the tVWFpp is indicated by triangles, and binding to the VWFpp in normal pooled plasma (NP) is indicated by circles.

Since our polyclonal antibodies were not suitable to measure VWFpp levels in plasma, we had to use commercial antibodies in the development of a rapid lateral flow assay.

5.3 The development of a rapid VWFpp lateral flow assay (LFA)

In order to develop a rapid VWFpp assay for the diagnosis of type 1 VWD, the next step was to introduce and develop a lateral flow assay.

5.3.1 LFA with CLB-Pro 35 as capture antibody and VWF/VWFpp polyclonal antibody as detection antibody

As described in the methodology section, we used commercially available antibodies in the LFA. Colour change was observed on both the test and control lines. Thus a complex was formed between the standard plasma and the two antibodies.

A standard curve was set with the WHO 6th IS for FVIII/VWF in plasma using four dilutions (0 %, 12.5 %, 25 % and 50%). However, the same colour intensity was observed on the test line for all the dilutions. Figure 5.7 shows the dip strip that was used to set the standard curve. No concentration dependent colour change was observed on the test line for each of the different dilutions.

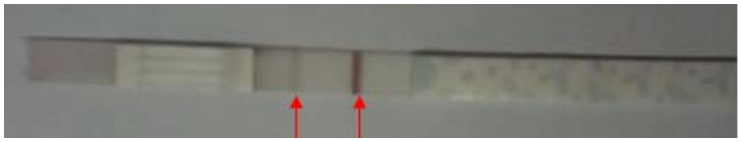


Figure 5.7 Dip strip result when CLB-Pro 35 was used as the capture antibody and VWF/VWFpp polyclonal antibody was used as the detection antibody.
A lighter colour was observed on test line and a darker colour on control line.

The antibodies were then swopped, the VWF/VWFpp polyclonal antibody was used as the capture and CLB-Pro 35 was used as the detection antibody since there was no concentration dependent colour change on the test line.

5.3.2 LFA with VWF/VWFpp polyclonal antibody as capture antibody and CLB-Pro 35 as detection antibody

After the capture and detection antibodies were swopped, colour change was only observed on the control line, but no test line appeared (figure 5.8).

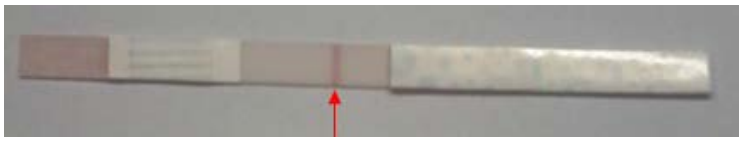


Figure 5.8 Dip strip result when VWF/VWFpp polyclonal antibody was used as the capture antibody and CLB-Pro 35 was used as the detection antibody.
A lighter colour was observed on control line only, but no test line was visible.

In order to test if the VWF/VWFpp polyclonal antibody was properly biotinylated, an ELISA plate was coated with different concentrations of this antibody, and streptavidin-HRP was used as the detection antibody. No concentration dependent colour change could be observed (figure 5.9). The biotinylation of this antibody was therefore not successful.

Figure 5.9 Biotinylation determination of the VWF/VWFpp polyclonal antibody. *This was performed as a normal ELISA using streptavidin-HRP as the detection antibody. The polyclonal antibody was added to the ELISA plate (A1-D1) wells using different dilutions. No concentration dependent binding was observed. The biotinylation of the VWF VWFpp polyclonal antibody was done using the ab201795-Biotin type A fast conjugation kit (Abcam, UK).*

Even with a concentrated VWF/VWFpp polyclonal antibody that was biotinylated using a different biotinylation kit, colour change was only observed on control line and not on the test line. (figure 5.10). The rapid lateral flow assay results confirmed that the biotinylated VWF/VWFpp polyclonal antibody could not be used as the capture antibody in this type of assay.

Figure 5.10 Rapid lateral flow assay results of the concentrated VWF/VWFpp polyclonal antibody.
The results confirm that this antibody cannot be used as the capture antibody.

An ELISA was then performed to determine if the biotinylated VWF/VWFpp polyclonal antibody could detect VWFpp in plasma. There seemed to be some

binding of the VWF/VWFpp polyclonal antibody to the VWFpp in WHO 6th IS for FVIII/VWF in plasma (figure 5.11). This is however not sufficient for developing a lateral flow assay.

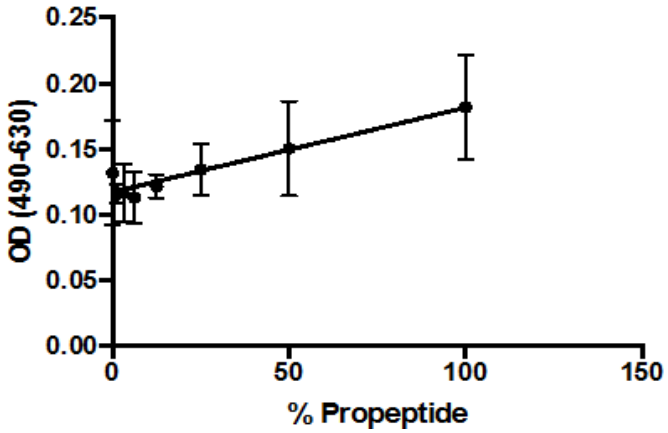


Figure 5.11 Binding of the biotinylated VWF/VWFpp polyclonal antibody to VWFpp in the WHO 6th IS for FVIII/VWF in plasma.

This standard curve shows that there was some binding of the VWF/VWFpp polyclonal antibody to the VWFpp, but however not specific and strong enough to be used in a lateral flow assay. The biotinylation of the VWF/VWFpp polyclonal antibody this time was done using the Lightning-link rapid biotin conjugation Kit type A (Innova biosciences, Cambridge, UK).

The last part of this study focuses on the validation of a rapid VWFpp ELISA using the commercially available monoclonal antibody pair to the VWFpp.

5.4 The development and validation of a rapid VWFpp ELISA

5.4.1 The development of a rapid VWFpp ELISA

A concentration dependent colour change was observed with the original 2 hour method and 30 minute incubation times (figure 5.12). The colour intensity of the reaction decreased with reduction in incubation time.

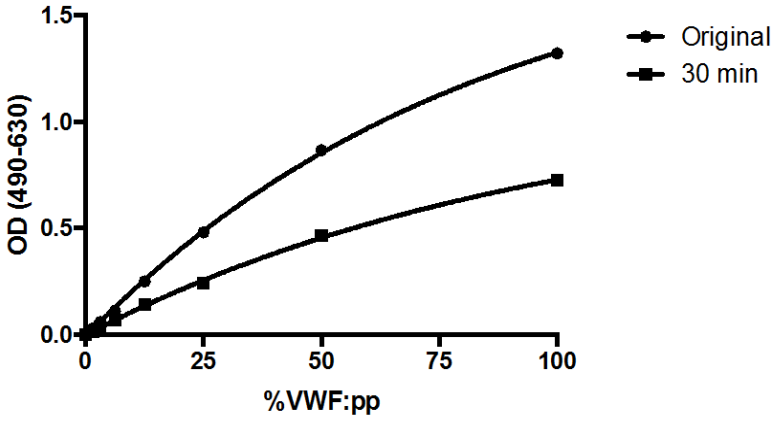


Figure 5.12 Comparison of the original 2 hours method to the 30 minutes incubation times for development of rapid VWFpp ELISA.

The standard curves were set up using the WHO 6th IS for FVIII/VWF in plasma. The lowest VWFpp concentration of 1.5625% was detected by both assays. The original 2 hours method is indicated by circles, and 30 minutes incubation time method is indicated by squares.

5.4.2 The validation of a rapid VWFpp ELISA

Twenty type 1 VWD samples were tested using the two different incubation times. The VWFpp/VWF:Ag ratios of the patients were determined based on the results from these incubation times. Similar results were obtained from these two methods (table 5.3). Patient 2, 5, 6, 10 and 14 showed an increased VWFpp/VWF:Ag ratio that was greater than 2 with both methods.

Table 5.3 Results of the type 1 VWD patients tested with the original and 30 minutes methods.

Patient	VWFpp Original method	VWFpp 30 minutes method	VWF:Ag	VWFpp/VWF:Ag ratio Original method	VWFpp/VWF:Ag ratio 30 minutes method
1	50	48	40	1.25	1.20
2	91	106	36	2.53	2.94
3	56	52	49	1.14	1.06
4	57	57	43	1.33	1.33
5	69	71	25	2.76	2.84
6	47	47	23	2.04	2.04
7	34	34	44	0.77	0.77
8	44	40	27	1.63	1.48
9	70	80	40	1.75	2.00
10	42	40	11	3.82	3.64
11	65	68	34	1.91	2.00
12	32	29	25	1.28	1.16
13	35	40	43	0.81	0.93
14	38	32	13	2.92	2.46
15	24	20	44	0.55	0.45
16	74	81	41	1.80	1.98
17	48	45	36	1.33	1.25
18	45	40	47	0.96	0.85
19	49	46	37	1.32	1.24
20	51	51	32	1.59	1.59

Normal VWFpp and VWF:Ag levels range between 50-150 %. A VWFpp/VWF:Ag ratio more than 2.0 indicates increased VWF clearance.

The original method was compared to the 30 minute method by using linear regression analysis (figure 5.13).

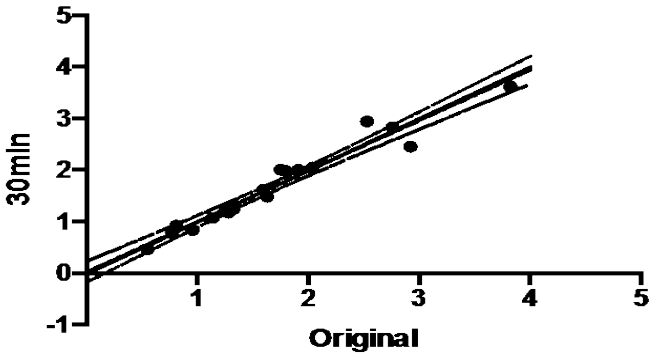


Figure 5.13 Linear regression showing a comparison between the original 2 hours method and the 30 minutes method.

The linear regression was constructed using the VWFpp/VWF:Ag ratio of 20 type 1 VWD patients. A 95 % confidence interval is shown by the broken lines. An R square value of 0.95 was obtained. The linear regression equation is Y=0.98X+0.02. The slope is 0.98 (0.87 to 1.09).

Deming regression of the original method and 30 minutes method is shown in figure 5. 14.

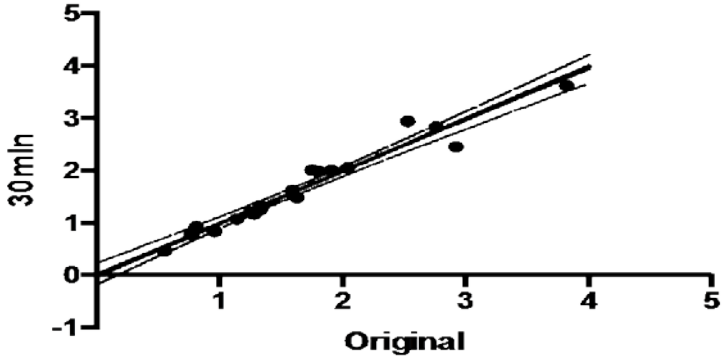


Figure 5.14 Deming regression showing a comparison between the original 2 hours method and the 30 minutes method.

The Deming regression was constructed using the VWFpp/VWF:Ag ratio of 20 type 1 VWD patients. A 95 % confidence interval is shown by the broken lines. The Deming regression equation is Y=1.00X-0.02. The slope is 1.00 (0.89 to 1,12).

We used both Linear and Deming regression analysis to compare the two assays to each other, in order to prove that the 30-minute method is as effective as the original two hours method.

The Bland-Altman plot of the comparison shows the difference vs. average of the two methods using the VWFpp levels of the 20 type 1 VWD patients (figure 5.15). The bias between the two methods was determined at - 0.19.

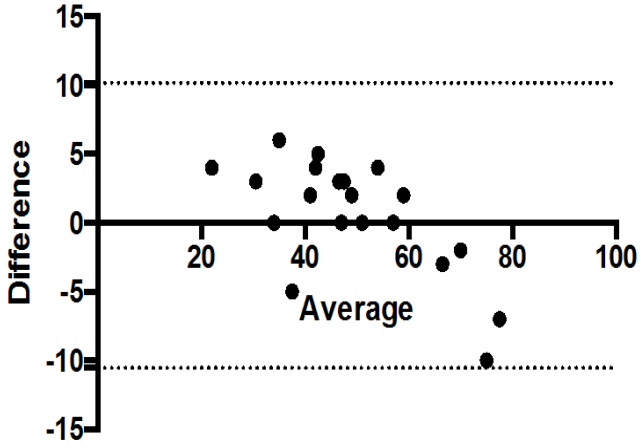


Figure 5.15 Difference vs. average: Bland-Altman plot showing a comparison between the original 2 hours method and the 30 minutes method.
The Difference vs. average: Bland-Altman plot was constructed using the VWFpp levels of the 20 type 1 VWD patients. A 95 % limits of agreement is indicated by the dotted lines.

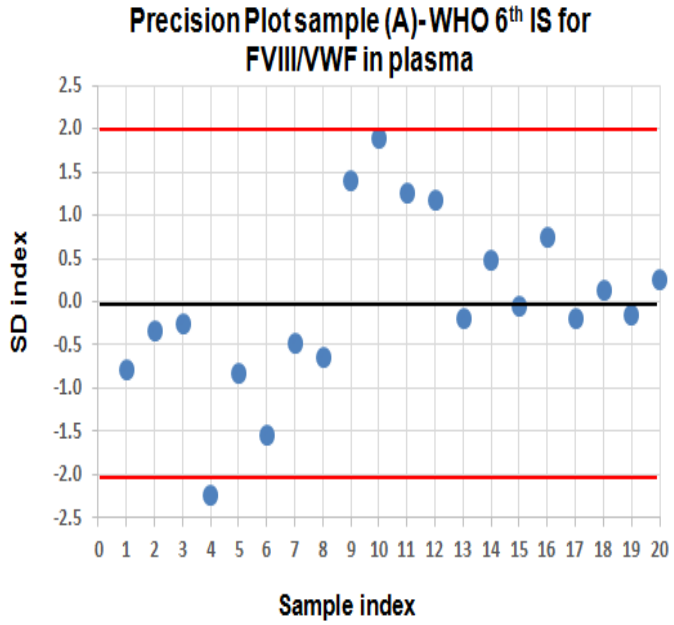
After the comparison of the two methods, the rapid 30 minute VWFpp ELISA was validated over five consecutive days. Inter-assay precision data over the 5 consecutive days for sample (A)- WHO 6th IS for FVIII/VWF in plasma and sample (B)- type 1 VWD patient sample is shown in table 5.4.

Table 5.4 Inter-assay precision of (A)- WHO 6th IS for FVIII/VWF in plasma and (B)- patient sample over 5 consecutive days.

Samples	(A)- WHO 6th IS for FVIII/VWF in plasma	(B)- type 1 VWD patient plasma
Mean (%)	92.16	91.97
SD	9.16	5.41
% CV	9.94	5.88
Target mean	103	
N	20	
Margin of error	4.01	
% Bias	1.94	
TEA %	18.33	

SD= standard deviation, % CV= % coefficient of variation, N= number of times sample was run or tested, TEA %= total allowable error %.
(Assigned VWFpp value based on WHO 6th IS for FVIII/VWF in plasma is 103 %)

Precision plots (over the 5 consecutive days) of the two samples (A) and (B) are shown in figure 5.16.



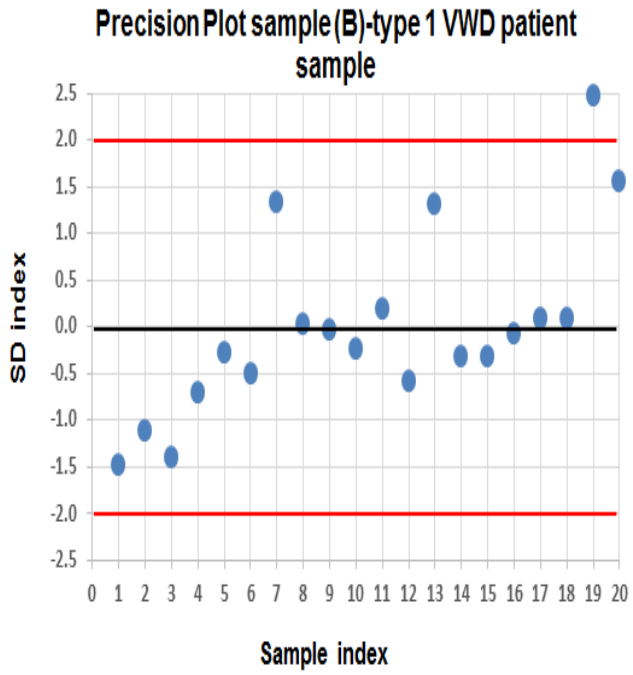


Figure 5.16 Precision plots of sample (A)- WHO 6th IS for FVIII/VWF in plasma and sample (B)-type 1 VWD patient sample.

Sample (A) and (B) were both tested four times on each day over the 5 consecutive days.

Intra-assay precision was also evaluated by determining the mean, SD and % CV as shown in table 5.5.

Table 5.5 Intra-assay precision of (A)- WHO 6th IS for FVIII/VWF in plasma and (B)- patient sample over 5 consecutive days.

		% CV
Mean (A)	92.16	9.54
SD (A)	8.79	
Mean (B)	92.19	4.70
SD (B)	4.33	

The % CV for sample (A) was 9.54 and 4.70 for sample (B). SD= standard deviation, % CV= % coefficient of variation, (A)= WHO 6th IS for FVIII/VWF in plasma sample, (B)= type 1 VWD patient sample.

Chapter 6: Discussion

The treatment strategy for individuals with an increased clearance rate of VWF differs from those with normal clearance rates. The ratio between the VWFpp and the VWF:Ag is used to identify conditions of reduced VWF half-life, such as type 1 VWD with increased clearance of VWF (Hubbard *et al.*, 2012; Meiring et al., 2009).

There is only one commercial assay (CLB-Pro 35 and CLB-Pro 14.3 mAb, Cellsciences) available to measure VWFpp levels in human plasma. This assay is not only expensive to be used in developing countries but is also very time consuming. The VWFpp assay is expensive as it uses monoclonal antibodies. Mammalian cells are commonly used for the expression of monoclonal antibodies because of their capability to perform post-translational modifications (Spadiut *et al.*, 2014). However, mammalian cells have disadvantages, such as slow growth rate and low expression yield. These factors make mammalian cell cultivation expensive and the cost of manufacturing is further increased by expensive media and the difficult culturing techniques (Cha *et al.*, 2005).

In a previous study, our laboratory aimed to develop a cost-effective VWFpp ELISA using ScFvs that were selected by phage display technology. One of the main advantages of phage display is the production of ScFvs that bind to a specific antigen, which can be performed within a couple of weeks (Carmen and Jermutus, 2002). However, complete characterization of the ScFvs and subsequent applications are usually hampered due to low yield of protein by *E. coli* (Kamionka, 2011). The initial aim of this study was to express the ScFvs using a yeast host-vector system in order to validate the cost-effective VWFpp ELISA.

Yeast host–vector systems have been successfully used in recombinant protein expression. However, some proteins cannot be expressed with the existing systems, thus there is a need for new yeast expression systems. Many systems for recombinant protein production have been developed, but none are universally applicable (Masaki *et al.*, 2012). In this study, a patented *Yarrowia lipolytica* expression system (Bragg *et al.*, 2017) was used to express the two ScFvs. The

VWFpp was expressed first, as it is not commercially available. Unfortunately, neither the VWFpp nor the two ScFvs could be expressed. This is because the VWFpp is too large for easy expression as it has a molecular weight of 95 kDa (Sadler, 1991). Furthermore, the VWFpp consists of 2 homologous cysteine-rich D domains, with 32 cysteines in each of these domains (Rosenberg *et al.*, 2002). Intra-chain disulfide bonds form between cysteines in a protein (Shewry and Tatham, 1997). It is known that a primary bottleneck in recombinant protein production is due to the presence of the disulfide bond structure (MacDonald *et al.*, 2016).

Next, two polyclonal antibodies were produced against a truncated form of the VWFpp. Since the VWFpp is large and contains a number of cysteines with intra-chain disulfide bonds, a truncated form was expressed in *E. coli,* and the two polyclonal antibodies were produced in rabbits. As with phage display, the cost for the production of polyclonal antibodies is also low (Lipman *et al.*, 2005).

An interesting finding was that these two polyclonal antibodies only detected the truncated VWFpp, but they could not detect the VWFpp in normal pooled plasma. Therefore, the polyclonal antibodies could not be used for the measurement of VWFpp levels in plasma. To confirm this, the commercial monoclonal antibody pair (CLB-Pro 35 and CLB-Pro 14.3) was tested for binding to the tVWFpp and to VWFpp in normal pooled plasma. The commercial antibody pair only binded to the VWFpp in normal pooled plasma, but could not bind to the truncated form of the VWFpp. It is not uncommon that monoclonal antibodies to a full length protein do not bind to truncated proteins due to the different tertiary structure of truncated proteins. Reduced antigenicity of the truncation thus affects epitope construction (Hosamani *et al.*, 2011).

The third effort was to develop a lateral flow assay (LFA) for the measurement of VWFpp levels in plasma, since the polyclonal antibodies could not be used to measure VWFpp levels. LFAs are low cost detection devices that are simple to use, rapid and portable. They are commonly used in biomedicine, agriculture, food and environmental sciences. The advantage of this assay in diagnostics is that patients can be rapidly diagnosed to receive the correct treatment on time (Koczula and Gallotta, 2016).

The LFA from Bioporto Diagnostics was used in this study, since this assay can be used as a semi-quantitative assay which is calibrated with a standard. The commercial CLB-Pro 35 antibody and a polyclonal antibody that detects either the mature VWF protein or the VWFpp were used as antibodies in the assay. Currently, colloidal gold is the most commonly used label in commercial LFAs. Although it can be prepared in the laboratory at low cost, many commercial sources are available. Colloidal gold has an intense colour and no development process is needed for visualization. Furthermore, it has high stability in both liquid and dried forms (Koczula and Gallotta, 2016). When the VWF/VWFpp polyclonal antibody was gold-labelled, an immune complex was formed between the two antibodies, however there was no concentration dependent effect on the test line. The gold-labelled antibody did not bind specific to the VWFpp as it can bind to both the full-length VWF protein and to the VWFpp. It is also known that polyclonal antibodies show higher cross reactivity, since they consist of a heterogeneous mixture of antibodies that bind to multiple epitopes on their target proteins (Nollens *et al.*, 2008). Even when the antibodies were swopped by biotinlylating the VWFpp polyclonal antibody and using it as the capture antibody, and then gold-labelling CLB-Pro 35 and using it as the detection antibody, no concentration-dependent colour change was observed on the test line. This LFA could therefore not be validated. Most rapid assays currently only use monoclonal antibodies (Chen *et al.*, 2014; Kusano *et al.*, 2007). There are however no other available commercial monoclonal antibodies to the VWFpp. This rapid VWFpp strip assay would be successful if two different monoclonal antibodies that bind to different epitopes on the VWFpp could be used. The commercial monoclonal antibody pair (CLB-Pro 35 and CLB-Pro 14.3) could not be used together, since CLB-Pro 14.3 is HRP-conjugated and such antibodies cannot be used in this LFA. This is because an HRP-conjugated antibody cannot be gold-conjugated and the detection antibody needs to be gold-conjugated.

In the last part of this study, a rapid sandwich-style ELISA was developed and validated. This test is almost similar to the conventional assay kit from Cellsciences, but the incubation times were much shorter (30 minutes instead of 2 hours). The same antibodies and antibody concentrations were used in the rapid assay. A good correlation was found between the two methods, with an R square value of 0.95. The linear regression equation was y= 0.98X+0.02. The slope of this linear regression

was 0.98, which is close to 1 that is also included within the 95 % confidence interval. The y-intercept of this linear regression was 0.02, thus close to zero, and is also included within the 95 % confidence interval.

The validation data performed over 5 consecutive days using two samples, showed an inter-assay CV of 9.94 % for the first sample and 5.88 % for the other one, and an intra-assay CV of 9.54 % for the first sample and 4.70 % for the second sample. These coefficient of variation values are both within acceptable criteria as they are less than 15 % according to the Food and Drug Administration guideline (2013). The intra-assay precision plots showed that most of the data-points of both samples were within the 2SD range.

Despite all the challenges that were faced in this study, a rapid VWFpp ELISA using the commercial antibody pair clone CLB-Pro 35 and CLB-Pro 14.3 was developed and validated. This rapid ELISA test has equal sensitivity and precision as the commercial ELISA kit method and it is 78 % faster than the commercial method. Therefore, this assay can be used to rapidly diagnose patients with increased VWF clearance.

Chapter 7: Conclusion

In this study, several attempts were made to produce antibodies to the VWFpp. The success of this study was hampered due to a lack of a functional antigen. However, a rapid VWFpp ELISA with reduced incubation times using the commercial antibody pair was developed and validated. The commercially available assay is very time consuming. It uses two hour incubation times and our rapid ELISA method uses 30 minute incubation times. This rapid ELISA test showed similar sensitivity and precision as the commercial ELISA kit method and can therefore be used to rapidly diagnose patients with increased VWF clearance. Furthermore, the reporting of patient results to clinicians can be done much faster, as clinicians need immediate answers on the diagnosis of their patients.

Chapter 8: Future studies

In a follow-up study, monoclonal antibodies to the VWFpp will be selected in order to develop a rapid lateral flow assay or a rapid ELISA to measure VWFpp levels in plasma. The cell lines of these monoclonal antibodies can be reused, which will make this assay to be more cost-effective than the commercial assay.

The rapid ELISA assay can be further improved by pre-coating the ELISA microplates in a storage buffer so that the assay can be used on demand.

References

't Hoen P.A.C., Jirka S.M.G., Ten Broeke B.R., Schultes E.A. Aguilera B., Pang K.H., Heemskerk H., Aartsma-rus A., Van Ommen G.J., Den Dunnen J.T. (2012). Phage display screening without repetitious selection rounds. Analytical Biochemstry. 421: 622-631.

Abcam. Sandwich ELISA. Avaliable from: http://www.abcam.com/ps/pdf/protocols/Sandwich_ELISA.pdf. [Accessed on: 16/09/2016].

Allen S., Abuzenadah A.M., Hinks J., Blagg J.L., Gursel T., Ingerslev J., Goodeve A.C., Peake I.R., Daly M.E. (2000). A novel von Willebrand disease-causing mutation (Arg273Trp) in the von Willebrand factor propeptide that results in defective mutimerization and secretion. Blood. 96: 560-568.

American society for microbiology. Antimicrobial Agents and Chemotherapy. Development of Anti-infectives using phage display: biological agents against bacteria, viruses, and parasites. [Image]. Available from: http://aac.asm.org/content/56/9/4569/F3.expansion.html. [Accessed on: 06/02/2018].

Bahadir E.B., Sezgintürk M.K. (2016). Lateral flow assays: Principles, designs and labels. Trends in Analytical Chemistry. 82: 286-306.

Berntorp E. (2007). Erik von Willebrand. Thrombosis Research. 120: S3-S4.

Bharati K.P., Prashanth U.R. (2011). von Willebrand disease: An overview. Indian Journal of Pharmaceutical Sciences. 73(1): 7-16.

Bioporto diagnostics. gRAD OneDetection Kit. gRAD(1)2015-09. [Image]. Available from: http://www.bioporto.com/Files/Images/Fra%20Files/Marketing-material/IFU-gRAD-OneDection-Kit-Nov-2015_3.pdf . [Accessed on: 11/05/2017].

Boster Biological Technology. ELISA handbook. Principle, troubleshooting, sample preparation, and assay protocols. Available from: http://www.bosterbio.com/media/pdf/ELISA_Handbook.pdf. [Accessed on: 17/09/2016].

Bowie E.J., Solberg Jr L.A., Fass D.N., Johnson C.M., Knutson G.J., Stewart M.L., Zoecklein L.J. (1986). Transplantation of normal bone marrow into a pig with severe von Willebrand's disease. Journal of Clinical Investigation. 78: 26-30.

Bragg R.R., Boucher C.E., Theron C.W., Hitzeroth A.C. (2017). Production of polypeptides relevant to human and animal health using Yarrowia Lipolytica. United States Patent Application Publication. US 2017/0002044 A1.

Branchford B.R., Di Paola J. (2012). Making a diagnosis of VWD. Hematology. 161-167.

Burd E.M. (2010). Validation of laboratory-developed molecular assays for infectious diseases. Clinical Microbiology Reviews. 23(3): 550-576.

Burton D.R. (1995). Phage display. Immunotechnology. 1: 87-94.

Canacopegdl. ELISA test. [Image]. Available from: http://canacopegdl.com/synonym/ELISA-test.html. [Accessed on: 07/02/2018].

Cannon III R.O. (1998). Role of nitric oxide in cardiovascular disease: focus on the endothelium. Clinical Chemistry. 44(8)B: 1809-1819.

Carmen S., Jermutus L. (2002). Concepts in antibody phage display. Briefings in Functional Genomics and Proteomics. 1(2): 189-203.

Casa L.D.C., Deaton D.H., Ku D.N. (2015). Role of high shear rate in thrombosis. Journal of Vascular Surgery. 61: 1068-1080.

Casari C., Lenting P.J., Wohner N., Christophe O.D., Denis C.V. (2013). Clearance of von Willebrand factor. Journal of Thrombosis and Haemostasis. 11(1): 202-211.

Cha H.J., Shin H.S., Lim H.J., Cho H.,S., Dalal N.N., Pham M.Q., Bentley W.E. (2005). Comparative production of human interleukin-2 fused with green fluorescent protein in several recombinant expression systems. Biochemical Engineering Journal. 24: 225-233.

Chen H., Ou Q., Tang Y., Gao X., Wu L., Xue C., Yu C., Cui J., Diao Y. (2014). Development and evaluation of a DAS-ELISA for rapid detection of Tembusu virus using monoclonal antibodies against the envelope protein. PLOS ONE. 9(5): e96366.

Clinical and Laboratory Standards Institute. (2003). Collection, transport, and processing of blood specimens for testing plasma-based coagulation assays. Available from: http://www.zxyjhjy.com/upload/attached/file/20170406/20170406163530_9760.pdf. [Accessed on: 12/06/2018].

Crawley J.T.B., Scully M.A. (2013). Thrombotic thrombocytopenic purpura: basic pathophysiology and therapeutic strategies. Hematology. 292-299.

Davies J.A., Collins P.W., Hathaway L.S., Bowen D.J. (2008). von Willebrand factor: evidence for variable clearance *in vivo* according to Y/C1584 phenotype and ABO blood group. Journal of Thrombosis and Haemostasis. 6: 97-103.

Dayananda K.M., Gogia S., Neelamegham S. (2011). *Escherichia coli*-derived von Willebrand factor-A2 domain fluorescence/förster resonance energy transfer proteins that quantify ADAMTS13 activity. Analytical Biochemistry. 410: 206-213.

De Wit T.R., Van Mourik J.A. (2001). Biosynthesis, processing and secretion of von Willebrand factor: biological implications. Best Practise and Research Clinical Haematology. 14: 241-255.

Denis C.V., Christophe O.D., Oortwijn B.D., Lenting P.J. (2008). Clearance of von Willebrand factor. Thrombosis and Haemostasis. 99: 271-278.

Di Stasio E., Lancellotti S., Peyvandi F., Palla R., Mannucci P.M., De Christofaso R. (2008). Mechanistic studies on ADAMTS13 Catalysis. Biophysical Journal. 95: 2450-2461.

European Medicines Agency. (2006). Part I: validation of analytical procedures: definitions and methodology. ICH harmonised tripartite guideline. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002662.pdf. [Accessed on: 06/07/2016].

Fairbanks G., Steck T.L., Wallach D.F.H. (1971). Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry. 10(13): 2606-2617.

Favaloro E.J. (2001). Appropriate laboratory assessment as a critical facet in the proper diagnosis and classification of von Willebrand disorder. Best Practice and Research Clinical Haematology. 14 (2): 299-319.

Favaloro E.J. (2011). Rethinking the diagnosis of von Willebrand disease. Thrombosis Research. 127 (2): S17-S21.

Favaloro E.J. (2016). Towards personalised therapy for von Willebrand disease: a future role for recombinant products. Blood Transfusion. 14: 262-276.

Favaloro E.J., Mohammed S. (2014). Towards improved diagnosis of von Willebrand disease: Comparative evaluations of several automated von Willebrand factor antigen and activity assays. Thrombosis Research. 134: 1292-1300.

Federici A.B. (2006). VWF propeptide: a useful marker in VWD. Blood. 108(10): 3229-3230.

Federici A.B. (2009). Classification of inherited von Willebrand disease and implications in clinical practice. Thrombosis Research. 124 (1): S2-S6.
Flood V.H. (2014). New insights into the genotype and phenotype of VWD. Hematology. 531-535.

Food and Drug Administration. (2013). Guidance for industry bioanalytical method validation. Available from: http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm368107.pdf. [Accessed on: 07/07/2016].

Frankel D.S., Meigs J.B, Massaro J.M., Wilson P.W.F., O'Donnell C.J., D'Agostino R.B., Tofler G.H. (2008). von Willebrand factor, type 2 diabetes mellitus, and risk of cardiovascular. The Framingham offspring study. Circulation. 118: 2533-2539.

Gardner M.D., Chion C.K.N.K., de Groot R., Shah A., Crawley J.T.B., Lane D.A. (2008). A functional calcium binding site in the metalloprotease domain of ADAMTS13. Blood. 1-20.

GE Healthcare. (2010). Strategies for protein purification. Handbook. Available from: http://wolfson.huji.ac.il/purification/PDF/Others/GE_StratProtPurificHanbook.pdf. [Accessed on: 09/04/2017].

GE Healthcare. (2015). Protein purification methods. Handbook. Available from: http://www.processdevelopmentforum.com/ppts/posters/Protein_purification_methods-_overview,_29155460.pdf. [Accessed on: 30/04/2017].

Genecards. VWF gene (protein coding). [Image]. Available from: http://www.genecards.org/cgi-bin/carddisp.pl?gene=VWF. [Accessed on: 10/08/2016].

Gera N., Hussain M., Rao B.M. (2013). Protein selection using yeast surface display. Methods. 60: 15-26.

Gill J.C. (2014). Diagnosis of von Willebrand disease in people with type O blood. Clinical Advances in Hematology and Oncology. 12(2): 119-121.

Goodeve A., Eikenboom J., Castaman G., Rodeghiero F., Federici A.B., Batlle J., Meyer D., Mazurier C., Goudemand J., Schneppenheim R., Budde U., Ingerslev J., Habart D., Vorlova Z., Holmberg L., Lethagen S., Pasi J., Hill F., Soteh H.M., Baronciani L., Hallden C., Guilliatt A., Lester W., Peake I. (2007). Phenotype and genotype of a cohort of families historically diagnosed with type 1von Willebrand disease in the European study, molecular and clinical markers for the diagnosis and management of type 1 von Willebrand disease (MCMDM-1VWD). Blood. 109: 112-121.

Gragnano F., Sperlongano S., Golia E., Natale F., Bianchi R., Crisci M., Fimiani F., Pariggiano I., Diana V., Carbone A., Cesaro A., Concilio C., Limongelli G., Russo M., Calabrò P. (2017). The role of von Willebrand factor in vascular inflammation: from pathogenesis to targeted therapy. Mediators of Inflammation. 1-13.

Haberichter S.L. (2015a). von Willebrand factor propeptide: biology and clinical utility. Blood. 126 (15): 1753-1761.

Haberichter S.L. (2015b). VWF propetide in defining VWD subtypes. Blood. 125: 2882-2883.

Haberichter S.L., Balisteri M., Christopherson P., Morateck P., Gavazova S., Bellissimo D.B., Manco-Johnson M.J., Gill J.C., Montgomery R.R. (2006). Assay of the von Willebrand factor (VWF) propeptide to identify patients with type 1von Willebrand disease with decreased VWF survival. Blood. 108: 3344-3351.

Haberichter S.L., Jacobi P., Montgomery R.R. (2003). Critical independent regions in the VWF propeptide and mature VWF that enable normal VWF storage. Blood. 101: 1384-1391.

Hanly W.C., Artwohl J.E., Bennett B.T. (1995). Review of polyclonal antibody production procedures in mammals and poultry. ILAR Journal. 37(3): 93-118.

Hassan R., Yusof W.A.W., Hussain N.H.N., Abdullah W.Z. (2012). Single-center experience of von Willebrand disease (VWD) among patients with menorrhagia: a diagnosis which could be missed. Indian Journal of Hematology and Blood Transfusion. 28 (3): 157-161.

Health and Human Services. (2014). Evaluation of a von Willebrand factor and von Willebrand factor propeptide in models of vascular endothelial activation, perturbation, and/or injury. Toxicologic Pathology. 42(4): 672-683.

Hollestelle M.J., Donkor C., Mantey E.A., Chakravorty S.J., Craig A., Akoto A.O., O'Donnell J., Van Mourik J.A., Bunn J. (2006). von Willebrand factor propeptide in malaria: evidence of acute endothelial cell activation. British Journal of Haematology. 133: 562-569.

Hosamani M., Shimizu S., Hirota J., Kokuho T., Kubota T., Watanabe S., Ohta M., Muneta Y., Inumaru S. (2011). Expression and characterization of Bluetongue virus serotype 21 VP7 antigen: C-terminal truncated protein has significantly reduced antigenicity. The Journal of Veterinary Medical Science. 73(5): 609-613.

Hospital Physician Hematology Board Review Manual. (2014). von Willebrand disease: approach to diagnosis and management. Hematology. 9(1): 1-12.

Hubbard A.R., Hamill M., Eikenboom H.C.J., Montgomery R.R., Mertens K., Haberichter S. (2012). Standardization of von Willebrand factor propeptide: value assignment to the WHO 6th IS Factor VIII/von Willebrand factor, plasma (07/316). Journal of Thrombosis and Haemostasis. 10: 959-960.

Kamionka M. (2011). Engineering of therapeutic proteins production in *Escherichia coli*. Current Pharmaceutical Biotechnology. 12: 268-274.

Keesler D.A., Flood V.H. (2018). Current issues in diagnosis and treatment of von Willebrand disease. Research and Practice in Thrombosis and Haemostasis. 2:34-41.

Khow O., Suntrarachun S. (2012). Strategies for production of active eukaryotic proteins in bacterial expression system. Asian Pacific Journal of Tropical Biomedicine. 2(2): 159-162.

Kobayashi T., Wada H., Nishioka N., Yamamoto M., Matsumoto T., Tamaru T., Nomura S., Masuya M., Mori Y., Nakatani K., Nishikawa M., Katayama N., Nobori T. (2008). ADAMTS13 related markers and von Willebrand factor in plasma from patients with thrombotic microangiopathy (TMA). Thrombosis Research. 121: 849-854.

Koczula K.M., Gallotta A. (2016). Lateral flow assays. Essays in Biochemistry. 60: 111-120.

Kong B., Guo G.L. (2014). Soluble expression of disulfide bond containing proteins FGF15 and FGF19 in the cytoplasm of *Escherichia coli*. PLOS ONE. 9(1): e85890.

Kumari A., Boronian K., Kunze G., Gupta R. (2015). Extracellular expression of YlLip 11 with a native signal peptide from *Yarrowia Lipotytica* MSR80 in three different yeast hosts. Protein Expression and Purification. 110: 138-144.

Kusano N., Hirashima K., Kuwahara M., Narahara K., Imamura T., Mimori T., Nakahira K., Torii K. (2007). Immunochromatographic assay for simple and rapid detection of *Satsuma dwarf virus* and related viruses using monoclonal antibodies. Journal of General Plant Pathology. 73: 66-71.

Laffan M., Brown S.A., Collins P.W., Cumming A.M., Hill F.G.H., Keeling D., Peake I.R., Pasi K.J. (2004). The diagnosis of von Willebrand disease: a guideline from the UK haemophilia centre doctors' Organization. Haemophilia. 10: 199-217.

Lavanya G., Sunil M., Eswarudu M.M., Eswaraiah M.C., Harisudha K., Spandana B., N. (2013). Analytical method validation: an updated review. International Journal of Pharmaceutical Sciences and Research. 4(4): 1280-1286.

Ledford-Kraemer M.R. (2010). Analysis of von Willebrand factor structure by multimer analysis. American Journal of Hematology. 85: 510-514.

Leenaars M., Hendriksen C.F.M. (2005). Critical steps in the production of polyclonal and monoclonal: evaluation and recommendations. ILAR journal. 46(3): 269-279.

Lenting P.J., Van Schooten C.J.M., Denis C.V. (2007). Clearance mechanisms of von Willebrand factor and factor VIII. Journal of Thrombosis and Haemostasis. 5: 1353-1360.

Leyte A., Voorberg J., Van Schijndel H.B., Duim B., Pannekoek H., Van Mourik J.A. (1991). The pro-polypeptide of von Willebrand factor is required for the formation of a functional Factor VIII-binding site on the mature von Willebrand factor. Biochemical Journal. 274: 257-261.

Lillicrap D. (2005). The molecular genetics of von Willebrand disease. Haematologica Reports. 1 (6): 3-8.

Lillicrap D. (2007). von Willebrand disease-phenotype versus genotype: deficiency versus disease. Thrombosis Research. 120: S11-S16.

Lin-Cereghino J., Wong W. W., Xiong S., Giang W., Luong L.T., Vu J., Johnson S. D., Lin-Cereghino G. P. (2005). Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast Pichia pastoris. Biotechniques 38(1): 44–48.

Lipman N.S., Jackson L.R., Trudel L.J., Weis-Garcia F. (2005). Monoclonal versus polyclonal antibodies: distinguishing characteristics, applications, and information resources. ILAR Journal. 46(3): 258-268.

Lumenlearning. Polyclonal and monoclonal antibody production. [Image]. Available from: https://courses.lumenlearning.com/microbiology/chapter/polyclonal-and-monoclonal-antibody-production/. [Accessed on: 31/08/2017].

MacDonald N.J., Nguyen V., Shimp R., Reiter K., Herrera R., Burkhardt M., Muratova O., Kumar K., Aebig J., Rausch K., Lambert L., Dawson N., Sattabongkot J., Ambroggio X., Duffy P.E., Wu Y., Narum D.L. (2016). Structural and immunological characterization of recombinant 6-cysteine domains of the *Plasmodium falciparum* sexual stage protein Pfs230. The Journal of Biological Chemistry. 291(38): 19913-19922.

Magari R.T. (2002). Statistics for laboratory method comparison studies. Biopharm. 28-32.

Mancuso D.J., Tuley E.A., Westfield L.,A., Worrall N.K., Shelton-Inloes B.B., Sorace J.M., Alevy Y.G., Sadler J.E. (1989). Structure of the gene for human von Willebrand factor. The Journal of Biological Chemistry. 264(33): 19514-19527.

Marianor M., Zaidah A.W., Maraina C.H.C. (2015). von Willebrand factor propeptide; a potential disease biomarker not affected by ABO blood groups. Biomarker Insights. 10:75-79.

Masaki K., Tsuchioka H., Hirano T., Kato M., Ikeda H., Iefuji H. (2012). Construction of a new recombinant protein expression system in the basidiomycetous yeast *Cryptococcus* sp. strain S-2 and enhancement of the production of a cutinase-like enzyme. Applied Genetics and Molecular Biotechnology. 93: 1627-1636.

Matsukawa M., Kaikita K., Soejima K., Fuchigami S., Nakamura Y., Honda T., Tsujita K., Nagayoshi Y., Kojima S., Shimomura H., Sugiyama S., Fujimoto K., Yoshimura M., Nakagaki T., Ogawa H. (2007). Serial changes in von Willebrand factor-cleaving protease (ADAMTS13) and prognosis after acute myocardial infarction. The American Journal of Cardiology. 100: 758-763.

Meiring M., Badenhorst P.N., Kelderman M. (2009). Laboratory diagnosis of von Willebrand disease. European Haematology. 33-36.

Meiring M., Coetzee M., Kelderman M., Badenhorst P. (2011). Laboratory diagnosis and management of von Willebrand disease in South Africa. Seminars in Thrombosis and Hemostasis. 37(5): 576-580.

Meiring M., Haupt L., Conradie C., Joubert J. (2017). Challenges in the laboratory diagnosis and management of von Willebrand disease in South Africa. Annals of Blood. 2: 19

Meiring M., Webb M., Goedhals D., Louw V. (2012). HIV-associated thrombotic thrombocytopenic purpura-what we know so far. European Oncology and Haematology. 8(2): 89-91.

Mikhail S., Kouides P. (2010). von Willebrand disease in the pediatric and adolescent population. Journal of Pediatric and Adolescent Gynecology. 23: S3-S10.

Montgomery R.R., Zimmerman T.S. (1978). von Willebrand's disease antigen II. A new plasma and platelet antigen deficient in severe von Willebrand's disease. Journal of Clinical Investigation. 1498-1507.

NanoComposix. (2016). Lateral flow assay development guide. V 1.0. Handbook. Available from: https://cdn.shopify.com/s/files/1/0257/8237/files/BioReady_Lateral_Flow_Handbook_v-1.0.pdf?4600895763011236291. [Accessed on: 04/08/2017].

National Institutes of Health. (2008). Protein production and purification. Nature Methods. 5(2): 135-146.

National Institutes of Health. (2011). von Willebrand disease. Genetics in Medicine. 13(5): 365-376.

NCBI. von Willebrand factor preproprotein [homo sapiens]. [Image]. Available from: http://www.ncbi.nlm.nih.gov/protein/NP_000543.2. [Accessed on: 17/08/2016].

Ng C., Motto D.G., Di Paola J. (2015). Diagnostic approach to von Willebrand disease. Blood. 125: 2029-2037.

Nicaud J., Madzak C., Van den Broek P., Gysler C., Duboc P., Niederberger P., Gaillardin C. (2002). Protein expression and secretion in the yeast *Yarrowia Lipotytica*. FEMS Yeast Research. 2: 371-379.

Nilsson I.M. (1999). The history of von Willebrand disease. Haemophilia. 5: 7-11.

Nollens H. H., Ruiz C., Walsh M.T., Gulland F.M.D., Bossart G., Jensen E.,D., McBain J.F., Wellehan J.F.X. (2008). Cross-reactivity between Immunoglobulin G antibodies of whales and dolphins correlates with evolutionary distance. Clinical and Vaccine Immunology. 15(10): 1547-1554.

OIE Terrestrial Manual. (2013). Chapter 1.1.5. Principles and methods of validation of diagnostic assays for infectious diseases. Available from: http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2008/pdf/1.01.05_VALIDATION.pdf. [Accessed on: 21/09/2016].

Payandeh M., Rahimi Z., Kansestani A.N., Hemmati S., Aleyasin M., Zare M.E, Nouri Z., Hashemian A.H., Gohardehi F. (2013). Clinical features and types of von Willebrand disease in women with menorrhagia referred to hematology clinic of Kermanshah. International Journal of Hematology- Oncology and Stem Cell Research. 7 (2): 1-5.

Peyvandi F., Garagiola I., Baronciani L. (2011). Role of von Willebrand factor in the haemostasis. Blood Transfusion. 9: S3-S8.

Pierce. (2003). Protein stability and storage. Technical resource. Available from: http://wolfson.huji.ac.il/purification/PDF/StorageProteins/PIERCE_ProteinStorage.pdf . [Accessed on: 07/02/2018].

Pimanda J., Hogg P. (2002). Control of von Willebrand factor multimer size and implications for disease. Blood Reviews. 16: 185-192.

Posthuma-Trumpie G.A., Korf J., Van Amerongen A. (2009). Lateral (immuno)assay: its strengths , weaknesses, opportunities and threats. A literature survey. Analytical and Bioanalytical Chemistry. 393: 569-582.

Pozzi N., Lancellotti S., De Cristofaro R., De Filippis V. (2012). Modeling ADAMTS13-von Willebrand factor interaction: implications for oxidative stress-related cardiovascular diseases and type 2A von Willebrand disease. Biophysical Chemistry. 160: 1-11.

Preston R.J.S., Rawley O., Gleeson E.M., O'Donnell J.S. (2013). Elucidating the role of carbohydrate determinants in regulating haemostasis – insights and opportunities. Blood. 1-30.

Purvis A.R., Sadler J.E. (2004). A covalent oxidoreductase intermediate in propeptide-dependent von Willebrand factor multimerization. The Journal of Biological Chemistry. 279(48): 49982-49988.

Rafdzah Z., Bulgiba A., Ismail N.A. (2013). Method comparison studies in medicine. JUMMEC. 16(1): 1-7.

Rajendran P., Rengarajan T., Thangavel J., Nishigaki Y., Sakthisekaran D., Sethi G., Nishigaki I. (2013). The vascular endothelium and human diseases. International Journal of Biological Sciences. 9(10): 1057-1069.

Robichon C., Luo J., Causey T.B., Benner J.S., Samuelson J.C. (2011). Engineering *Escherichia coli* BL21(DE3) derivate strains to minimize *E.coli* protein contamination after purification by immobilized metal affinity chromatography. Applied and Environmental Microbiology. 77(13): 4634-4646.

Rodeghiero F. (2013). von Willebrand disease: pathogenesis and management. Thrombosis Research. 1: S47-S50.

Rosenberg J.B., Haberichter S.L., Jozwiak M.A., Vokac E.A., Kroner P.A., Fahs S.A., Kawai Y., Montgomery R.R. (2002). The role of the D1 domain of the von Willebrand factor propeptide in multimerization of VWF. Blood. 100: 1699-1706.

Ruggeri Z.M. (1999). Structure and function of von Willebrand factor. Thrombosis and Haemostasis. 82: 576-584.

Ruggeri Z.M. (2003). von Willebrand factor. Current Opinion in Hematology. 10: 142-149.

Ruggeri Z.M. (2007). The role of von Willebrand factor in thrombus formation. Thrombosis Research. 120. S5-S9.

Ruggeri Z.M., Ware J. (1993). von Willebrand factor. Faseb journal. 7: 308-316.

Sadler J.E. (1991). von willebrand factor. The Journal of Biological Chemistry. 266(34): 22777-22780.

Sadler J.E., Gralnick H.R. (1994). A new classification for von Willebrand disease. Blood. 84(3): 676-679.

Sajid M., Kawde A., Daud M. (2015). Designs, formats and applications of lateral flow assay: a literature review. Journal of Saudi Chemical Society. 19: 689-705.

Sambrook J., Russel D. (2001). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Sanders Y.V, Groeneveld D., Meijer K, Fijnvandraat K., Cnossen M.H.,Van der Bom J.G., Coppens M., De Meris J., Laros-van Gorkom B.A.P.,Mauser-Bunschoten E.P., Leebeek F.W.G., Eikenboom J, and the WiN study group. (2015). von Willebrand factor propeptide and the phenotypic classification of von Willebrand disease. Blood. 125 (19): 3006-3013.

Scheja A., Ákesson A., Geborek P., Wildt M., Wollheim C.B., Wollheim F.A., Vischer U.M. (2001). von Willebrand factor propeptide as a marker of disease activity in systemic sclerosis (scleroderma). Arthritis Research. 3: 178-182.

Schneppenheim R. (2011). The pathophysiology of von Willebrand disease: therapeutic implications. Thrombosis Research. 128 (1): S3-S7.

Setlai B.D.L. (2014). Development of an ELISA (enzyme linked immunosorbent assay) to measure VWF porpeptide levels in plasma. Masters dissertation. University of the Free State.

Sharma R., Flood V.H. (2017). Advances in the diagnosis and treatment of von Willebrand disease. Blood. 1-23.

Shewry P.R., Tatham A.S. (1997). Disulphide bonds in wheat gluten proteins. Journal of Cereal Science. 25: 207-227.

Spadiut O., Capone S., Krainer F., Glieder A., Herwig C. (2014). Microbials for the production of monoclonal antibodies and antibody fragments. Trends in Biotechnology. 32(1): 54-60.

Spasovski G., Vanholder R., Allolio B., Annane D., Ball S., Bichet D., Decaux G., Fenske W., Hoorn E. J., Ichai C., Joannidis M., Soupart A., Zietse R., Haller M., Van der Veer S., Van Biesen W., Nagler E. (2014). Clinical practice guideline on diagnosis and treatment of hyponatraemia. European Journal of Endocrinology. 170 (3): G1-G47.

Strong J. (2006). von Willebrand disease and pregnancy. Current Obstetrics and Gynaecology. 16: 1-5.

Sumpio B.E., Riley J.T., Dardik A. (2002). Cells in focus: endothelial cell. The International Journal of Biochemistry and Cell Biology. 34: 1508-1512.

Thermo Scientific. (2011). Thermo Scientific Pierce assay development technical handbook. Version 2. Available from: https://tools.thermofisher.com/content/sfs/brochures/1602127-Assay-Development-Handbook.pdf. [Accessed on: 16/09/2016].

Trombetta C.M., Perini D., Vitale L., Cox R.J., Stanzani V., Piccirella S., Montomoli E. (2015). Validation of a single radial haemolysis assay: a reliable method to measure antibodies against influenza viruses. Journal of Immunological Methods.422:95-101.

Tsai H., Sarode R., Downes K.,A. (2003). Ultralarge von Willebrand factor multimers and normal ADAMTS13 activity in the ambilical cord blood. Thrombosis Research. 108: 121-125.

Valentijn K.M., Eikenboom J. (2013). Weibel-Palade bodies: a window to von Willebrand disease. Journal of Thrombosis and Haemostasis. 11: 581-592.

Van Mourik J.A., Boertjes R., Huisveld I.A., Fijnvandraat K., Pajkrt D., Van Genderen P.J.J., Fijnheer R. (1999). von Willebrand factor propeptide in vascular disorders: a tool to distinguish acute and chronic endothelial cell perturbation. Blood. 94(1): 179-185.

Van Schooten C.J., Shahbazi S., Groot E., Oortwijn B.D., Van den Berg H.M., Denis C.V. Lenting P.J. (2008). Macrophages contribute to the cellular uptake of von Willebrand factor and factor VIII in vivo. Blood. 112: 1704-1712.

Van Schooten C.J.M., Denis C.V., Lisman T., Eikenboom J.C.J., Leebeek F.W. (2007). Variations in glycosylation of von Willebrand factor with O-linked sialylated T antigen are associated with its plasma levels. Blood. 109: 2430-2437.

Verbalis J.G., Goldsmith S.R., Greenberg A., Korzelius C., Schrier R.W., Sterns R.H., Thompson C.J. (2013). Diagnosis, evaluation, and treatment of hyponatremia: expert panel recommendations. The American Journal of Medicine. 126: S1-S42.

Veterian Key. Bleeding and hemostasis. [Image]. Available from: https://veteriankey.com/bleeding-and-hemostasis/. [Accessed on: 02/02/2018].

Villar A., Jimenez-Yuste V., Quintana M., Hernandez-Navarro F. (2002). The use of haemostatic drugs in haemophilia: desmopressin and antifibrinolytic agents. Haemophilia. 8: 189-193.

Villaverde A., Carrio M.M. (2003). Protein aggregation in recombinant bacteria: biological role of inclusion bodies. Biotechnology Letters. 25: 1385-1395.

Vischer U.M., Emeis J.J., Bilo H.J.G., Stehouwer C.D.A., Thomsen C., Rasmussen C., Hermansen K., Wollheim C.B., Ingerslev J. (1998). von Willebrand factor (VWF) as a plasma marker of endothelial activation in diabetes: improved reliability with parallel determination of the VWF propeptide (VWF:AgII). Thrombosis and Haemostasis. 80: 1002-1007.

Vischer U.M., Ingerslev J., Wollheim C.B., Mestries J., Tsakiris D.A., Haefeli W.E., Kruithof E.K.O. (1997). Acute von Willebrand factor secretion from the endothelium in vivo: assessment through plasma propeptide (VWF:AgII) levels. Thrombosis and Haemostasis. 77(2): 387-393.

World Health Organization. (2011). Laboratory quality standards and their implementation. Available from: http://apps.who.int/medicinedocs/documents/s22409en/s22409en.pdf?ua=1. [Accessed on: 19/03/2018].

World Health Organization. (2017). Cardiovascular diseases (CVDs). Available from: http://www.who.int/mediacentre/factsheets/fs317/en/. [Accessed on: 05/02/2018].

Yawn B.P., Nichols W.L., Rick M.E. (2009). Diagnosis and management of von Willebrand disease: guidelines for primary care. American Academy of Family Physicians. 80 (11): 1261-1268.

Yuzbasheva E.Y., Yuzbashev T.V., Gvilava I.T., Sineoky S.P. (2012). Protein display on the *Yarrowia lipolytica* yeast cell surface using the cell wall protein YlPir1. Applied Biochemistry and Microbiology. 48(7): 650-655.

Zhang Z., Kuipers G., Niemiec Ł., Baumgarten T., Slotboom D.J., De Gier J., Hjelm A. (2015). High-level production of membrane proteins in *E.coli* BL21(DE3) by omitting the inducer IPTG. Microbial Cell Factories. 14(142): 1-11.

Zheng X., Chung D., Takayama T.K., Majerus E.M., Sadler J.E. Fujikawa K. (2001). Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura. Journal of Biological Chemistry. 276: 41059-41063.

Appendix A: VWFpp levels with the Rapid ELISA

Raw data for inter- and intra-assay precision

	(A)	(B)
DAY 1	85.13	83.99
	89.28	85.88
	90.04	84.37
	71.74	88.15
DAY 2	84.71	90.47
	78.18	89.23
	88.00	99.18
	86.35	92.12
DAY 3	105.04	91.82
	109.61	90.70
	103.90	92.95
	103.14	88.82
DAY 4	90.54	99.09
	96.69	90.20
	91.90	90.20
	99.09	91.56
DAY 5	90.56	92.44
	93.57	92.44
	90.93	105.36
	94.71	100.40

(A)= WHO 6th IS for FVIII/VWF in plasma sample, (B)= type 1 VWD patient sample.