# THE CORRELATION BETWEEN THE ABO BLOOD GROUP, PLASMA VON WILLEBRAND FACTOR AND THE RISTOCETIN COFACTOR ACTIVITY

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BLOEMFONTEIN

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## DECLARATION OF INDEPENDENT WORK

I, **MOJALEFA BRIAN SETLAI**, hereby declare that this research project submitted to the Central University of Technology, Free State, for the degree MAGISTER TECHNOLOGIAE: BIOMEDICAL TECHNOLOGY, is my own independent work that has not been submitted to any institution before, by me or any other person in fulfilment of the requirements for the attainment of any qualification.

Signature of Student

31/08/2020
Date

## VERKLARING VAN ONAFHANKLIKE WERK

Ek, **MOJALEFA BRIAN SETLAI**, verklaar hiermee dat die navorsingsprojek wat vir die verwerwing van MAGISTER TECHNOLOGIAE: BIOMEDIESE TEGNOLOGIE aan die Sentrale Universiteit vir Tegnologie, Vrystaat, wat deur my voorgelê word, my selfstandige werk is, en nie voorheen deur my of enige ander persoon by enige ander instelling, ter verwerwing van enige kwalifikasie voorgelê is nie.

Handtekening van Student

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## Abbreviations

| | | |
|---|---|---|
| 1. | BSA | Bovine saline albumin |
| 2. | cDNA | Coding deoxyribonucleic acid |
| 3. | CPD | Citrate phosphate dextrose |
| 4 | DDAVP | 1-Deamino-8-D-arginine vasopressin |
| 5. | DNA | Deoxyribonucleic acid |
| 6. | F VIII:Ag | Factor VIII coagulant antigen |
| 7. | F VIII:C | Factor VIII coagulant activity |
| 8. | FWP | Fixed washed platelets |
| 9. | GP1b | Glycoprotein 1base |
| 10. | HCl | Hydrochloric acid |
| 11. | HMW | High molecular weight |
| 12. | OPD | Ortho-phenylenediamine |
| 13. | PNP | Pooled normal plasma |
| 14. | PRP | Platelet rich plasma |
| 15. | vWF:RCo | Ristocetin cofactor activity |
| 16. | SD | Standard deviation |
| 17. | TSC | Trisodium citrate |
| 18. | UDP-Gal | UDP-galactose |
| 19. | UDP-GalNac | UDP-N-acetyl-galactosamine |
| 20. | vWD | von Willebrand disease |
| 21. | vWF | von Willebrand factor |
| 22. | vWF:Ag | von Willebrand factor antigen level |

## SUMMARY

This project was a scientific analytical study to determine the correlation between the ABO blood group, plasma von Willebrand factor level (vWF:Ag) and ristocetin cofactor activity (vWF:RCo). A total of 200 blood samples were drawn from healthy volunteers. Fifty samples each from A, B, AB and O blood groups were used for both plasma vWF:Ag levels and vWF:RCo activity assays and analysis of the samples ran over a period of five days. Volunteers donated blood in citrate phosphate dextrose (CPD) bags at the blood bank. One unit of platelets was collected by means of plateletphoresis from the platelet donor.

The assays for plasma vWF:Ag levels and the vWF:RCo activity using the in-house method, were done. The statistical analysis of these assays' results were carried out by employing the Kruskal–Wallis test to determine the difference among the blood groups, and the Spearman correlation to determine the correlation between the two variables of vWF, which are vWF:Ag and vWF:RCo activity.

Based on the outcome of the study results, it was concluded that only O blood group influenced both parameters of the plasma vWF and not the entire ABO blood group as previously reported. The conclusion could be supported by comparing these results with previous reports.

Different reference ranges for vWF:Ag level and vWF:RCo activity were established for the different ABO blood groups. Theoretically, failure to use blood group-specific ranges would place plasma vWF levels of individuals with blood group O below the normal range, leading to over-diagnosis of vWD, and under diagnosis in group A, B, and AB, where in these groups more attention needs to be paid to vWF:RCo activity if plasma vWF is normal. Regardless of the patient's blood group and the established reference ranges, a certain level of plasma vWF:Ag is needed in the body to maintain normal haemostasis.

The established reference ranges showed that there was no clinically significant lower cut-off value for the different blood groups. It was therefore concluded that the diagnosis of von Willebrand disease based on blood group-specific vWF:Ag levels and vWF:RCo activity without regard to family and bleeding history of the patient, was detrimental and it was not recommended.

## OPSOMMING

Hierdie projek is 'n wetenskaplike analitiese studie om die verband tussen die ABO bloedgroep, plasma von Willebrand faktor vlak (vWF:Ag) en ristosetien ko-faktor aktiwiteit (vWF:RCo) te bepaal. In totaal is 200 bloedmonsters van gesonde vrywillige skenkers bekom. Vyftig monsters elk van A, B, AB en O bloedgroepe is gebruik om beide die vWF:Ag vlakke in die plasma en vWF:RCo-aktiwiteitbepalings te meet. Die bloed is in bloedskenkersakkies met sitraat-fosfaat-dektrose (CPD) versamel. Een eenheid plaatjiekonsentraat is deur middel van plaatjieferese geneem.

Analise van plasma vWF:Ag, en die vWF:RCo-aktiwiteite deur die in-huis metode, was uitgevoer. Die statistiese analise van albei resultate was uitgevoer deur die Kruskal–Wallis toets om die verskil tussen die bloedgroepe te bepaal, en die Spearman korrelasie om die verband tussen vWF:Ag en vWF:RCo-aktiwiteite te bepaal.

Hieruit kan afgelei word dat die parameters van vWF slegs by bloedgroep O van toepassing is en nie by al die ABO-bloedgroepe soos voorheen gedokumenteer is nie. Verskillende verwysingswaardes vir vWF:Ag-vlakke en VWF:RCo-aktiwiteite is met hierdie studie volgens die ABO-bloedgroepe ingedeel. Teoreties

beteken dit dus dat indien bloedgroep-spesifieke-verwysingswaardes nie gebruik word nie, sou meer mense met verlaagde plasma vWF:Ag-vlakke by individue met bloedgroep O gediagnoseer word, dus meer mense met von Willebrand se siekte en daar teenoor 'n onderdiagnose van von Willebrand se siekte by groepe A, B en AB individue. By bloedgroepe A, B en AB individue sou dus meer aandag gegee moes word aan die VWF:RCo-aktiwiteit indien die plasma vWF normaal voorkom. Afgesien van die pasiënt se bloedgroep en verwysingswaardes soos vasgestel in hierdie studie moet 'n minimum vlak van plasma vWF:Ag vir normale hemostase gehandhaaf word.

Die verwysingswaardes soos vasgestel dui daarop dat daar geen kliniese betekenisvolle verlaagde afsnywaardes by die verskillende bloedgroepe bepaal is nie. Dit kan dus bevestig word dat die diagnose van von Willebrand se siekte gebaseer op die bloedgroep-spesifieke-vWF:Ag-vlakke en vWF:RCo-aktiwiteite sonder inagneming van familiegeskiedenis en 'n bloedingsneiging by die pasiënt, verwarring kan veroorsaak, en dus nie aanbeveel word nie.

# Chapter 1

## Introduction

von Willebrand disease (vWD) is the most frequently inherited bleeding disorder affecting nearly one percent of the world population (Rodeghiero, Castaman, Tosetto, Batlle, Baudo, Cappelletti, Casana, De Bosch, Eikenboom, Federici, Lethagen, Linari & Srivastava, 2005). It is caused by qualitative or quantitative defect of the von Willebrand factor (vWF). The clinical manifestations of vWD are brought about by a decrease in the plasma vWF level (vWF:Ag) or/and the reduction of two of the established functions of vWF namely, its role in supporting the adhesion of platelets to damaged blood vessels, and its function as a carrier and protector of factor VIII from proteolytic degradation (Colman, Hirsh, Marder & Salzman, 1994).

von Willebrand disease is subdivided into three groups, which are vWD type 1 where there is partial quantitative deficiency of vWF, vWD type 2 associated with qualitative defect of vWF, and vWD type 3 in which there is a complete lack of vWF and consequently, a markedly reduced levels of factor VIII (Pasi, Collins, Keeling, Browns, Cumming, Dolan, Hay, Hill, Laffan & Peake, 2004). The diagnosis of vWD is based on the determination of the plasma vWF:Ag level, bleeding time, activated partial thromboplastin time, factor VIII coagulant activity (FVIII:C), the ristocetin

cofactor activity (vWF:RCo) and/or collagen binding activity (vWF:CB), vWF:Multimer analysis, platelet function analysis and platelet count (Favaloro, Bonar, Kershaw, Sioufi, Hertzberg, Street, Lloyd & Marsden, 2004). Ristocetin cofactor activity is the measure of the normal function of vWF by measuring vWF's ability to bind to platelet glycoprotein 1b (GP1b), causing platelet aggregation in the presence of ristocetin during *in vitro* laboratory testing. Ristocetin is an antibiotic that was introduced as an antibiotic into clinical practice in the late 1950s. It is similar to vancomycin in structure and antimicrobial mechanism of action (Colman *et al*., 1994). It was observed that in the presence of vWF, ristocetin induced macroscopic platelet agglutination *in vivo* and *in vitro*, therefore it was subsequently withdrawn from clinical use because recipients developed thrombocytopenia. That led to the development of vWF:RCo assay, which is used *in vitro* to determine the qualitative normal function of vWF (Turecek, Siekmann & Schwarz, 2002).

The diagnosis of vWD is also based on platelet vWF measurements, its ability to interact with its platelet receptors, and the analysis of the multimeric composition of vWF. Several variables that can affect the vWF:RCo activity include the responsiveness of the platelets, plasma vWF:Ag level, platelet GP1b status, the pH of the platelet-rich plasma and the platelet count (Colman *et al.,* 1994). Several factors complicate the diagnosis of vWD and these include; (1) vWF is an acute phase reactant

protein that is rapidly released from storage sites during stress, exercise, pregnancy or infusion of adrenalin, all of which induce an increase in the level of plasma vWF:Ag, (2) the ABO blood group has a significant influence on plasma vWF:Ag levels. According to Gill, Endres-Brooks, Bauer, Marks (Jr.) and Montgomery (1987), individuals with blood group O have the lowest levels of plasma vWF:Ag, followed by group A, then group B and lastly group AB with the highest vWF:Ag levels. A plasma vWF deficiency was indicated in earlier studies in vWD patients, but not in normal individuals and patients with haemophilia A. Patients with haemophilia A were found to have low plasma levels of factor VIII and normal to increased levels of vWF:Ag.

Of the three types of vWD that are present, type 1 is the most frequently encountered. It comprises of 80% to 90% cases of vWD, yet it is the most frequently missed diagnosis of a bleeding disorder because patients do not bleed spontaneously as in type 2 and type 3. Women with vWD are most often diagnosed because they may notice increased menstrual bleeding (Mitchell, 2003). Most individuals do not know that they are carriers of the vWD. In most cases, the physician picks up vWD while a patient is being examined for other illnesses. Patients often start to bleed after a major operation or tooth extraction (Castaman, Federici, Rodeghiero & Mannucci, 2003). Studies by Gill *et al*. (1987) and Sweeney and Hoernig (1992) have shown that the ABO blood group of an individual

influences the plasma vWF:Ag level. According to Gill *et al.* (1987), there may be symptomatic vWD patients with decreased concentrations of structurally and functionally normal vWF because of the blood group O type. Some individuals of blood group AB with a genetic defect of vWF may have the diagnosis overlooked because vWF:Ag levels are elevated due to their blood type (Gill *et al.*, 1987). The qualitative deficiency of vWF is detected by adding ristocetin to vWF in the presence of the platelets, to measure its vWF:RCo activity.

Intensive studies have been conducted on the association of ABO blood group with plasma vWF:Ag level, and it was established that the ABO blood group influences these levels (Sweeney & Hoernig, 1992). However, there has not been an extensive study that has been conducted on the correlation of vWF:RCo activity and the ABO blood group (Colman *et al.,* 1994). In this study, vWF:RCo activity was expected to show the same trend results and vary according to the blood group type the same as vWF:Ag levels do because it measures the functional activity of vWF. The Ethics Committee from The University of Free State approved the study project and gave permission to conduct the study on the correlation between vWF:RCo activity and the ABO blood group during the meeting held on 21 May 2002, on the condition that Informed Consent is available in the preferred language of the trial person. The reference number for the study is ETOVS 92/02.

***The aim of the study***

The aim of the study was to establish the correlation between the ABO blood group, plasma vWF:Ag levels and vWF:RCo activity, by determining whether the vWF:RCo activity was influenced by the ABO blood group type in the same manner as the plasma vWF:Ag level, as was previously reported in the literature about vWF:Ag. The aim was also to determine the different reference ranges for the different ABO blood groups for both plasma vWF:Ag level and the vWF:RCo activity, and their significance in the diagnosis of vWD.

***Objectives of the study***

- Validate the in-house vWF:RCo assay method for sensitivity, reproducibility, and accuracy against the commercial kit method.
- Determine the correlation between the plasma vWF:Ag level and the ABO blood group and compare the results to the previous reports by Gill *et.al.* (1987)
- Determine the correlation between the vWF:RCo activity and the ABO blood groups by running vWF:RCo activity assay on different ABO blood groups.
- Determine the significance and applicability of the established plasma vWF:Ag level and vWF:RCo activity reference ranges for the different ABO blood groups when investigating vWD.

# Chapter 2

# Literature Review

## 2.1 von Willebrand disease

### 2.1.1 Background and history

von Willebrand disease was discovered in 1926 by Dr. Eric von Willebrand on a young girl who was evaluated for bleeding disorders, and it was named after him. Eric von Willebrand first described a bleeding disorder that manifested as an excessive menstrual bleeding and bleeding from mucous membranes on this young girl who subsequently died from these bleedings. von Willebrand disease was initially described as an atypical variant of haemophilia despite its different clinical and laboratory manifestations (Ratnoff & Forbes, 1996). Eric von Willebrand assessed the patient and other members of her family from the Aland Islands and discovered that they had prolonged bleeding times despite having normal platelet counts, in contrast to the normal bleeding times found in haemophilia patients. He therefore concluded that the patients were suffering from a qualitative disorder of platelet function, however, he could not determine whether the abnormality in the platelet function was intrinsic or extrinsic to the platelets (Colman *et al.,* 1994).

It was later observed that transfusion of fresh normal blood to vWD patients decreased coagulation times. Other studies showed improvement of the coagulation abnormality and the prolonged bleeding time after transfusion of fresh frozen plasma. Transfusion of normal platelets to vWD patients did not increase factor VIII nor shorten the bleeding time. This indicated that patients with vWD were deficient in a plasma factor necessary for the normalization of the bleeding time (Beutler, Lichtman, Coller & Kipps, 1995). von Willebrand's early attempts to define an *in vitro* abnormality in platelet physiology or function were unsuccessful. The role of vWF in haemostasis process, was more clearly understood after it was discovered that the ristocetin induced platelet aggregation in the presence of normal concentration of vWF:Ag (Colman *et al.,* 1994).

### 2.1.2 Classification of von Willebrand disease

According to Pasi *et al*. (2004), the current classification of vWD was introduced in 1994 (Sadler, 1994). von Willebrand disease is classified into two major categories with three principal types of vWD been defined. The two major categories are characterized by quantitative defects that are divided into partial deficiency (type 1) and severe deficiency (type 3) of plasma vWF:Ag, and qualitative defects (type 2), which is divided into four subcategories, type 2A, type 2B, type 2M and type 2N (Castaman *et al*., 2003).

**vWD type 1:** It is characterized by partial quantitative deficiency of qualitatively normal plasma vWF. It is the most frequently encountered vWD type (80% to 90% cases of vWD) and its transmission is autosomal dominant. vWD type 1 is the most common genetic form of vWD. An individual with vWD type 1 has generally mild clinical symptoms beside partially decreased plasma vWF:Ag level, which include low or normal FVIII:C, decreased vWF:RCo and all the multimeric forms are present and normal (Rodeghiero *et al*., 2005). It is the safest and the easiest form of vWD to treat since 1-Deamino-8-D-arginine vasopressin (DDAVP) or desmopressin, causes the release of the structurally normal vWF from endothelial stores (Pasi *et al*., 2004).

von Willebrand disease Vicenza is a variant of vWD type1 although it has also been classified under type 2M vWD. Patients with this variant have normal platelet vWF, plasma vWF and ultralarge plasma vWF multimers like those usually found in platelets (Sadler, 2005). It is caused by the mutation R1205H in the vWF D3 domain, and the mutation does not significantly affect vWF synthesis, multimerization or secretion (Sadler, 2005).

**vWD type 2**: It is the second most common form of vWD and is characterized by qualitative defect of vWF. The patients have a relatively normal to reduced plasma levels, with functional defect in their circulating

protein. The defect is often located in the multimeric structure. vWD type 2 constitutes about 20% to 30% cases and its transmission is autosomal dominant. There are four main subtypes of vWD type 2, each characterized by a different structural and functional defect, namely, type 2A, type 2B, type 2M, and type 2N (Israels & Israels. 2002).

In vWD type 2A, the large and intermediate plasma vWF and platelet vWF high molecular weight (HMW) multimers are absent, with the decrease in the platelet-dependent function (Castaman *et al*., 2003). The FVIII:C activity and the plasma vWF:Ag levels are usually low to normal, but platelet vWF:RCo activity is reduced, suggesting a dysfunctional molecule (Pasi *et al*., 2004). These patients usually have prolonged bleeding times and moderate to severe bleeding symptoms. Because of the abnormality of the molecule, the increase in the vWF:Ag level during stress or pregnancy does not correct the abnormal multimers or increase their function. Thus, these patients may have more difficulty with intrapartum bleeding or post surgical bleeding (Colman *et al*., 1994). Treatment with DDAVP in vWD type 2A does not correct the functional abnormality although there is an increase in the level of abnormal vWF:Ag and has variable clinical effects (Pasi *et al*., 2004). vWD type 2A was the first type 2 variant to be defined at the molecular level. Since the first report of an abnormal deoxyribonucleic acid (DNA) sequence, a cluster of mutations in the intron 28 has been reported (Colman *et al*., 1994). It is caused by

mutations in vWF domain A2 (Sadler, 2005). When the initial two mutations were reported, it was thought that one of the mutations produced full-length vWF multimers while the other produced only the smaller multimers, suggesting that vWD type 2A could be caused by abnormal proteolysis of full-length vWF or smaller multimers produced through an ineffective multimerization process (Colman *et al.*, 1994).

It is now known that some of these mutations prevent vWF from escaping from the rough endoplasmic reticulum. Since these mutations occur on the N-terminal portion of vWF, abnormal multimerization involving the N-terminal disulfide bonds is presumed to occur, resulting in only small forms appearing in the culture supernatant (Colman *et al.*, 1994). vWD type 2A may be considered as a nonspecific defect in multimer size, and diagnosis of this subtype depends on the analysis of multimeric distribution. According to Sadler (1994), at least two different pathophysiological mechanisms are known to cause vWD that would be classified as type 2A; i.e., impaired biosynthesis of large vWF multimers, and increased degradation of large vWF multimers in the plasma (Sadler, 1994).

The third most common variant of vWD is type 2B. vWD type 2B is characterized by the vWF's increased affinity for platelet GP1b. The abnormality leads to heightened and spontaneous binding of the vWF molecules to the surface of non-activated platelets and normal platelets,

and subsequently results in accelerated clearance of these normal platelets and the removal of the largest vWF multimers from circulation (Israels & Israels, 2002). vWD type 2B is associated with the loss of HMW vWF multimers and mild thrombocytopenia that result from this rapid clearance of vWF-platelet complex from the circulation (Pasi *et al*., 2004). The mutations in vWD type 2B occur in vWF domain A1 that bind to platelet GP1b binding-site (Castaman *et al*., 2003). The largest plasma vWF multimers are absent in vWD type 2B because they are bound to the platelets (Sadler, 1994). The plasma vWF in vWD type 2B, binds to normal platelets in the presence of lower concentrations of ristocetin than what normal vWF does (Beutler *et al*., 1995).

von Willebrand disease type 2M is a qualitative variant with defective interactions between vWF and platelets but not because of the loss of HMW plasma vWF multimers (Pasi *et al*., 2004). The clinical symptoms of vWD type 2M show increased bleeding time, normal platelet count, decreased vWF:RCo activity and normal plasma vWF:Ag, FVIII:C and multimer analysis (Israels & Israels, 2002). vWD type 2M is inherited as an autosomal dominant trait, with the mutations also occurring at vWF A1 domain (Israels & Israels, 2002). The multimer distribution may be normal or even show larger than normal multimers. Despite the multimer distribution, the presence of a structural or functional defect indicates that the multimers contain qualitatively abnormal vWF subunits (Sadler, 1994).

The vWF multimers in type 2M vWD show sensitivity to proteolysis and are cleared faster from plasma than multimers of normal plasma (Beutler *et al.*, 1995, Sadler 2005).

von Willebrand disease type 2N is another variant of vWD type 2 that result from defective binding of plasma vWF to factor VIII with resultant low levels of factor VIII (Pasi *et al.*, 2004). Its inheritance is usually autosomal recessive due to mutations in the vWF-binding site for factor VIII in the vWF D'D3 domains (Israels & Israels, 2002). Because the abnormal plasma vWF have markedly decreased affinity for factor VIII, the plasma level of factor VIII is usually reduced (Israels & Israels. 2002). There is a decreased susceptibility to proteolysis of plasma vWF in vWD type 2N (Beutler *et al.*, 1995).

**vWD type 3**: It is a severe bleeding disorder characterized by a total lack of vWF in both the plasma and the cellular components and consequently the plasma levels of factor VIII are markedly reduced (Pasi *et al.*, 2004). It is encountered in 10% of the cases of vWD and its transmission is autosomal recessive. Patients present with bleeding tendencies very early in life and both the vWF:Ag and vWF:RCo activity are less than 5% of the normal value. Consanguinity is common in kindreds with this variant, and careful study of the parents often reveals reduced levels of vWF:Ag even though they are usually clinically asymptomatic. Thus, this type has also

been referred to as recessive-type vWD (Colman *et al.*, 1994). These patients have little or no intracellular or extracellular vWF and therefore do not respond to DDAVP (Colman *et al.*, 1994).

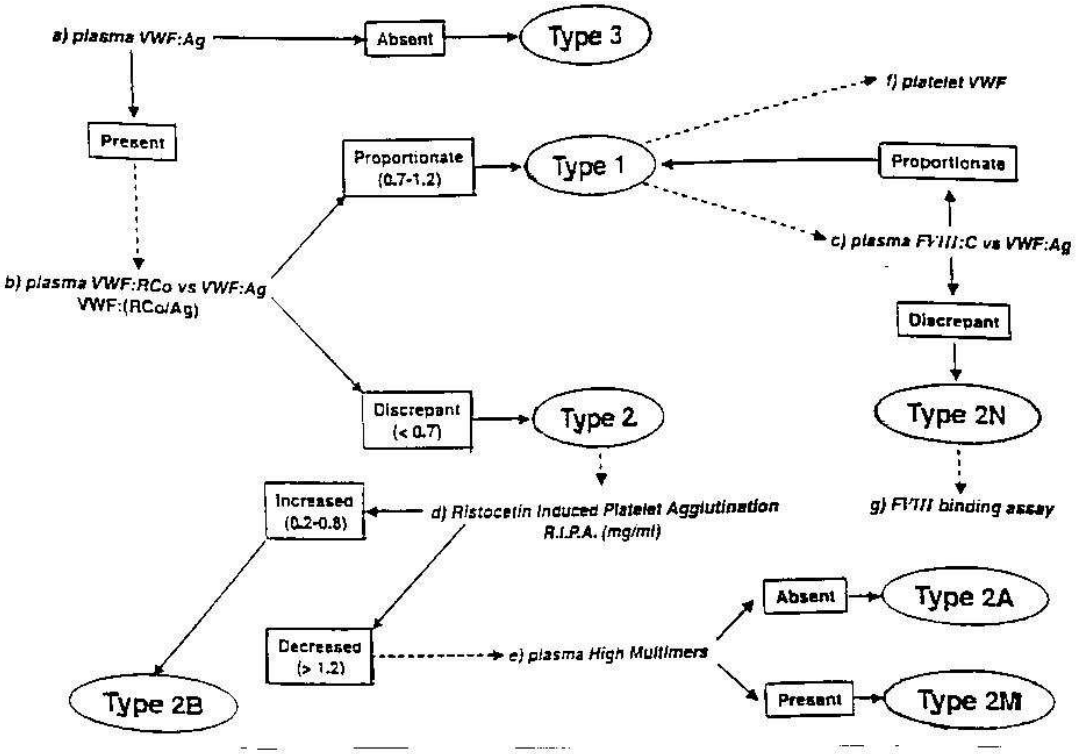



Figure 2.1: Schematic flow chart of the laboratory diagnosis of von Willebrand disease's subtypes: (a) vWD type 3 can be diagnosed when there is undetectable levels of plasma vWF:Ag; (b) When vWF is present but there is a proportional reduction in both its plasma levels and the vWF:RCo, with a RCo/Ag ratio of > 0.7 and FVIII:C levels equal or higher than vWF:Ag, it indicates vWD type 1. If the ratio is < 0.7, it indicates vWD type 2; (c) In a case where FVIII:C levels are lower than the plasma vWF:Ag, vWD type 2N is diagnosed but it should be confirmed by FVIII binding assay (g); (d) vWD type 2B is diagnosed if the RIPA is increased (< 0.8mg/ml), and if it is decreased (> 1.2mg/ml) vWD type 2A or 2M is diagnosed; (e) vWD type 2A and type 2M are diagnosed according to the presence or absence of the HMW vWF multimers; (f) vWD type 1 can be further characterized by the platelet vWF content (Castaman *et al.*, 2003).

### 2.1.3 Clinical manifestation and features of von Willebrand disease

Patients with vWD often suffer from easy bruising, gastrointestinal bleeding, epistaxes, prolonged bleeding from minor cuts, menorrhagia, and excessive but not life-threatening bleeding after trauma or surgery. In women, the most frequent symptom is menorrhagia, which may be severe and out of proportion to other haemorrhagic symptoms. von Willebrand disease should be prominently considered in the differential diagnosis of menorrhagia (Baindur, Shetty & Pathare, 2000). If untreated, menorrhagia may lead to iron deficiency and anaemia.

Menorrhagia probably accounts for the disproportionate number of women who are referred for evaluation of von Willebrand disease. Menstruation is seldom a cause of severe blood loss, although menorrhagia is common. Menorrhagia is a common symptom in females with vWD. It occurs in more than 50% of women with vWD and may be the only clinical manifestation of the disease. It can be managed by oral contraceptive estrogen and progesterone combinations (Kadir, Economides, Sabin, Owens & Lee, 1998). Menorrhagia is often severe, and before hormonal therapy was available, it often required extreme treatment such as ovarian ablation by radiation, a practice not currently recommended (High, 1999). Interestingly, pregnancy in women with vWD is usually well tolerated because plasma vWF:Ag and factor VIII levels are increased during that

period, reaching a peak in the third trimester and falling rapidly postpartum (Kouides, Phatak, Burkart, Braggins, Cox, Bernstein, Belling, Holmberg, Maclaughlin & Howard, 2000). In patients with vWD type 1, this rise is associated with a reduction in haemorrhagic symptoms. In patients with variants such as vWD type 2B or platelet-type, pseudo-vWD, the elevated levels of abnormal vWF:Ag may increase the spontaneous binding to platelets and result in more marked thrombocytopenia (Pasi *et al*., 2004). In vWD type 3, factor VIII and plasma vWF:Ag levels do not increase during pregnancy and vWF concentrate are required to cover delivery or caesarean section. While characteristic bleeding symptoms occur in patients with vWD, the absence of bleeding symptoms does not rule out this diagnosis during pregnancy (Kouides *et al*., 2000).

The haemorrhagic tendency in vWD is highly variable and depends on the type and severity of disease. Patients with vWD type 1 and 2 may have mild bleeding symptoms characterized by haemorrhage from delicate mucocutaneous tissues. Delayed haematoma formation and haemathroses, characteristic of haemophilia A, are not prominent features in these patients. Post-traumatic, post surgical and dental bleeding may be severe and can be the presenting manifestation of von Willebrand disease (Castaman *et al*., 2003). Patients with vWD type 1 and type 2 lead relatively normal lives with a normal life expectancy. Patients with severe vWD type 3 have a bleeding tendency that clinically resembles

severe haemophilia A. Some of these patients develop antibodies to vWF, which inhibit its platelet adhesion-promoting property and cause rapid removal of infused material from the circulation (Colman *et al*., 1994). In the studies by Miller, Graham, Goldin, and Elston (1979), Goldin, Elston, Graham, and Miller (1980) and Abildgaard, Suzuki, Harrison, Jefcoat and Zimmerman (1980), approximately one-half of patients with documented vWD type 1 or type 2 had no history of bleeding despite reduced factor VIII or vWF:Ag levels on at least some occasions (Miller *et al*., 1979; Goldin *et al.,* 1980; Abildgaard *et al.,* 1980). Patients with severe vWD type 3 have a severe haemorrhagic tendency. Spontaneous haemorrhage from mucous membrane and the gastrointestinal tract can be frequent and may be life threatening. Usually, haemorrhage after dental and other surgical procedures can be controlled only by replacement therapy (Zhang, Josephs, & Zhou, 1999). Because of the low factor VIII levels, deep haematoma and joint haemorrhages similar to those in haemophilia may occur in vWD type 3 (Zhang *et al*., 1999).

Unlike congenital vWD, acquired vWD is a rare condition that occurs most commonly in patients of advanced age. Most reported cases of acquired vWD have been associated with autoimmune or clonal hematoproliferative disorders (Tefferi & Nichols, 1997). Autoimmune disorders associated with acquired vWD include systemic lupus erythematosus, hypothyroidism and antiphospholipid syndrome, while disorders such as neoplasia,

multiple myeloma, lymphoma and Waldenström's macroglobulinemia are associated with clonal hematoproliferative disorders (Tefferi & Nichols, 1997). Acquired vWD may result from a reduced rate of vWF synthesis, from secondary vWF abnormalities, or the presence of an autoantibody that either interferes with vWF function or promotes rapid clearance by binding to vWF and removing it (Israels & Israels, 2002). The mechanism of acquired vWD involves inhibition of vWF as part of a generalized autoimmune reaction, or development of specific anti-vWF antibodies (Tefferi & Nichols, 1997). von Willebrand factor is a reactive protein and therefore its level increases when there is damage to the vascular endothelium in the post-operative period, during infection, in cancer, and in renal hepatic disorders (Blann, 1993).

Angiodysplasia refers to small telangiectasias in the wall of the small intestine and colon that occur in some patients with vWD, more commonly in those who are over 50 years of age and typically in those with vWD type 3 (Quick, 1967). Although it is not clear whether these disorders are linked, the presence of angiodysplasia in vWD can be a serious and disabling complication. Bleeding from angiodysplasia may present as melena or occult gastrointestinal bleeding, but typically presents with recurrent episodes of acute blood loss (Quick, 1967). Cycles of haematochezia resulting in hypotension and cessation of bleeding, followed days to weeks later by haematochezia, hypotension and

cessation of bleeding, may occur. Colonoscopy may reveal a bleeding site but more often shows multiple telangiectasias without a defined source of bleeding (Ahr, Rickles & Hoyer, 1977). Rarely, bleeding can be brisk enough to be visible by angiography. Operative intervention is not recommended unless a source of recurrent bleeding has been definitively identified (Ramsey, MacLeod, Buist & Heading, 1975). Pickering, Brody & Barret (1981) and Froom, Margulis & Grenadier (1981) reported occurrence of increased frequency of mitral valve prolapse in vWD, perhaps as a linked mesenchymal disorder. However, a subsequent report described a normal frequency of mitral valve prolapse in patients with vWD (Kuhsel, Polster & Rudiger, 1983).

Inhibitors to vWF in patients with vWD are allo-antibodies, usually IgG, which inhibit the haemostatic function of vWF. They are uncommon and occur only in patients with severe vWD type 3 disease, among whom the prevalence of inhibitors has been estimated to be 7% to 8% (Lopez-Fernandez, Martin & Berges, 1988). There is almost always a prior history of exposure to exogenous vWF, either as plasma cryoprecipitate, or factor VIII concentrates. Patients with deletion of the vWF gene as the cause of their disease may be at a higher risk for the development of inhibitors (Shelton-Inloes, Chehab & Mannucci, 1987; Ngo, Glotz, Koziol, Lynch, Gitschier, Ranieri, Ciavarella, Ruggeri & Zimmerman, 1988). A familial tendency to inhibitor formation has also been noted by Ruggeri, Ciavarelli

& Mannucci (1979). The presence of an inhibitor to vWF in a patient with vWD is suggested by the failure to respond clinically to replacement therapy, by decreased recovery of infused vWF, and by lack of correction of the bleeding time (Ruggeri *et al.*, 1979).

Confirmation of the presence of an antibody requires the demonstration of an inhibitor of ristocetin-induced platelet aggregation in the patient's plasma. In most cases, antibodies to vWF also appear to inhibit factor VIII activity (Fricke, Brinkhous, Garris & Roberts, 1985). However, some antibodies do not inhibit the function but promote accelerated clearance of transfused vWF. Those antibodies must be demonstrated by mixing the patient's antibodies with normal vWF and demonstrating antigen-antibody binding (Lopez-Fernandes *et al.*, 1988).

Bleeding complications in the newborn are rare because the stress of labour and delivery cause a rise in vWF:Ag level. This often confuses the diagnosis when the infant’s plasma is sampled immediately after birth as the vWF:Ag level may be within the normal range and not reflect the same reduced level subsequently found in vWD patients (Colman *et al.*, 1994). Similarly, levels of vWF:Ag and factor VIII are elevated in the newborn infants, particularly with vaginal delivery. This increase probably reduces the likelihood of bleeding but makes the diagnosis of vWD difficult in the new-borns. An infant with vWD type 2B may have thrombocytopenia, but

usually does not have clinically significant bleeding. In infants at risk for severe vWD type 3, laboratory testing usually reveals undetectable levels of vWF:Ag and vWF:RCo activity. Haemorrhage in these infants requires correction of both the factor VIII and vWF defects (Colman *et al.*, 1994).

## 2.2 von Willebrand factor

### 2.2.1 Synthesis of von Willebrand factor

von Willebrand factor is an important haemostatic factor synthesized in endothelial cells and megakaryocytes as a 2813 amino acid precursor molecule (Turecek *et al.*, 2002). Endothelium forms the inner lining of all blood vessels and therefore, is in a direct contact with the blood. Upon vascular injury, the arrest of bleeding is subsequently controlled by vasoconstriction of the blood vessels, formation of a platelet plug, and clot formation. Because of that and the synthesis and secretion of the haemostatic components, the endothelium is able to modulate coagulation and fibrinolysis (Verweij, 1988). The presence of vWF in platelet $\alpha$-granules originates from megakaryocytes (Krizek & Rick, 2000). Once bound to the subendothelium, platelets secrete substances, which promote the release of vWF from the $\alpha$-granules and facilitate further platelet aggregation (Verweij, 1988). Beside its presence in the endothelium cells, megakaryocytes, platelets, subendothelium, and in the

circulating blood, vWF can also be associated with the external endothelial cell surface. von Willebrand factor is initially synthesized as a pre-pro-polypeptide. During its transport to the outside of the cell, the single-chain polypeptides are assembled into multimers. Plasma vWF consists of a heterogeneous series of multimers, composed of single-type glycoprotein subunit, linked together by disulfide bonds. Verweij (1988) showed the haemostatic potency of vWF to increase with the increasing multimer size. Therefore, the multimeric assembly of vWF is a crucial aspect in vWF biosynthesis.

### 2.2.2 Structure of von Willebrand factor

A coding deoxyribonucleic acid (cDNA) encoding the message for vWF was first isolated in 1985 (Ratnoff & Forbes, 1996). It is located on band 21 of chromosome 12 and contains more than 250-kilo bases. The precursor molecules of vWF form dimers within the endoplasmic reticulum through disulfide bonds between their C-terminal cystine knot domains. The pro-vWF dimers are transported to the Golgi apparatus where the propeptide facilitates the formation of disulfide-linked vWF multimers intermediate with the D'D3 domain of vWF (Sadler, 2005). The precursor molecule of vWF consists of a 22 amino acid signal peptide, followed by a 741 amino acid propeptide, and then by 2050 amino acids of the natural protein (Turecek *et al.*, 2002). The amino acid sequence of vWF is rich in

repetitive and secondary structure, and defines four distinctive domains that are repeated two to four times. There are three A-domains, three B-domains, two C-domains and four D-domains (Verweij, 1988). von Willebrand factor's complex secondary structure is formed and maintained by numerous disulfide bonds.

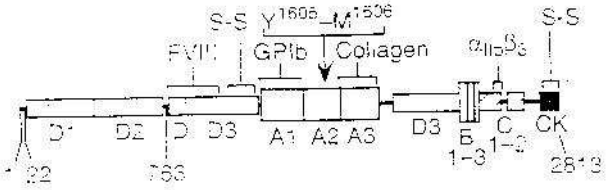



Figure 2.2: Diagrammatic representation of von Willebrand factor structure showing A to D domains and the binding sites for FVIII, platelet GP1b, collagen, and platelet integrin $\alpha_{IIb}\beta_3$, and intersubunit disulfide bonds (S-S) (Sadler, 2005)

There are 169 cysteinyl residues in the mature protein and they are largely clustered in the carboxy and amino terminals, where they contribute to dimer and multimer formation respectively (Ratnoff & Forbes, 1996). von Willebrand factor circulates as polymerized monomers linked by carboxy terminus to carboxy terminus and by amino terminus to amino terminus. The platelet GP1b is the receptor for the vWF during platelet aggregation. It is absent in the platelets of patients with the hereditary disorder, Bernard-Soulier syndrome. Platelets from these patients do not react with ristocetin even though the plasma levels of vWF are normal, because ristocetin binds to both vWF and platelet GP1b. The platelet GP1b-binding site is located within the A1 domain of vWF, at amino acids 509 to

695, within the disulphide loop. The vWF-binding site for factor VIII is located in the amino terminal 272 amino acids on the vWF D'D3 domain. von Willebrand factor binds to the acid portion of the factor VIII light chain, and binding involves both hydrophobic and electrostatic interactions between the two proteins. von Willebrand factor binding site for collagen is located at the A3 domain (Ratnoff & Forbes, 1996).

### 2.2.3 Release of von Willebrand factor

The transport of vWF from the Golgi apparatus to the outside of the cell can follow two routes. von Willebrand factor can either be secreted continuously (constitutive pathway), or it can be stored in rod-shaped secretory organelles and released upon command (regulated pathway). The majority of the newly synthesized proteins are secreted constitutively, which take at least two hours. About 5% of the newly synthesized vWF follow the regulated pathway. Some of the vWF is stored in the platelet $\alpha$-granules and the rod-shaped organelles called Weibel-Palade bodies that are specific for endothelial cells for later secretion (Sadler, 2005). Release of vWF from Weibel-Palade bodies can be induced by treatment of the cells with secretagogues such as thrombin, phorbol esters and calcium ionophore A23187 (Verweij, 1988). Once endothelium cells receive a stimulus, vWF is rapidly released at a rate that is much higher than that required for the synthesis and secretion of vWF by nonstimulated cells.

### 2.2.4 Function of von Willebrand factor

von Willebrand factor is an adhesive protein present in human plasma and has at least two different functions (Turecek *et al*., 2002). When secreted, it interacts with platelets to cause adhesion to surfaces. It is a large plasma glycoprotein that promotes the adhesion of platelets to the vessel wall after a vascular injury (Verweij, 1988). Under normal circumstances, platelets do not interact with the vessel wall, however, upon vascular injury platelets readily adhere to the subendothelium, which becomes exposed after the endothelium detaches. This site of the endothelium is highly thrombogenic (Verweij, 1988). The adhesion of the platelets to the subendothelium is the initial step in platelet plug formation.

Platelet adhesion is proportional to the vWF:Ag level. Patients with a low plasma vWF:Ag level will have decreased or normal vWF:RCo activity, while patients with no vWF, or with a functional defect in vWF and a normal or high plasma vWF:Ag level, will have decreased vWF:RCo activity (Nishino, Girma, Rothschild, Fressinaud & Meyer, 1989). According to Ratnoff and Forbes (1996), Howard and Firkin observed that the addition of normal plasma to that of a vWD patient produces platelet agglutination. The function of vWF can be best described as forming a molecular bridge between the platelet and the vessel wall. Besides being a haemostatic factor itself, vWF also stabilizes plasma factor VIII during

secondary haemostasis (Krizek & Rick, 2000). von Willebrand factor acts as a protective carrier for factor VIII in the coagulation process (Matsui, Shimoyama, Matsumoto, Fujimura, Takemoto, Sako, Hamako & Titani, 1999). A deficiency in vWF results in a haemostatic defect, reduced plasma levels of factor VIII, which though synthesized normally, has a shorter half-life in the absence of its carrier, and a prolonged bleeding time as platelets fail to adhere to the cut edges of the small vessels (Hoffbrand & Lewis, 1989). von Willebrand factor protects factor VIII from proteolytic degradation (Blann, 1993), and the complex can bind directly to platelet and cellular surfaces, localizing factor VIII to sites where it can participate directly in coagulation and thrombus formulation (Ratnoff & Forbes, 1996).

### 2.3 Ristocetin Cofactor assay

The determination of vWF:Ag levels and its functional activity is important in the diagnosis of vWD. Ristocetin cofactor activity is the functional activity of vWF based on the property of ristocetin to agglutinate platelets in the presence of vWF (Castaman *et al*., 2003). According to Turecek *et al*. (2002), the vWF:RCo assay is currently the most common *in vitro* test used to determine the functional activity of plasma vWF of patients with vWD. Previously the vWF:RCo assay was said to be the most reliable and specific test for the diagnosis of vWD because it appeared to be most closely related to the biological activity of vWF (Fleming, 1995), but that is

no longer the case. Based on the outcome of their recent studies, Casonato, Pontara, Bertomoro, Sartorello, Cattini and Girolami (2001) suggested that vWF:CB be substituted for vWF:RCo. Turecek *et al.* (2002) also mentioned in his article that vWF:CB assays, based on the enzyme-linked immunosorbent assay (ELISA) technique that measure the interaction of vWF and collagen, are an alternative analytic procedures based on a physiological function than that of the vWF:RCo procedure.

Ristocetin was shown to agglutinate normal platelets *in vitro*, but caused little or no aggregation to platelets from patients with vWD. The defect could be corrected with normal or haemophilic plasma and could be blocked by specific monoclonal antibodies directed against vWF. Even after fixation, platelets still agglutinate in response to ristocetin in the presence of vWF, demonstrating that this event does not require metabolically active platelets. This observation permitted the development of a quantitative assay, the ristocetin cofactor assay (Colman *et al.*, 1994). The addition of ristocetin to normal platelet-rich plasma causes platelet clumping, and the amount or rate of clumping is reduced in patients with vWD. Plasma vWF does not bind to its platelet receptor platelet GP1b unless it is structurally modified by binding to subendothelial connective tissue structures such as collagen. Ristocetin is thought to mimic this modifying action of subendothelium on vWF. Ristocetin binds to both vWF and platelet GP1b. The platelet receptor for vWF during this aggregation

process is platelet GP1b, a glycoprotein receptor absent from the platelets of patients with the hereditary disorder Bernard-Soulier syndrome (Colman *et al*., 1994). Platelets from these patients do not aggregate with ristocetin, even though their plasma have normal vWF:Ag levels. Ristocetin is thought to induce a change in surface charge or structure that is either directly associated with the bridge between the ligand and the receptor or, perhaps more likely, modifies the structure of the ligand or receptor so as to facilitate spontaneous interaction. It can also precipitate proteins such as fibrinogen and vWF, thus it can bind to specific sites on vWF, causing a conformational change that facilitates the interaction of vWF with platelet GP1b (Colman *et al*., 1994).

Previous studies have demonstrated that the concentration of ristocetin necessary to induce vWF-binding to platelets, and the concentration necessary to cause ristocetin-induced precipitation of plasma proteins, are identical to the concentration required for ristocetin to spontaneously dimerize itself (Colman *et al*., 1994). Hyperresponsiveness to ristocetin-induced platelet aggregation may sometimes reflect platelet or vWF abnormalities. Historically, vWF:RCo assay was the first assay employed to evaluate the plasma vWF activity, however, it is a difficult and not well standardized test, and its reproducibility is low. Nonetheless, it is currently the only one able to measure vWF-GP1b interaction (Casonato *et al*., 2001). Present methods for the vWF:RCo assay use freshly prepared

platelets. Because the procedure for the preparation of platelets is tedious and there is a possibility of considerable variation from one platelet preparation to the other, proper standardization of the vWF:RCo assay is difficult, therefore presenting major practical disadvantages in performance and reproducibility (Miller, Platt, Daniele & Kaczor, 2002), (Turecek *et al.*, 2002).

## 2.4 The ABO blood group

### 2.4.1 Background and history

The first human blood transfusion was performed by Blundell on December 22, 1818 (Erskine & Wiener, 1973). He injected blood from several donors into his patient using a syringe, over a period of 30 to 40 minutes. The patient improved temporarily but relapsed and died 56 hours after the transfusion. Blundell continued with his experiments, unfortunately only four out of twenty transfusions were successful. It was only after the discovery of the ABO blood grouping in 1901 by Karl Landsteiner that a major breakthrough in human blood transfusion occurred (Harmening, 1989).

Landsteiner indicated that blood group differences could explain the serious reactions to blood transfusions that occurred even when human

instead of animal donors were used (Erskine & Wiener, 1973). He took samples of blood from six colleagues, separated the serum and prepared saline suspensions of the red cell. Each serum was mixed with each cell suspension; in some mixtures the red cells were agglutinated while in others they were not agglutinated. Based on the reactions, Landsteiner was able to divide human beings into three distinct blood groups (Race & Sanger, 1975).

Table 2.1: Serological reactions and the identification of ABO blood groups

| **Serological reactions** | | **Blood group Cell suspensions** | **Antigens on the cells** | **Antibodies in the serum** |
|---|---|---|---|---|
| **Anti-A** | **Anti-B** | | | |
| 0 | 0 | O | None | anti-A, anti-B |
| +++ | 0 | A | A | anti-B |
| 0 | +++ | B | B | anti-A |
| +++ | +++ | AB | A and B | None |

Two antigens on the red blood cells were recognized and they were termed A and B. The individuals with A antigen on their red cells are called A group and those with B antigen are called B group. Those with neither A nor B are called group O. The AB group was the fourth group discovered in 1902 by von Decastello and Sturli who were both Landsteiner's pupils (Harmening, 1989). The AB group individuals have both A and B antigens on their red cells.

### 2.4.2 Heredity and genetics of the ABO blood group

The ABO gene is located on the terminal portion of the long arm of chromosome 9 (q34.1 -q34.2). The ABO system comprises of the three major alleles A, B and O. Only one of these can occupy the locus on the chromosome (Harmening, 1989). The inheritance of A and B alleles are co-dominant and O is recessive, therefore when A or B is inherited, it is always expressed. O is only expressed when inherited in double dose (homozygous) and the genotype is OO. It is not known whether group A (or B) person is homozygous AA (or BB), or heterozygous AO (or BO), and this can only be established by constructing a family tree (Issitt & Anstee, 1998). Only the phenotype is known at the initial stage.

Blood group A individuals have the A allele encoding an $\alpha$-3-GalNAc transferase that uses the H structure as acceptor. B individuals have the B allele encoding an $\alpha$-3-Gal transferase working on the same acceptor. Blood group O individuals have none of these two enzyme activities and express the unmodified H structure on erythrocytes (Cartron & Rouger, 1995). The cDNA of the O allele has a single base deletion at the beginning of the gene corresponding to amino acid 87, which produce a shift in the reading frame inducing the introduction of a few wrong amino acid and the abrupt stop of the chain at the position corresponding to amino acid 117, by the appearance of stop codon. This aborted protein

has no A or B transferase activity because it lacks the COOH-terminal portion of the protein where the catalytic domain of the A and B glycosyltransferases is located. More than 90% of blood group O people have this stop codon in double dose (Cartron & Rouger, 1995).

### 2.4.3 Structure and biosynthesis of the ABO blood group determinants

Studies have shown that the A, B, and H oligosaccharide determinants, which are the antigenic/molecules of the ABO system, are expressed not only by the erythrocyte, but also by other tissues (Cartron & Rouger, 1995). These antigens have been shown to exist as water-soluble molecules. The antigenic moieties of the ABO blood group system are composed of oligosaccharide molecules. These antigenic molecules of the ABO blood groups are found as terminal modifications on structurally complex oligosaccharide determinants, which are in turn expressed by soluble glycosphingolipids, soluble-free oligosaccharide, and membrane-associated structures (Cartron & Rouger, 1995). The membrane-associated entities include integral membrane proteins, and membrane-associated glycolipids, that are in turn displayed on the surface of the red cell. The synthesis of the A, B, and H molecules is determined by several distinct genetic loci. These loci encode glycosyltransferases that act in sequence to catalyze the synthesis of these oligosaccharide determinants (Figure 2.3) (Cartron & Rouger, 1995).

The presence of the A or B allele dictates the structure of the corresponding A or B glycosyltransferase that converts H substance to either A or B antigen. The A and B antigens are located on the outer surface of the red cell membrane. Group O individuals carry only H substance (Israels & Israels, 2002).

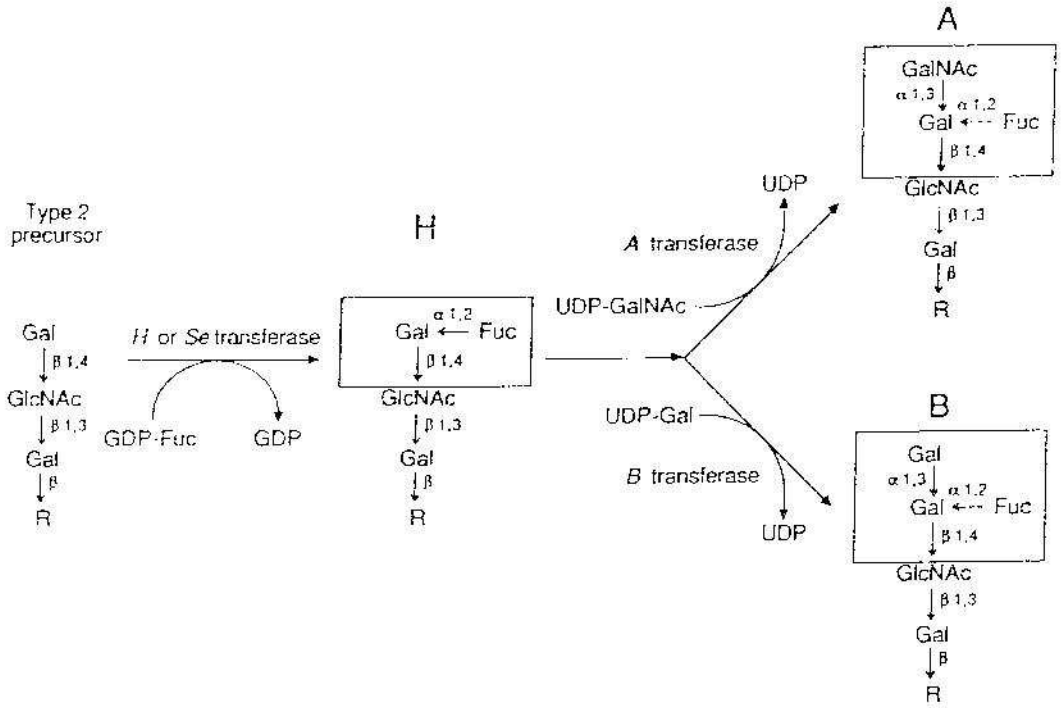



Figure 2.3: Biosynthetic pathway for blood group A, B, and H synthesis: The H oligosaccharide molecule serves as a substrate for the glycotransferase encoded by the A blood group locus [A-transferase; α-(1,3)-N-acetylgalactosaminyl-transferase], or by the B blood group locus [B-transferase; α-(1,3)-galactosyltransferase]. These enzymes form the A and B blood group antigenic determinants respectively. The blood group A transferase uses UDP-N-acetyl-galactosamine (UDP-GalNac) as its sugar nucleotide donor, while the blood group B transferase requires UDP-galactose (UDP-Gal). The R indicates the glycoconjugate substructure that consists of N-linked, O-linked, or lipid-linked glycoconjugates (Cartron & Rouger, 1995).

von Willebrand factor is a glycoprotein, with a great diversity of oligosaccharide structures, including molecules similar to blood group A, B, and H (O) antigens (Souto, Almasy, Muñiz-Diaz, Soria, Borrell, Bayén, Mateo, Madoz, Stone, Blangero & Fontcuberta, 2000). The antigens of the ABO blood group are covalently linked to the oligosaccharide side-chains of vWF (Sarode, Goldstein, Sussman, Nagel & Tsai, 2000). It is possible that the blood group determinants may affect the processing, the release, or perhaps the catabolism of vWF and by so doing, they may influence its plasma concentration (Souto *et al.*, 2000).

Other investigators studied the genetic linkage between the ABO blood group and the vWF:Ag levels and previous analysis suggested that the loci for vWF and ABO blood groups were not linked (Gill *et al.*, 1987). This conclusion was substantiated by the recent location of the gene for vWF on chromosome 12, whereas the gene for ABO blood group determination is on chromosome 9. Thus, a direct linkage on the significant variation in plasma vWF:Ag concentration among individuals of different ABO blood group could not be explained (Gill *et al.*, 1987).

# Chapter 3

# Materials and Methods

## 3.1 Introduction

The research involved a scientific analytical study to determine the correlation between the ABO blood group, vWF:Ag level, and vWF:RCo activity. A total of 200 blood samples were drawn from healthy volunteers of the Bloemfontein blood bank. Samples were taken to Universitas hospital for analysis, and 50 samples each from A, B, AB and O blood groups were used for both plasma vWF:Ag levels and vWF:RCo activity assays. Blood samples obtained from the blood bank were ideal for this study because the volunteers' ABO blood groups were already known.

## 3.2 Materials

### 3.2.1 Study subjects

The two hundred donors who were divided into four groups of 50 A, 50 B, 50 O and 50 AB blood groups, were all from Caucasian population. The platelet donor was a healthy 24 years old male of African descent, non-smoker and non-drinker.

***Inclusive and exclusive criteria for the donors:***

- Pregnant women were excluded because the plasma vWF:Ag level increases during pregnancy.
- Only the blood from volunteers who have donated at least three or more units of blood was used. The reason was to eliminate the stress induced raised levels of vWF:Ag since it is an acute phase reactant protein that is rapidly released from storage sites during stress, as is often the case with new donors.
- Samples from donors who were on medication were excluded from this study.
- Only donors with no known bleeding tendency disorders could donate blood or platelets for this study.

### 3.2.2 Reagents

i. von Willebrand factor assay kit (Diagnostic Stago)

ii. Fixed washed platelets

iii. Ristocetin

iv. Bovine saline albumin (BSA)

v. Tris buffer

vi. Pooled normal plasma (PNP)

### 3.2.3 Instrumentation

Table 3.1: Apparatus that were used for both the plasma vWF:Ag and the vWF:RCo activity assays

| Apparatus | Description | Brand | Supplier |
|---|---|---|---|
| Microplate reader | EL 312e. | Biotek | Biotek Instruments |
| Platelet aggregometer | Monitor IV Plus | Helena | Helena Laboratories |
| pH meter | Micro pH 2000 | Crison | Crison Instruments |
| Weighing scale | E 2000D | Sartorius Excellence | Zeiss West Germany (Pty) Ltd. |
| Cobe Spectra | LRS™ System | | Viking Medical & Scientific (Pty) Ltd. |

## 3.3 Methods

### 3.3.1 Sample collection and preparation

***Blood donors:***

The blood was drawn into citrate phosphate dextrose (CPD) bags at the blood bank. From the CPD bag line, 4.5ml of blood was drawn into the 3.2% trisodium citrate (TSC). The samples were then collected and taken to Universitas hospital. They could not be analyzed immediately, therefore they were centrifuged at 1 400 g-value and the plasma was separated

from the cells and stored at -80ºC. The citrate in the TSC and CPD function as an anticoagulant. The blood bank used CPD because the phosphate in CPD creates a less acidic medium than TSC, thus improving cell viability.

***Platelet donor:***

The platelets were obtained by means of plateletpheresis. Collect-and-return lines of the Cobe Spectra apheresis machine were connected to 16g infusion catheters that were inserted into the donor's antecubital veins of both the left and right arms. One unit of platelets was collected, while the rest of the blood cells were circulated back into the donor's blood circulation system.

***Sample analysis:***

The analysis of the samples ran over the period of five days. Each day forty samples from one group were analyzed for vWF:Ag levels and vWF:RCo activity (day 1 40 Os; day 2 40 As; day 3 40 Bs; day 4 40 Abs). On the firth day, the remaining ten samples from each group were analyzed. The two assays were run concurrently.

### 3.3.2 Validation of the in-house vWF:RCo assay method

Every laboratory seeks to improve its methodology as technology advances, but at the same time looks at its expenditure and cost effectiveness, therefore new or alternative assays are introduced. The proposed method may be more accurate, more sensitive or less expensive than the current one. The proposed method may be a kit provided by a commercial company, a method described in a journal, or a test developed within the laboratory itself (in-house method). However, the new method (in this study; the in-house vWF:RCo assay) cannot be accepted on faith, it must be tested and shown to meet the criteria established by the laboratory.

To validate the in-house method, it was compared to current assay method (commercial kit vWF:RCo assay method provided by Scientific group Ltd.), which, together with plasma vWF:Ag assay, were used as a reference test methods.

***Procedure for the validation***

Ten serial dilutions were prepared from normal plasma to obtain concentrations ranging from 200% to 20% in increments of 20%. Both methods (kit and in-house) for the vWF:RCo activity assay specify that a 50% dilution (1:2) of plasma must be made (with tris buffer for the

commercial kit, and with BSA albumin for the in-house method) to obtain 100% concentration. The specified concentrations of 200% to 20% were prepared as follows:

| *Tube nō* | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| *Conc%* | 200 | 180 | 160 | 140 | 120 | 100 | 80 | 60 | 40 | 20 |
| *Plsm ml* | Pure | 0.9 | 0.8 | 0.7 | 0.6 | 0.5 | 0.4 | 0.3 | 0.2 | 0.1 |
| *BSA/Tris ml* | | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 |

The plasma vWF:Ag and the vWF:RCo assays using both the commercial kit method and the in-house method for the vWF:RCo assay, were performed on these dilutions.

In this correlation study, the paired results were plotted on separate graphs, with the vWF:Ag assay as the x-axis and the vWF:RCo assays as y-axis. The regression line was calculated and both methods were compared. The regression line has a slope of a certain correlation coefficient ($r^2$ value) that is calculated to state agreement between the results of the two methods. In the regression method, the values on the y-axis are presented as the dependent variable where certain predictions are expected. In other words, regression measures how close do the values lie to the estimated regression line. An $r^2$ value of 1.0 represents perfect correlation between the two methods.

Lineweaver-Burke plot measures the deviation of the results from the target value. It was drawn to compare both methods' results in order to determine how much do they deviate from vWF values. Unlike the Levey-Jennings chart, Lineweaver-Burke plot measures the deviation by using the difference between the two sets of values instead of the standard deviation (SD). It is accomplished by plotting the mean of the two sets of results on the x-axis and the difference between the two values on the y-axis. The closer the difference is to zero, the less the deviation from the target values and visa versa. The negative difference means the values are greater than the target values, while the positive difference means the values are less than the target values.

The in-house method was performed on two successive days to determine the effect of freezing and thawing on the vWF:RCo activity.

### 3.3.3 In-house Ristocetin Cofactor activity assay

***Introduction***

This particular assay was developed after it was found that most of the patients that were screened for vWD had prolonged bleeding times while the concentrations of factor VIII coagulant activity (FVIII:C), factor VIII coagulant antigen (FVIII:Ag), and FVIII:vWF:RCo were decreased. In haemophilic patients, bleeding times, FVIII:Ag and FVIII:vWF:RCo were

normal, whereas FVIII:C was decreased. Ristocetin cofactor activity measures the functional activity of vWF (Hoffbrand & Lewis, 1989).

***Principle***

Washed platelets agglutinate in the presence of ristocetin when normal plasma is added as a source of vWF. Formalin-fixed washed platelets are used in the assay. This aggregation follows a dose response curve dependent on the amount of ristocetin cofactor activity in the plasma (Dacie & Lewis, 2001).

***Reagent Preparation*** (Chanarin, 1989)

i. Saline - Ready for use.

ii. Ristocetin - One vial of 15mg/l was reconstituted with 0.75ml of distilled water to obtain required concentration of 10g/l.

iii. Fixed washed platelets (FWP):

a) One unit of platelets was collected on the Cobe Spectra apheresis machine.

b) It was divided into 50ml Falcon tubes and was then incubated for one hour at 37°C.

c) Platelet rich plasma (PRP) was diluted with equal volumes of 2% formalin in normal saline.

d) The mixture was placed at 4°C and left undisturbed for 18 hours.

e) The platelets were centrifuged at 2000g for 20 minutes at 4°C.

f) Supernatant was drawn off with a syringe and replaced with cold normal saline to resuspend the platelets using a syringe with a needle. It was ensured that no platelet clumps were present.

g) Sedimentation and resuspension in fresh cold normal saline was repeated three times.

h) The sedimented platelets were resuspended in saline containing bovine albumin (5.0g of bovine albumin added to 2.2g of sodium chloride in one liter of 50mM Tris pH 7.4), and the platelet count was adjusted to be between 300 and 500 X $10^9$/l.

i) 3ml of FWP were aliquoted into 5ml plastic test tubes and stored at 80°C until they were required for the test.

iv. Bovine Saline Albumin (BSA):

a) 50 mM tris buffer pH 7.4 - 6.06g of tris was dissolved in 990ml of distilled water. The pH was adjusted to 7.4 with HCl and then made up to 1 liter.

b) 5g of bovine serum albumin were dissolved in tris buffer. The mixture left at 2°C to 8°C for 24 hours.

v. Standard plasma and control

Pooled normal plasma (platelet poor plasma) that was used for our daily internal quality control for coagulation tests, was used as both the standard and the control for the in-house vWF:RCo assay. Pooled

normal plasma was obtained from healthy donors by following the same criteria that was described in chapter 3 section 3.2.1.

Standard curves for each batch of tests were constructed from serial dilutions starting from 1:2 to 1:16, with concentrations of 100%, 50%, 25% and 12.5% respectively. Pure plasma was included as a starting calibration point with the concentration of 200%. For this study, the standard plasma was arbitrarily designated the calibration value of 100% at 1:2 dilution (Chanarin, 1989). Dating from as far back as 1978, when Zuzel *et al.* (1978) did a study on methods for measuring vWF:RCo activity, he designated normal pooled plasma with 100% vWF activity. Other investigators who did the same, included, Ermens *et al.* (1995), Casonato *et al.* (2000), and Favaloro *et al.* (2005) to mention few. However, with the kit assay each batch of standard is precalibrated and may not necessarily be 100%.

***Preparation of working reagents, test and standard samples*** (Chanarin, 1989)

i. All plasma samples were thawed at 37°C before they were analyzed.

ii. Thawed stock ristocetin was diluted to 10g/l in distilled water.

iii. FWP were thawed at 37°C for five minutes after the removal from -80°C storage, and then reconstituted with 6ml of BSA. They were left

to equilibrate at room temperature for 30 minutes before been used and maintained at that temperature during the assay.

iv. A 'blank' dilution consisting of 300µl of BSA and 100µl of FWP was prepared.

***Procedure***

1. The standard curves were prepared from serial dilutions as follows:

| Plastic tube | | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|
| BSA | (µl) | - | 500 | 500 | 500 | 500 |
| Plasma | (µl) | 500 | - | - | - | - |
| Mix and transfer | (µl) | 500 | 500 | 500 | 500 | 500 |
| Concentration | (%) | 200 | 100 | 50 | 25 | 12.5 |

2. 1:2 dilutions of the test plasmas (200µl of BSA and 200µl of plasma) were prepared.
3. The aggregometer was switched on 30 minutes before it was used.
4. The optical density readings and the chart recorder were set.
5. The measuring conditions of the aggregometer were set - all aggregations were run for four minutes.
6. 100µl of FWP and 300µl of BSA was placed in a cuvette - this was the 100% aggregation sample ("blank").
7. 200µl of each standard dilution was added to 250µl of FWP.
8. 50µl of ristocetin was added to the above mixture to activate the aggregation.

9. The aggregometer printed the slope of the aggregation together with its slope value when the test was completed.
10. The above procedure was repeated for all the standard plasma dilutions, as well as the test and the control plasma dilutions.
11. Five calibration points were plotted on the log-log graph paper using the slope values that were obtained from different vWF:RCo concentrations of 200%, 100%, 50%, 25%, and 12.5%, to construct exponential graph. The vWF:RCo values of the tests and controls were obtained by plotting the measured slope on the x-axis against the vWF:RCo concentration on the y-axis, on the exponential graph.

### 3.3.4 von Willebrand factor assay

***Principle***

A microplate with wells coated with specific rabbit anti-human von Willebrand factor antibodies capture the vWF to be measured. Rabbit anti-vWF antibody coupled with peroxidase binds to the remaining free antigenic determinants of vWF forming a "sandwich". The bound enzyme peroxidase is then revealed by its activity in a predetermined time on the substrate ortho-phenylene diamine in the presence of hydrogen peroxide. Hydrochloric acid is added to this complex to stop the reaction; the intensity of the colour produced bears a direct relationship with the vWF concentration initially present in the plasma (Dacie & Lewis, 2001).

***Reagent preparation (kit insert)***

Asserachrome plasma vWF:Ag assay kits from Diagnostica Stago were used. The assay kits were stored at 2ºC to 8ºC. Unused and the already in-use plasma vWF:Ag assay kits were stable for the period of the specified expiry date on the box at this temperature range. One vWF:Ag assay kit is sufficient for three analytical runs of 50 tests each, which were run in duplicate, the control and calibration included.

*The vWF:Ag assay kit contains:*

i. Reagent 1: aluminium pouch that contained two strips of 16 wells. Each well was coated with specific rabbit anti-human vWF antibody fragments, established and hermetically sealed. The strips were ready for usage.

ii. Reagent 2: specific rabbit anti-human vWF antibody coupled with peroxidase, and then freeze-dried with stabilizers.

Half an hour before it was used, each vial was reconstituted with 8ml of diluted reagent 4. Once reconstituted the reagent is stable for 24 hours at 2ºC to 8ºC.

iii. Reagent 3a: tablet that contained 2mg ortho-phenylene diamine (OPD, 2HCl).

iv. Reagent 3b: tablet that contained 5mg urea peroxide as a source of hydrogen peroxide.

OPD/$H_2O_2$ substrate was prepared just before use. For two strips, two

tablets of reagent 3a and two tablets of reagent 3b were added to 8ml of distilled water. The OPD/$H_2O_2$ solution is stable for one hour at room temperature.

v. Reagent 4: 10-fold concentrated phosphate buffer. The reagent was diluted 1:10 with distilled water. Diluted reagent 4 is stable for 15 days at 2ºC to 8ºC.

vi. Reagent 5: 20-fold concentrated washing solution. The reagent was diluted 1:20 with distilled water and was then placed into the plastic wash bottle. Diluted reagent 5 is stable for 15 days at 2ºC to 8ºC.

vii. Reagent 6 (Standard plasma): lyophilized human plasma that contained a vWF:Ag level in the neighbourhood of 100% after it was reconstituted with 0.5ml of distilled water (the exact assay value was supplied with each specific kit).

viii. Reagent 7 (Control plasma): lyophilized human plasma that contained a known vWF value (assay value provided in the kit insert). Each vial was reconstituted with 0.5ml of distilled water. It was swirled gently to mix and allowed to stand for 10 minutes at room temperature before it was used. The reconstituted control plasma is stable for four hours at room temperature and for 12 hours at 2ºC to 8ºC.

***Procedure (kit insert)***

1. 1:51 dilution (50µl of standard plasma and 2.5ml of diluted reagent 4) of the standard plasma was prepared to obtain the starting concentration containing the highest calibrator value.

   Further double dilutions of the standard plasma were prepared in diluted reagent 4 as follows:

| | | | | | | |
|---|---|---|---|---|---|---|
| Std. plasma | (µl) | 50 | - | - | - | - |
| Diluted reagent 4 | (ml) | 2.5 | 1 | 1 | 1 | 1 |
| Mix and transfer | (ml) | 1 | 1 | 1 | 1 | 1 |
| Concentration | (%) | 100 | 50 | 25 | 12.5 | 6.25 |

2 1:51 dilutions (50µl of plasma and 2.5ml of diluted reagent 4) of the test plasma and control plasma were prepared.

3 200µl of each of the dilutions of the standard plasma, control and test plasmas were pipetted in duplicate into the pre-coated wells.

4 The wells were covered and incubated for two hours at room temperature.

5 After two hours, the solutions in the wells were thrown out and each well was filled with washing solution. This washing process was repeated three times. Each well had to be completely filled with washing solution and then completely emptied (the inverted strips were tapped several times on absorbent paper).

6 200µl of reagent 2 was pipetted into each well.

7 They were incubated for two hours at room temperature after which the washing process was repeated as described in step 5.

8 300µl of OPD/$H_2O_2$ substrate was pipetted into each well.

9 They were left at room temperature for ***exactly*** three minutes.

10 100µl of 1N HCl was added into each well to stop any further reactions and the strips were left to stand for ten minutes on the bench.

11 After ten minutes, the absorbency (optical density) was measured at 492 nm on the microplate reader.

12 Five calibration points were plotted on the log-log graph paper using the optical density (OD) values that were obtained from different vWF concentrations of 100%, 50%, 25%, 12.5%, and 6.25% (N.B: each batch lot provided the starting calibration value for the standard plasma), to construct a linear graph. The vWF values of the tests and controls were obtained by plotting the measured OD on the X-axis against the vWF concentration on the Y-axis, on the linear graph.

### 3.3.5 Statistical analysis

The data of the comparison and validation of the two vWF:RCo assay methods was analyzed by performing regression analysis and Lineweaver-Burke plots. The results of the study were submitted to the department of biostatistics at the University of the Free State for statistical analysis. According to the statistical report, the data did not follow the

normal distribution and therefore, the Kruskal – Wallis test was used to determine whether the observed difference among the blood groups was statistically significant. The Kruskal – Wallis test uses the p-value approach to test hypothesis. In the Kruskal – Wallis test, the null hypothesis ($H_0$) states that there is no significant difference between the two values for a given probability, while the alternative hypothesis ($H_1$) states that there is significant difference between the two values for a given probability. The p-value is referred to as the observed level of significance, which is the smallest level (critical p-value) at which $H_0$ can be rejected. If the calculated p-value is greater than or equal to the critical p-value, the $H_0$ is not rejected. In contrast, if the calculated p-value is smaller than the critical p-value, the $H_0$ is rejected. The critical p-value at the level of significant is 0.05, which is located as 0.025 on both tale-ends of the Gaussian distribution curve (Levine, Ramsey & Smidt, 2001).

Spearman correlation test was used to determine the correlation between the two variables of vWF, namely, vWF:Ag and vWF:RCo. The test uses the same principle as regression, except that the x-axis and the y-axis are independent of each other, and there are no estimates and expected predictions. The test just compares the variables and determines whether they correlate or not (Levine *et al.*, 2001).

The reference ranges were determined by calculating percentiles from the empirical distribution of the 50 observations from each ABO blood group. Just as the median splits the distribution graph into two equal sections, the percentile splits it into hundredths. The mean value of a normal distribution is also the 50$^{th}$ percentile, and so the mean can be read directly from the graph. It is known that from the 16$^{th}$ percentile to the 50$^{th}$ percentile is one SD, and likewise from the 50$^{th}$ percentile to the 84$^{th}$ percentile is one SD. Thus, to determine the SD, the value corresponding to the 16$^{th}$ percentile is subtracted from the value corresponding to the 84$^{th}$ percentile and the difference is divided by two to obtain the SD.

Alternatively, in a non-symmetrical distribution like the ones observed in the four ABO blood groups, the appropriate percentiles are calculated from the empirical distribution of the observations, and the value corresponding to the 2.5 percentile is subtracted from the value corresponding to the 97.5 percentile, and the difference is divided by four to obtain the SD value. The 2SD was used to establish the reference ranges of the four ABO blood groups (Levine *et al.*, 2001).

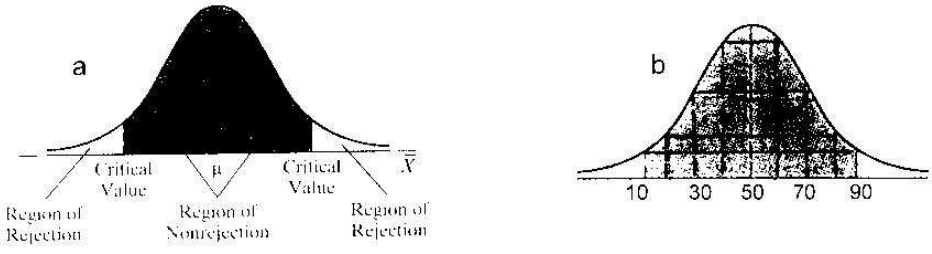



Figure 3.1: Normal distribution curve illustrating level of significance (a), and the different percentiles with confidence intervals (b)

# Chapter 4

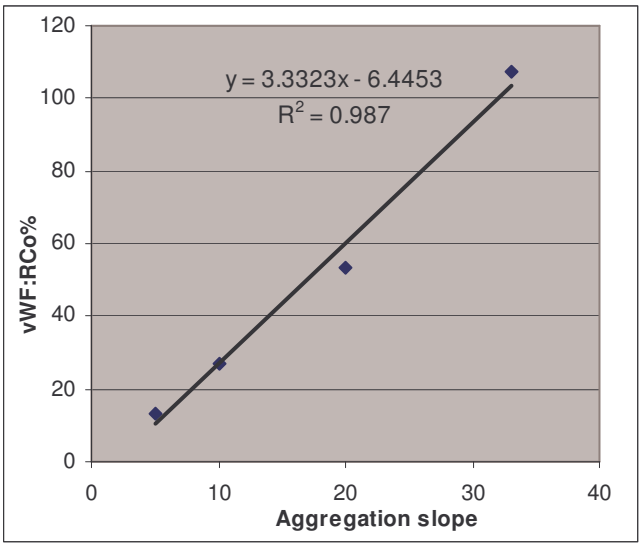
# Results and Discussion

## 4.1 Introduction

Two hundred samples (50 samples from each blood group) were analyzed for plasma vWF:Ag levels and vWF:RCo activity. The in-house vWF:RCo activity assay was suggested as an alternative method to be used for this project instead of the vWF:RCo assay kit method. This was done in consideration of the cost of the project due to the limited grants that was provided. To determine whether the in-house vWF:RCo assay method was reliable in terms of sensitivity, reproducibility and accuracy, it was compared with the kit vWF:RCo assay method, which was used as a reference method, and validated.

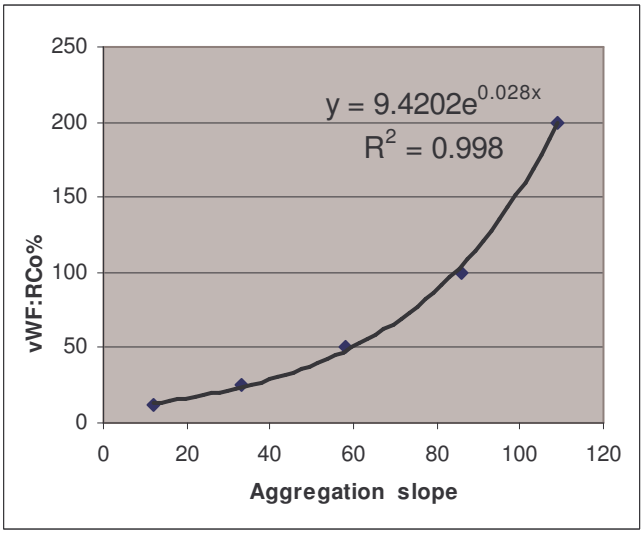
## 4.2 Results of the comparison between the in-house method and the kit method for the vWF:RCo activity assay

The plasma vWF values and both vWF:RCo assays values of the ten serial dilutions that were prepared from pooled normal plasma to obtain known concentrations ranging between 200% and 20% in increments of 20%, were read off their respective standard graphs in Figure 4.1.



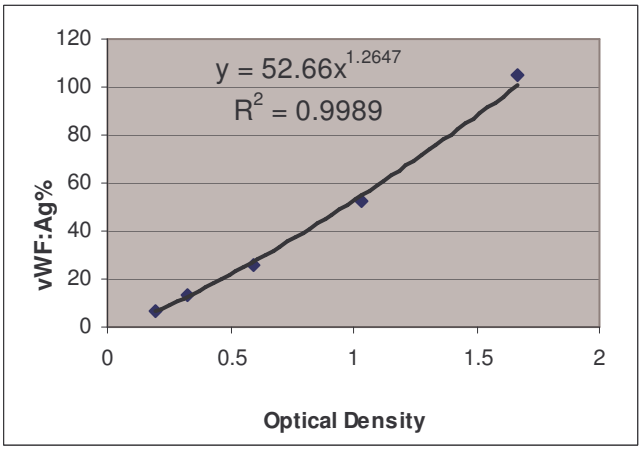

A

| STD dilutions% | Aggr. Slope |
|---|---|
| 107.1000 | 33 |
| 53.5500 | 20 |
| 26.7750 | 10 |
| 13.3875 | 5 |




B

| STD dilutions% | Aggr. Slope |
|---|---|
| 200 | 109 |
| 100 | 93 |
| 50 | 61 |
| 25 | 40 |
| 12.5 | 1 |




C

| STD dilutions% | OD |
|---|---|
| 105 | 1.6665 |
| 52.5 | 1.0335 |
| 26.25 | 0.5895 |
| 13.125 | 0.3280 |
| 6.5625 | 0.1915 |

Figure 4.1: Standard calibration graphs obtained by the kit vWF:RCo assay (A), the in-house vWF:RCo assay (B), and the plasma vWF:Ag assy.(C)

Table 4.1: The results of both the kit and the in-house vWF:RCo activity assays when compared with the known serial dilution values as well as plasma vWF values

| A | B% | C | D% | E | F% | G | H% |
|---|---|---|---|---|---|---|---|
| 1 | 200 | 61 | 197 | 110 | 205 | 2.1075 | 135 |
| 2 | 180 | 57 | 183 | 98 | 146 | 2.0065 | 127 |
| 3 | 160 | 52 | 167 | 96 | 138 | 1.9660 | 124 |
| 4 | 140 | 55 | 177 | 99 | 151 | 1.8325 | 113 |
| 5 | 120 | 47 | 150 | 92 | 124 | 1.6915 | 102 |
| 6 | 100 | 46 | 147 | 89 | 114 | 1.5440 | 91 |
| 7 | 80 | 42 | 134 | 73 | 73 | 1.3010 | 73 |
| 8 | 60 | 32 | 100 | 59 | 49 | 1.0450 | 56 |
| 9 | 40 | 24 | 74 | 48 | 36 | 0.7485 | 37 |
| 10 | 20 | 10 | 27 | 30 | 22 | 0.4490 | 19 |
| control | 100 | | | 89 | 114 | 1.3375 | 79 |

**A** - Test tube number

**B** - Concentrations of different serial dilutions that were prepared from PNP.

**C** - Aggregation slopes obtained by using the commercial vWF:RCo assay kit.

**D** - vWF:RCo activity values determined from the aggregation slopes in C.

**E** - Aggregation slopes obtained by using the in-house vWF:RCo assay.

**F** - vWF:RCo activity values determined from the aggregation slopes in E.

**G** - The values of the OD of the serial dilutions measured by the microplate reader for determining the plasma vWF:Ag levels.

**H** The plasma vWF values determined from the OD in G.

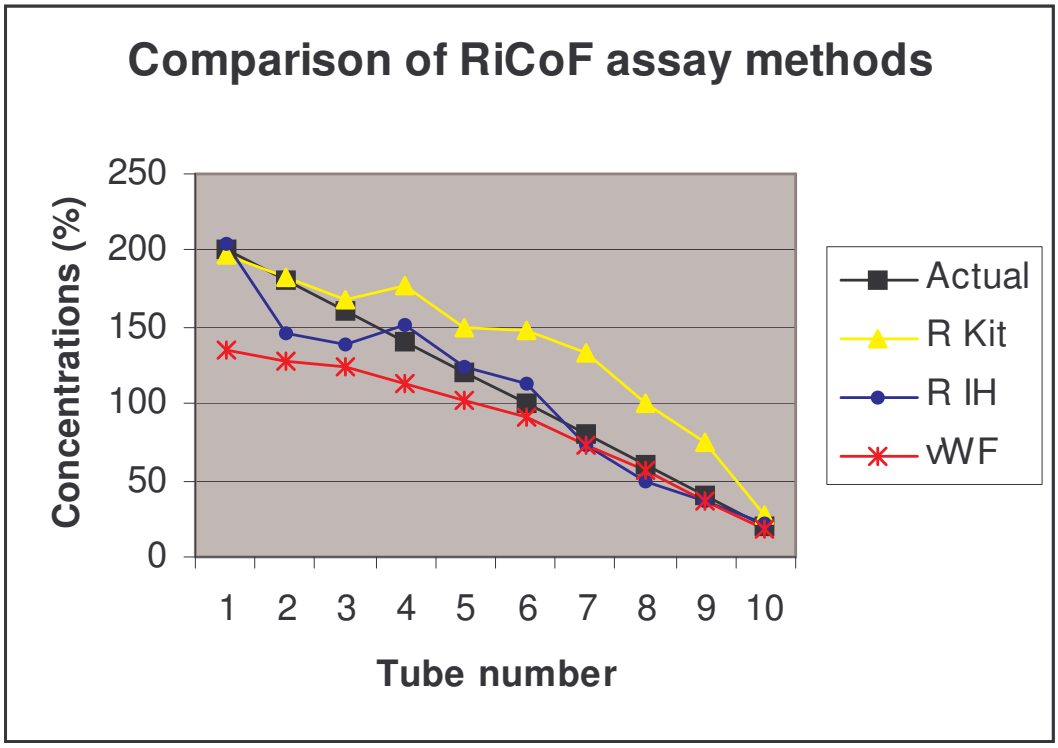



Figure 4.2: Comparison between the kit and the in-house vWF:RCo assay method's performances relative to plasma vWF concentration

Table 4.1 results were illustrated in Figure 4.2 where it was observed that vWF values diverged from the actual concentrations from about 80%. At higher concentrations, the determined vWF values increasingly showed lower values than the actual concentration. This was not a problem from a diagnostic perspective as the vWD patients have a decreased vWF:Ag level. For research purposes however, this could be problematic as the results of people with vWF:Ag levels higher than 80% would be lower than the actual values. The vWF:RCo assay values from the in-house method correlated well with the actual values up to the concentration of 140%, but in the range between 150% and 200%, results obtained were not precise. The values obtained with the vWF:RCo assay kit method diverged from

the actual values from 20% to 150% and were significantly higher than the actual concentrations as well as the vWF values.

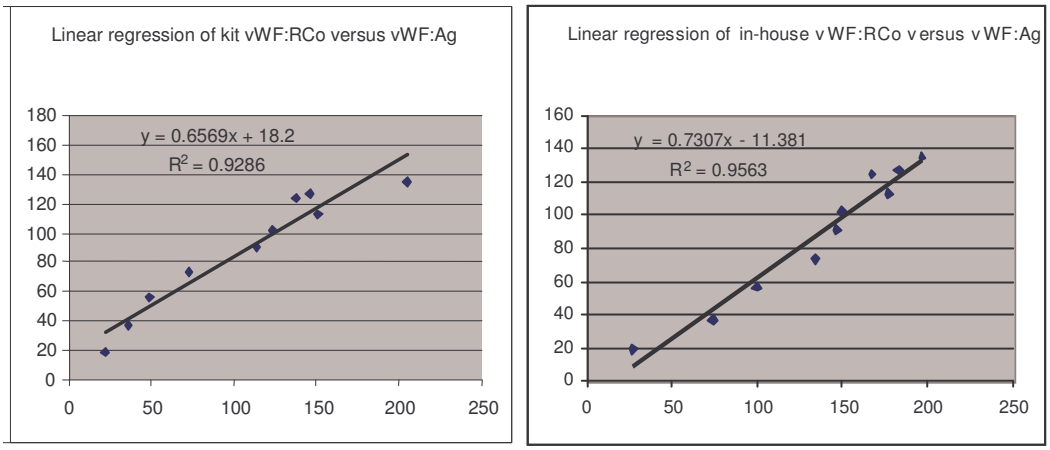



Figure 4.3: Linear regression between the in-house vWF:RCo activity and the kit vWF:RCo activity methods

Figure 4.3 illustrates how the vWF values correlated with the two assay methods of the vWF:RCo activity. Figure 4.3 demonstrates that although both methods correlated well with the vWF:Ag levels, the in-house method yielded better vWF:RCo activity results than the kit method, where the linear regression of vWF:RCo kit and vWF yielded correlation $r^2$ value of 0.93 while the linear regression of vWF:RCo in-house and vWF yielded correlation $r^2$ value of 0.96. The results also showed that although there was less fluctuation between the higher concentrations for the kit method, the values for the in-house method correlated better with the vWF value. This was important because the ratio between the vWF:Ag and vWF:RCo activity is used to diagnose the sub-types of von Willebrand disease. The normal range of the vWF:Ag/vWF:RCo activity ratio is 0.7 - 1.2

(Castaman *et al.*, 2003). Because of the divergence of vWF determinations from the actual values and the inaccuracy of the in-house vWF:RCo above 150%, this ratio should be used with caution. It can be used as a diagnostic tool to diagnose sub-classes of vWD disease, but it should not be interpreted in patients with normal vWF:Ag levels and vWF:RCo activity. Correlation analysis did not clearly explain the significance of the differences found between the in-house method and the kit method, therefore Lineweaver-Burke plots (Figure 4.4) were drawn to compare both methods' results to vWF values. Figure 4.4 illustrates that there was a good correlation between the in-house vWF:RCo values and vWF up to 100%, thereafter, at higher percentages the results diverged. This was caused by decreased sensitivity of the vWF:Ag assay at values above 100%. Divergence between vWF and vWF:RCo kit values was seen from 0 to 200%.

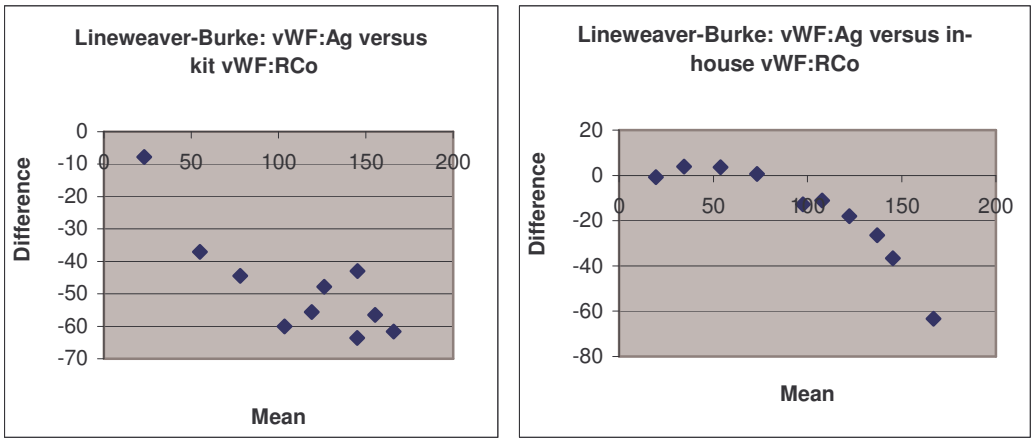
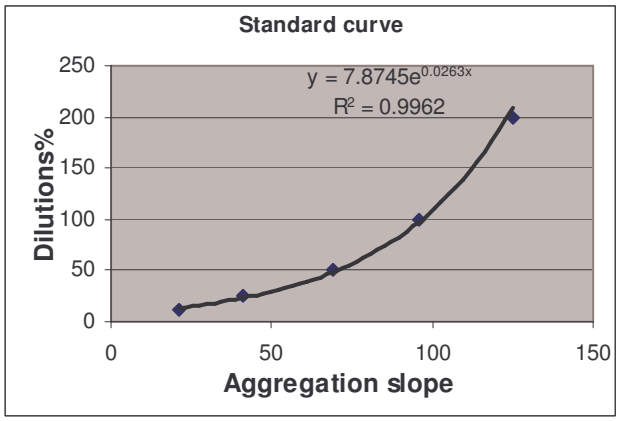



Figure 4.4: Comparison between the in-house and the kit vWF:RCo activity results with plasma vWF values using Lineweaver-Burke plot

Based on all the results that were obtained from the different comparison methods, it was decided that the in-house method using formalin fixed platelets could be used for this study. The high cost of commercial vWF:RCo assay kit was also considered. According to Allain, Cooper, Wagner and Brinkhous (1975), the use of formalin-fixed platelets is advantageous over fresh washed platelets because it excludes the possibility of variation of the response of the platelets (Zuzel, Nilsson & Aberg, 1978).

## 4.3 Validation results of the in-house vWF:RCo activity assay method

The same data that was used for comparing the two methods was used to validate the in-house method. The added data in the validation results was obtained when the in-house method was performed on two successive days to determine the effect of freezing and thawing on the vWF:RCo activity.




| STD dilutions% | Aggr. Slope |
|---|---|
| 200 | 125 |
| 100 | 96 |
| 50 | 69 |
| 25 | 41 |
| 12.5 | 21 |

Figure 4.5: Standard calibration graph by the in-house vWF:RCo assay for the dilutions that have been frozen overnight

Table 4.2: The validation results obtained after the in-house vWF:RCo assay was performed on the dilutions that have been frozen overnight

| A | B% | C | D% | E | F% | G% | H | I% |
|---|---|---|---|---|---|---|---|---|
| 1 | 200 | 110 | 205 | 121 | 192 | 198 | 2.1075 | 135 |
| 2 | 180 | 98 | 146 | 119 | 181 | 164 | 2.0065 | 127 |
| 3 | 160 | 96 | 138 | 115 | 162 | 150 | 1.9660 | 124 |
| 4 | 140 | 99 | 151 | 101 | 112 | 131 | 1.8325 | 113 |
| 5 | 120 | 92 | 124 | 98 | 103 | 113 | 1.6915 | 102 |
| 6 | 100 | 89 | 114 | 94 | 94 | 104 | 1.5440 | 91 |
| 7 | 80 | 73 | 73 | 84 | 73 | 73 | 1.3010 | 73 |
| 8 | 60 | 59 | 49 | 74 | 55 | 52 | 1.0450 | 56 |
| 9 | 40 | 48 | 36 | 50 | 29 | 33 | 0.7485 | 37 |
| 10 | 20 | 30 | 22 | 31 | 18 | 20 | 0.4490 | 19 |
| control | 100 | 89 | 114 | 94 | 94 | 104 | 1.3775 | 79 |

**A** - Test tube number

**B** - Concentrations of different serial dilutions that were prepared from PNP.

**C** - Aggregation slopes obtained by using the in-house vWF:RCo assay on freshly prepared PNP dilutions.

**D** - vWF:RCo activity values determined from the aggregation slopes in C.

**E** - Aggregation slopes obtained by using the in-house vWF:RCo assay on PNP dilutions that have been frozen overnight at -80ºC.

**F** - vWF:RCo activity values determined from the aggregation slopes in E.

**G** - The mean values of the two in-house vWF:RCo assay runs.

**H** - The values of the OD of the serial dilutions measured by the microplate reader for determining the plasma vWF:Ag levels.

**I** The plasma vWF values determined from the OD in H.

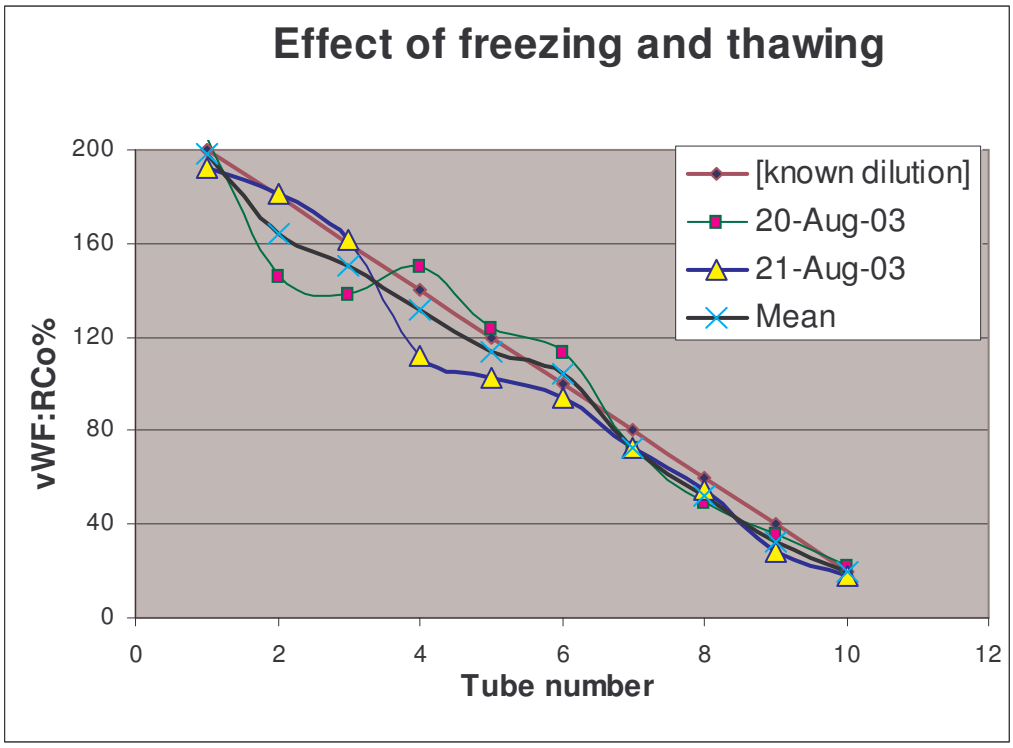



Figure 4.6: The effect of freezing and thawing on the vWF:RCo activity results

The other effects on the vWF:RCo activity results could have come from prolonged storage, and the freezing and thawing of the samples. However, the freezing and thawing effects were determined only on vWF:RCo assay because the plasma vWF:Ag levels could only be analyzed once. The results showed that at concentrations higher than 100%, there was a greater variation between the values obtained on the samples that have not been frozen than the ones that have been frozen and thawed (Table 4.2). A decrease in reproducibility at levels above 100% concentration was observed from these results. Reproducible results were achieved at concentrations below 100%, but at levels above 100% concentration, results varied significantly. However, looking at the

mean values, it was observed that the in-house vWF:RCo assay yielded linear results, which was not obtained with neither the kit vWF:RCo assay nor vWF determents. Figure 4.6 illustrates that the vWF:RCo activity was slightly decreased, even though it was not significant because the assayed values were within range of the known concentration dilutions. After the demonstration in 1971 by Howard and Firkin that ristocetin induced platelet aggregation, the vWF:RCo assay has been in use since 1975 with little change in methodology (Miller *et al.*, 2002).

Other investigators on von Willebrand disease encountered difficulties with vWF:RCo activity determinations. Ermens, de Wild, Vader and van der Graaf (1995) evaluated four agglutination assays for measurement of vWF. In the report, they stated that the imprecision of the aggregometer methods were probably because they required more manual steps. Zuzel *et al.* (1978) did a study on the methods for measuring vWF:RCo activity and found that neither storage of fresh plasma at room temperature for 24 hours nor storage of deep frozen plasma (-70ºC) for 60 days had any significant effect on the plasma vWF:RCo activity. On the other hand, freeze-drying caused a loss of activity. In his discussion, Zuzel *et al.* (2000) stated that what was regarded as optimal conditions for the quantitative assay of the vWF:RCo activity was based on empirical grounds.

## 4.4 Results of the study

The vWF:Ag assay and the in-house method for vWF:RCo activity assay were performed on each of the fifty samples of blood group O, A, B and AB. The standard graphs for each batch of test run, for both the plasma vWF:Ag assay and the in-house vWF:RCo assay are presented in appendixes 1 - 10. Individual results are presented in appendix 11. All the control values were within the stipulated ranges (kit insert).

Table 4.3: Plasma vWF:Ag and vWF:RCo activity median results with reference ranges

| | Group O | | Group A | | Group B | | Group AB | |
|---|---|---|---|---|---|---|---|---|
| | vWF:RCo% | vWF% | vWF:RCo% | vWF% | vWF:RCo% | vWF% | vWF:RCo% | vWF% |
| Median | 64.4 | 53.9 | 103.1 | 80.0 | 79.4 | 72.3 | 116.4 | 71.5 |
| MIN | 14.9 | 18.9 | 39.8 | 24.1 | 30.6 | 21.9 | 38.1 | 24.5 |
| MAX | 269.6 | 131.5 | 227.3 | 126.3 | 434.6 | 146.0 | 590.7 | 146.0 |
| Ref.range | 32 – 125 | 26 – 130 | 42 – 224 | 28 - 124 | 40 - 205 | 43 - 123 | 42 - 406 | 31 - 134 |

The statistics report of the study results in Table 4.3, showed that the results were consistent with the previous reports by Gill *et al.* (1987) that the ABO blood group affected plasma vWF:Ag levels. The median values of the four blood groups' results showed that group O had the lowest vWF:Ag level of 53.9%, followed by group AB with 71.5%, group B with 72.3% and group A with the highest vWF:Ag level of 80.0%. When looking at the vWF:RCo activity assay, it was again blood group O that had the lowest vWF:RCo activity of 64.4% followed by group B with

79.4%, group A with 103.1% and group AB with the highest vWF:RCo activity of 116.4%.

Because of the divergence from the actual levels of vWF:Ag above 80% in this study, the differences between blood groups might be larger than calculated, but in the light of the above, it would not affect diagnosis of vWD, and also, one dilution was used for the test. The results in Table 4.4 and 4.5 were interpreted by applying the Kruskal – Wallis test in the manner that was described in chapter 3, section 3.3.5. These results show the differences in the level of plasma vWF:Ag and vWF:RCo activity respectively among the different blood groups.

Table 4.4: The differences in the level of plasma vWF:Ag among different blood groups

| **The Kruskal – Wallis Test** | | |
|---|---|---|
| **Variable blood groups** | **Calculated p-value** | **Critical p-value (0.05)** |
| O & A | 0.0001 | smaller |
| O & B | 0.0005 | smaller |
| O & AB | 0.0052 | equal |
| A & B | 0.3520 | greater |
| A & AB | 0.1401 | greater |
| B & AB | 0.5672 | greater |

Table 4.4 shows that blood group O had a significant difference with other blood groups for vWF:Ag. The other results of blood groups A & B, A & AB and B & AB for vWF:Ag did not show significant difference, with the p-values of 0.3520, 0.1401 and 0.5672 respectively. While the sequence in

the previous studies was AB, B, A and O starting from highest to lowest for vWF:Ag (Gill *et al.*, 1987), our findings were A, B, AB and O.

Table 4.5: The differences in the vWF:RCo activity among different blood groups

| **The Kruskal – Wallis Test** | | |
|---|---|---|
| **Variable blood groups** | **Calculated p-value** | **Critical p-value (0.05)** |
| O & A | 0.0001 | smaller |
| O & B | 0.0396 | smaller |
| O & AB | 0.0001 | smaller |
| A & B | 0.0145 | smaller |
| A & AB | 0.3158 | greater |
| B & AB | 0.0023 | smaller |

Table 4.5 shows that there was significant difference among the blood groups for vWF:RCo, with exception of A & AB that had the p-value of 0.3158. The blood groups O & A, O & AB and B & AB for vWF:RCo had p-values of 0.0001, 0.0001 and 0.0023 respectively. The vWF:RCo activity results showed an almost similar trend as observed by Gill *et al.* (1987) for vWF:Ag levels, where blood group AB had the highest mean value for vWF:RCo assay, followed by blood group B, blood group A and then blood group O.

Table 4.6: The correlation between plasma vWF:Ag and vWF:RCo activity variables of the four blood groups

| Spearman Correlation Coefficient Test | |
|---|---|
| **Blood group** | **vWF:Ag and vWF:RCo** |
| O | -0.04070 |
| A | 0.31659 |
| B | 0.37919 |
| AB | 0.40704 |

The Spearman Correlation Coefficient test was also described in chapter 3 section 3.3.5. Table 4.6 shows that there was no good correlation between vWF:Ag and vWF:RCo in all the blood groups. In blood group O, there was no correlation, with $r^2$ value of -0.0407. Correlation coefficient means if the x-value increases with certain unit, the y-value should also increase with slightly more or less, or equal amount of that unit. However, in the light of the results presented in Table 4.6, that was not the case. These correlation coefficient results also indicate that if the vWF:Ag/vWF:RCo ratios were to be calculated, the study subjects in general would fall outside the ratio range of 0.7 – 1.2, with the majority of those coming from blood group AB with 32%, group O with 30%, group A with 26% and group B with 12%. The high percentage of these outside range ratios in blood groups AB and O were due to highly elevated vWF:RCo activities in the blood group AB, and reduced vWF:Ag in the blood group O.

Some of the values in these results were accepted as outliers, for an

example, the minimum and the maximum concentration values of all the blood groups, which at the end influenced the outcome of the mean concentration values. As seen in Appendix B, the results of samples 6, 35, 36 and 40 of blood group O, were examples that showed that some of the blood donors could be vWD type 1. Samples 4, 9, and 29 of blood group AB were examples that showed that some of the blood donors could be vWD type 2.

After statistical analysis revealed that the data were non-symmetrically distributed in all the blood groups for both variables, the median was used to determine the reference range of different blood groups, using percentiles. From these results, it was observed that there is no clinically significant lower cut-off value for the different blood groups. These reference ranges are also too large for normal samples. This can be attributed by the number of outliers that were used in the calculations of percentiles when reference ranges were determined. The use of different reference ranges for the different blood groups could be debatable because active levels of vWF:Ag determine the presence of vWD. A person of blood group O should clinically demonstrate the same effect of a circulating vWF:Ag level as a person of any other blood group with a similar circulating level of vWF:Ag.

# Chapter 5

## Conclusion

In the light of the outcome of this study, it was determined that plasma vWF:Ag levels of blood group O persons were significantly lower than persons with blood groups A, B and AB. The vWF:RCo activity was also determined to be lower in persons with blood group O than that of persons with the other ABO blood groups.

From the findings of these study it was concluded that; only blood group O has an influence on the levels of both plasma vWF:Ag and the vWF:RCo activity, and not the entire ABO blood group, as previously reported by Gill *et al.*(1987) and Sweeney and Hoernig (1992). The plasma vWF:Ag and the vWF:RCo activity in the blood groups A, B and AB showed not to be influenced by the ABO blood group status. This conclusion could be supported by comparing these results with the previous reports that there was no definite sequence to which blood group followed after blood group O with the lowest values of plasma vWF. Gill *et al.* (1987) found blood group O to have the lowest values, followed by group A, then group B and group AB with the highest values. Shima, Fujimura, Nishiyama, Tsujiuchi, Narita, Matsui, Titani, Katayama, Yamamoto & Yoshioka (1995) did a study on ABO blood group genotype and plasma vWF:Ag in normal individuals, and found that the non-O genotype blood groups had higher

plasma vWF:Ag and vWF:RCo activity levels than the O genotype blood groups. The OO genotype had the lowest mean values followed by BO, AO, AA, AB and BB genotype with the highest mean values. The values were 89.9U/dl, 100.5U/dl, 103.7U/dl, 113.3U/dl, 113.8U/dl and 114.5U/dl respectively for plasma vWF:Ag levels, and 82.2U/dl, 107.3U/dl, 110.6U/dl, 123.8U/dl, 124.3U/dl and 125.3U/dl respectively for vWF:RCo activity levels (Shima *et al.*, 1995).

When looking at the established reference ranges for both plasma vWF and vWF:RCo activity for the different ABO blood groups, it hypothetically means that failure to use blood group-specific ranges would place plasma vWF:Ag levels of individuals with blood group O below the normal range. However, these reference ranges did not show a significant lower cut-off value for the different blood groups, and furthermore they are too large for normal samples. The questions that arose were whether the diagnosis of vWD was influenced by the patient's ABO status, where it was found that more vWD type 1 patients were blood group O, and whether the blood group-specific reference ranges would provide a conclusive solution to the diagnosis of von Willebrand disease. It was therefore recommended that different reference ranges should not be used for different blood groups, because regardless of the patient’s blood group and the reference ranges, a certain level of plasma vWF:Ag is needed in the body to maintain normal haemostasis. The implication of the fact that persons with blood group O

have lower levels of plasma vWF:Ag and vWF:RCo, means that there is a higher incidence of vWD in persons with blood group O.

The overall goal of the objectives of this study was achieved; in which we found that only blood group O has a direct influence on the plasma vWF:Ag and the vWF:RCo activity levels, and the reference ranges have no clinical cut-off value and therefore could not be used for the diagnosis of vWD. Therefore, contributing to the quality of the diagnosis and treatment of von Willebrand disease.

Some of the shortcomings of the study were;

- It took a period of one and half month to obtain all 200 samples, therefore plasma from these samples had to be frozen at -80ºC before they could be analyzed. The effect of freezing and thawing had to be determined before the study could even commence, as some plasma was frozen longer than the others were.
- It was not possible to analyze all 200 samples in one day; therefore, there was a day-to-day variation in the outcome of the slopes of the standard curves. This meant that the values were not determined from the same standard curve.
- The plasma vWF:Ag assay: the tests were run in duplicate, however due to the budget that was limited only to 200 samples (one dilution in duplicate) by calculation, only one dilution (1:51) was used to

determine the standard curve, the test samples and the control values. Because of the same reason again, no sample could be analyzed twice for vWF:Ag assay.

- The in-house vWF:RCo assay: because of the tediousness of the manual labour that is involved in this assay the tests were run singularly, only one dilution (1:2) was used to determine the standard curve, the test samples and the control values. A time of no less than four hours was needed to prepare and complete a batch of forty samples, including the standard and the control. There are no commercially available controls and standards for the in-house vWF:RCo assay.
- We were unable to monitor the process of sample collection as it was done by the Bloemfontein Blood Bank staff.

The reasons for this effect of blood group O on vWF:Ag is still not clear, although it has been postulated that the processing, release, or catabolism of vWF may be affected by the blood group determinants, thus influencing its plasma concentration (Souto *et al.*, 2000). This sets base for further studies on this subject.

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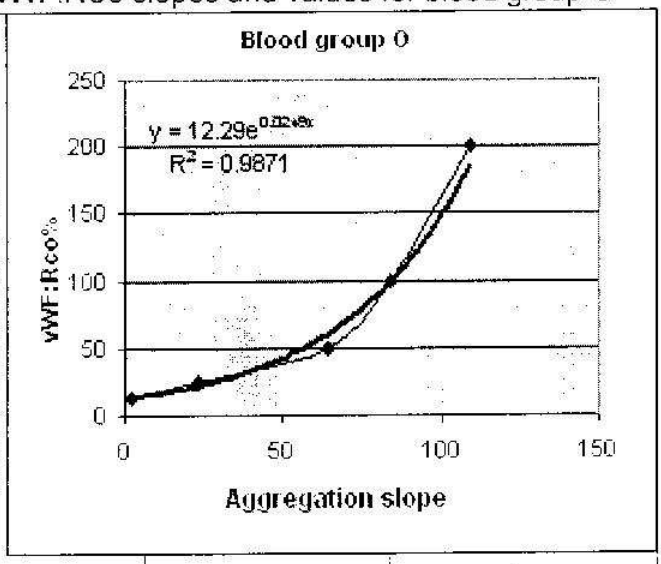
## Appendix 1

vWF:RCo Standard curve with the vWF:RCo slopes and values for blood group O

| *Slope* | *Std.Dilution* |
|---|---|
| 2 | 12.5 |
| 23 | 25 |
| 64 | 50 |
| 84 | 100 |
| 109 | 200 |

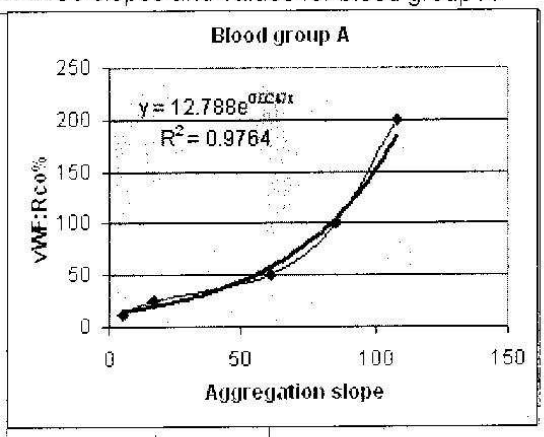
| Group O | Slope | vWF:RCo |
|---|---|---|
| 1 | 69 | 68.50 |
| 2 | 47 | 39.61 |
| 3 | 68 | 66.82 |
| 4 | 59 | 53.40 |
| 5 | 42 | 34.97 |
| 6 | 3 | 13.24 |
| 7 | 93 | 124.52 |
| 8 | 84 | 99.52 |
| 9 | 46 | 38.64 |
| 10 | 71 | 72.00 |
| 11 | 41 | 34.11 |
| 12 | 65 | 62.01 |
| 13 | 84 | 99.52 |
| 14 | 92 | 121.46 |
| 15 | 56 | 49.56 |
| 16 | 64 | 60.48 |
| 17 | 47 | 39.61 |
| 18 | 63 | 59.00 |
| 19 | 53 | 45.99 |
| 20 | 67 | 65.18 |
| 21 | 57 | 50.81 |
| 22 | 56 | 49.56 |
| 23 | 74 | 77.59 |
| 24 | 42 | 34.97 |
| 25 | 48 | 40.61 |
| 26 | 55 | 48.34 |
| 27 | 66 | 63.57 |
| 28 | 68 | 66.82 |
| 29 | 49 | 41.63 |
| 30 | 84 | 99.52 |
| 31 | 52 | 44.86 |
| 32 | 68 | 66.82 |
| 33 | 75 | 79.54 |
| 34 | 86 | 104.60 |
| 35 | 90 | 115.56 |
| 36 | 90 | 115.56 |
| 37 | 64 | 60.48 |
| 38 | 57 | 50.81 |
| 39 | 57 | 50.81 |
| 40 | 84 | 99.52 |
| 41 | 67 | 65.18 |



## Appendix 2

vWF:RCo Standard curve with the vWF:RCo slopes and values for blood group A

| *Slope* | *Std.Dilution* | |
|---|---|---|
| 5 | 12.5 | |
| 17 | 25 | |
| 61 | 50 | |
| 85 | 100 | |
| 108 | 200 | |
| **Group A** | **Slope** | **vWF:RCo** |
| 1 | 56 | 50.99 |
| 2 | 66 | 65.28 |
| 3 | 64 | 62.13 |
| 4 | 67 | 66.91 |
| 5 | 70 | 72.06 |
| 6 | 58 | 53.58 |
| 7 | 85 | 104.38 |
| 8 | 73 | 77.60 |
| 9 | 48 | 41.85 |
| 10 | 92 | 124.08 |
| 11 | 86 | 106.99 |
| 12 | 108 | 184.22 |
| 13 | 61 | 57.70 |
| 14 | 151 | 532.83 |
| 15 | 46 | 39.83 |
| 16 | 63 | 60.62 |
| 17 | 111 | 198.38 |
| 18 | 76 | 83.57 |
| 19 | 94 | 130.36 |
| 20 | 111 | 198.38 |
| 21 | 92 | 124.08 |
| 22 | 94 | 130.36 |
| 23 | 94 | 130.36 |
| 24 | 90 | 118.10 |
| 25 | 74 | 79.54 |
| 26 | 89 | 115.22 |
| 27 | 86 | 106.99 |
| 28 | 84 | 101.83 |
| 29 | 116 | 224.46 |
| 30 | 103 | 162.81 |
| 31 | 69 | 70.30 |
| 32 | 63 | 60.62 |
| 33 | 84 | 101.83 |
| 34 | 89 | 115.22 |
| 35 | 82 | 96.92 |
| 36 | 53 | 47.35 |
| 37 | 59 | 54.92 |
| 38 | 59 | 54.92 |
| 39 | 58 | 53.58 |
| 40 | 78 | 87.80 |
| 41 | 71 | 73.86 |

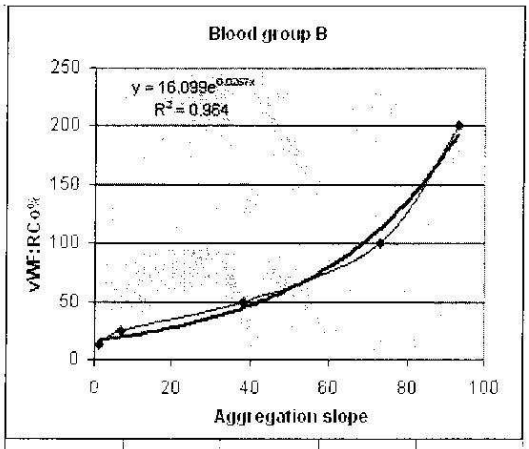


## Appendix 3

vWF:RCo Standard curve with the vWF:RCo slopes and values for blood group B

| *Slope* | *Std.Dilution* |
|---|---|
| 1 | 12.5 |
| 7 | 25 |
| 38 | 50 |
| 73 | 100 |
| 93 | 200 |

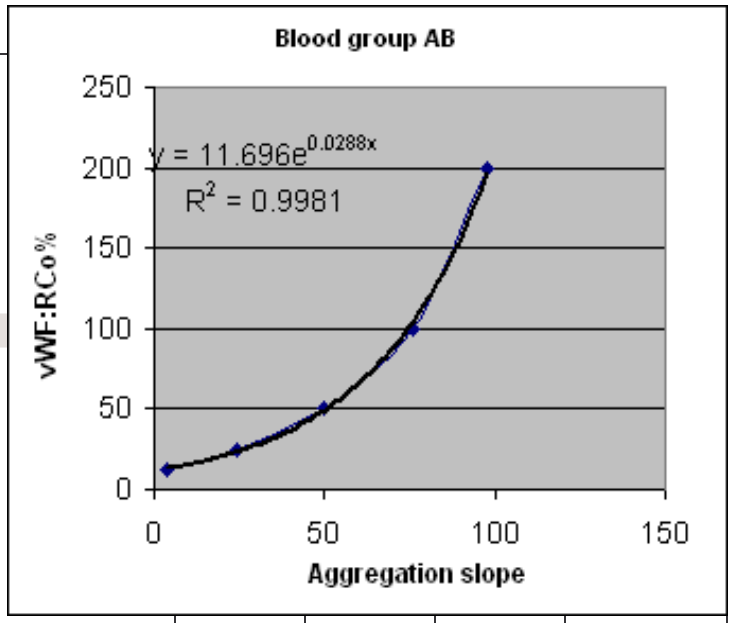
| Group B | Slope | vWF:RCo |
|---|---|---|
| 1 | 39 | 45.61 |
| 2 | 61 | 82.06 |
| 3 | 63 | 86.56 |
| 4 | 55 | 69.91 |
| 5 | 57 | 73.75 |
| 6 | 62 | 84.28 |
| 7 | 53 | 66.28 |
| 8 | 65 | 91.31 |
| 9 | 63 | 86.56 |
| 10 | 61 | 82.06 |
| 11 | 37 | 43.24 |
| 12 | 50 | 61.18 |
| 13 | 70 | 104.35 |
| 14 | 61 | 82.06 |
| 15 | 42 | 49.41 |
| 16 | 64 | 88.90 |
| 17 | 53 | 66.28 |
| 18 | 71 | 107.17 |
| 19 | 63 | 86.56 |
| 20 | 53 | 66.28 |
| 21 | 69 | 101.60 |
| 22 | 45 | 53.53 |
| 23 | 51 | 62.83 |
| 24 | 71 | 107.17 |
| 25 | 24 | 30.56 |
| 26 | 61 | 82.06 |
| 27 | 64 | 88.90 |
| 28 | 34 | 39.91 |
| 29 | 59 | 77.79 |
| 30 | 63 | 86.56 |
| 31 | 53 | 66.28 |
| 32 | 73 | 113.05 |
| 33 | 60 | 79.90 |
| 34 | 49 | 59.56 |
| 35 | 36 | 42.10 |
| 36 | 50 | 61.18 |
| 37 | 45 | 53.53 |
| 38 | 71 | 107.17 |
| 39 | 50 | 61.18 |
| 40 | 55 | 69.91 |
| 41 | 61 | 82.06 |



## Appendix 4

vWF:RCo Standard curve with the vWF:RCo slopes and values for blood group AB

| *Slope* | Std.Dilution |
|---|---|
| 4 | 12.5 |
| 24 | 25 |
| 50 | 50 |
| 76 | 100 |
| 98 | 200 |

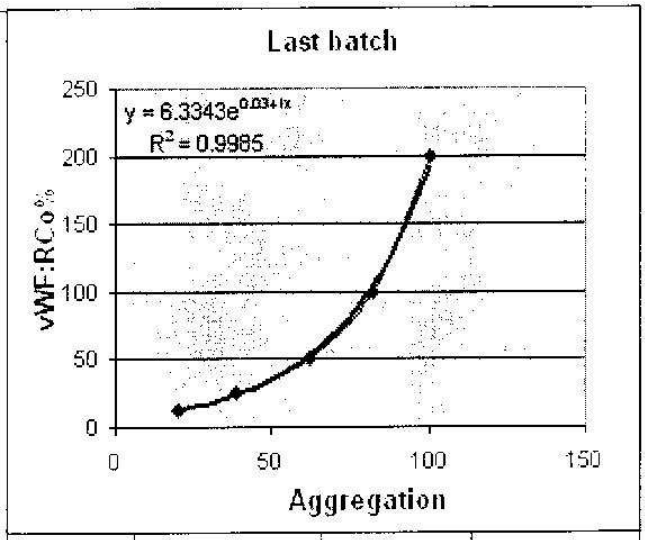



| Group AB | Slope | vWF:RCo |
|---|---|---|
| 1 | 67 | 80.55 |
| 2 | 58 | 62.16 |
| 3 | 81 | 120.55 |
| 4 | 51 | 50.81 |
| 5 | 105 | 240.63 |
| 6 | 90 | 156.22 |
| 7 | 57 | 60.39 |
| 8 | 96 | 185.68 |
| 9 | 46 | 43.99 |
| 10 | 94 | 175.29 |
| 11 | 79 | 113.80 |
| 12 | 65 | 76.04 |
| 13 | 97 | 191.11 |
| 14 | 83 | 127.70 |
| 15 | 55 | 57.01 |
| 16 | 58 | 62.16 |
| 17 | 79 | 113.80 |
| 18 | 51 | 50.81 |
| 19 | 84 | 131.43 |
| 20 | 67 | 80.55 |
| 21 | 41 | 38.09 |
| 22 | 47 | 45.28 |
| 23 | 44 | 41.53 |
| 24 | 47 | 45.28 |
| 25 | 64 | 73.88 |
| 26 | 90 | 156.22 |
| 27 | 82 | 124.07 |

## Appendix 5

vWF:RCo Standard curve with vWF:RCo the slopes and values for all the ABO blood groups

| *Slope* | *Std.Dilution* | |
|---|---|---|
| 20 | 12.5 | |
| 39 | 25 | |
| 62 | 50 | |
| 82 | 100 | |
| 100 | 200 | |
| **Group O** | **Slope** | **vWF:RCo** |
| 14 | 89 | 131.7465 |
| 36 | 89 | 131.7465 |
| 42 | 110 | 269.6108 |
| 43 | 61 | 50.70778 |
| 44 | 72 | 73.78676 |
| 45 | 47 | 31.45882 |
| 46 | 82 | 103.7702 |
| 47 | 75 | 81.73476 |
| 48 | 82 | 103.7702 |
| 49 | 83 | 107.3698 |
| 50 | 25 | 14.85712 |
| 6 | 81 | 100.2913 |
| **Group A** | | |
| 14 | 90 | 136.3165 |
| 15 | 41 | 25.63809 |
| 42 | 83 | 107.3698 |
| 43 | 85 | 114.9479 |
| 44 | 70 | 68.92227 |
| 45 | 94 | 156.2378 |
| 46 | 104 | 219.7256 |
| 47 | 84 | 111.0943 |
| 48 | 95 | 161.6574 |
| 49 | 93 | 150.9999 |
| 50 | 105 | 227.3474 |
| **Group B** | | |
| 1 | 34 | 20.1939 |
| 25 | 52 | 37.3069 |
| 28 | 40 | 24.7786 |
| 35 | 123 | 420.0103 |
| 42 | 70 | 68.9223 |
| 43 | 124 | 434.5797 |
| 44 | 88 | 127.3296 |
| 45 | 65 | 58.1182 |
| 46 | 74 | 78.9946 |
| 47 | 67 | 62.2202 |
| 48 | 102 | 205.2399 |
| 49 | 99 | 185.2821 |
| 50 | 75 | 81.7348 |



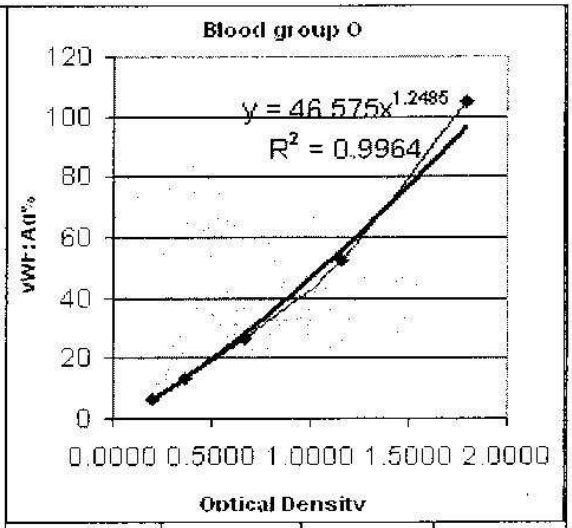

| **Group AB** | **Slope** | **vWF:RCo** |
|---|---|---|
| 28 | 79 | 93.6795 |
| 29 | 57 | 44.2422 |
| 30 | 92 | 145.9376 |
| 31 | 63 | 54.2867 |
| 32 | 79 | 93.6795 |
| 33 | 107 | 243.3935 |
| 34 | 103 | 212.3592 |
| 35 | 89 | 131.7465 |
| 36 | 75 | 81.7348 |
| 37 | 133 | 590.6829 |
| 38 | 82 | 103.7702 |
| 39 | 82 | 103.7702 |
| 40 | 92 | 145.9376 |
| 41 | 105 | 227.3474 |
| 42 | 122 | 405.9294 |
| 43 | 100 | 191.7091 |
| 44 | 93 | 150.9999 |
| 45 | 95 | 161.6574 |
| 46 | 89 | 131.7465 |
| 47 | 86 | 118.9353 |
| 48 | 82 | 103.7702 |
| 49 | 88 | 127.3296 |
| 50 | 103 | 212.3592 |

## Appendix 6

vWF Standard curve with the vWF OD and vWF:Ag values for blood group O

| *OD* | *Std. Dilution* |
|---|---|
| 1.7900 | 105 |
| 1.1570 | 52.5 |
| 0.6620 | 26.25 |
| 0.3670 | 13.125 |
| 0.2000 | 6.5625 |

| **Group O** | **OD** | **vWF:Ag** |
|---|---|---|
| Control | 1.3989 | 71 |
| 1 | 0.9850 | 46 |
| 2 | 0.9880 | 46 |
| 3 | 1.3480 | 68 |
| 4 | 0.4860 | 19 |
| 5 | 1.0520 | 50 |
| 6 | 0.9860 | 46 |
| 7 | 2.0420 | 114 |
| 8 | 1.5090 | 78 |
| 9 | 1.0240 | 48 |
| 10 | 1.3590 | 68 |
| 11 | 2.2970 | 132 |
| 12 | 0.6230 | 26 |
| 13 | 1.1400 | 55 |
| 14 | 1.1960 | 58 |
| 15 | 0.9790 | 45 |
| 16 | 1.4900 | 77 |
| 17 | 1.7090 | 91 |
| 18 | 1.0280 | 48 |
| 19 | 1.2320 | 60 |
| 20 | 1.2940 | 64 |
| 21 | 1.0860 | 52 |
| 22 | 1.4040 | 71 |
| 23 | 1.0890 | 52 |
| 24 | 1.0710 | 51 |
| 25 | 0.9860 | 46 |
| 26 | 1.0910 | 52 |
| 27 | 1.4920 | 77 |
| 28 | 1.2130 | 59 |
| 29 | 1.3900 | 70 |
| 30 | 0.9320 | 43 |
| 31 | 1.1590 | 56 |
| 32 | 0.9930 | 46 |
| 33 | 0.8540 | 38 |
| 34 | 1.3830 | 70 |
| 35 | 0.8520 | 38 |
| 36 | 0.9740 | 45 |
| 37 | 0.648 | 27 |
| 38 | 0.6550 | 27 |
| 39 | 1.0320 | 48 |
| 40 | 1.0070 | 47 |
| 41 | 0.9290 | 42 |

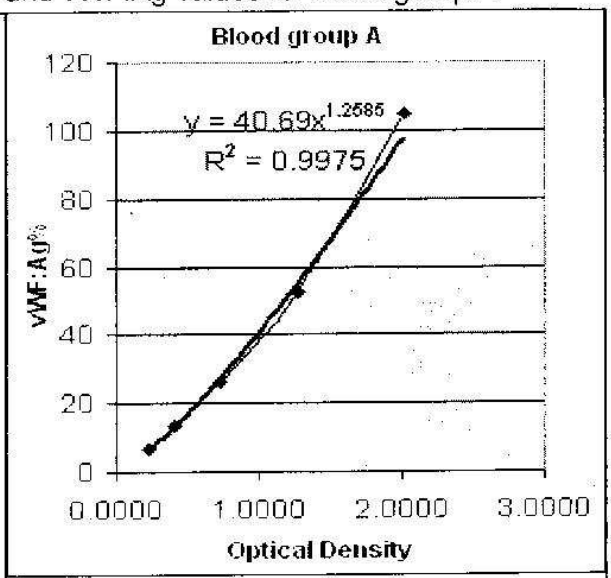


## Appendix 7

vWF Standard curve with the vWF OD and vWF:Ag values for blood group A

| *OD* | *Std. Dilution* |
|---|---|
| 2.0090 | 105 |
| 1.2800 | 52.5 |
| 0.7250 | 26.25 |
| 0.4160 | 13.125 |
| 0.2260 | 6.5625 |

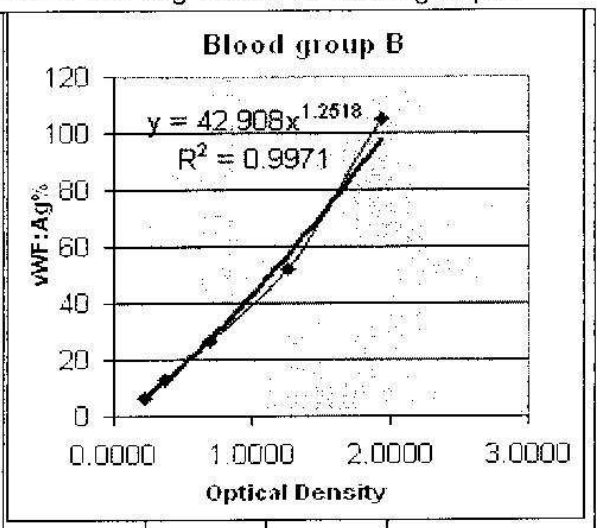
| Group A | OD | vWF:Ag |
|---|---|---|
| Control | 1.7340 | 81.3450 |
| 1 | 1.7655 | 83.2091 |
| 2 | 1.6555 | 76.7379 |
| 3 | 1.9095 | 91.8386 |
| 4 | 1.2130 | 51.8832 |
| 5 | 2.1165 | 104.5390 |
| 6 | 1.8475 | 88.1017 |
| 7 | 1.8775 | 89.9059 |
| 8 | 1.2435 | 53.5303 |
| 9 | 1.2400 | 53.3407 |
| 10 | 0.6590 | 24.0744 |
| 11 | 2.4595 | 126.2898 |
| 12 | 2.0420 | 99.9293 |
| 13 | 1.2675 | 54.8337 |
| 14 | 2.3790 | 121.1100 |
| 15 | 2.0870 | 102.7086 |
| 16 | 1.6835 | 78.3749 |
| 17 | 2.1855 | 108.8460 |
| 18 | 1.8175 | 86.3051 |
| 19 | 1.7290 | 81.0499 |
| 20 | 2.1425 | 106.1577 |
| 21 | 1.5845 | 72.6193 |
| 22 | 1.1340 | 47.6670 |
| 23 | 1.2160 | 52.0447 |
| 24 | 1.9285 | 92.9901 |
| 25 | 1.5000 | 67.7795 |
| 26 | 0.8115 | 31.2844 |
| 27 | 1.6170 | 74.4988 |
| 28 | 2.0740 | 101.9041 |
| 29 | 2.2345 | 111.9260 |
| 30 | 2.0990 | 103.4523 |
| 31 | 1.4420 | 64.4979 |
| 32 | 1.7085 | 79.8424 |
| 33 | 1.7145 | 80.1954 |
| 34 | 2.3075 | 116.5471 |
| 35 | 1.5365 | 69.8617 |
| 36 | 0.7340 | 27.5719 |
| 37 | 1.7025 | 79.4897 |
| 38 | 1.5155 | 68.6621 |
| 39 | 0.7805 | 29.7878 |
| 40 | 1.8805 | 90.0867 |
| 41 | 2.1500 | 106.6256 |



## Appendix 8

vWF Standard curve with the vWF OD and vWF:Ag values for blood group B

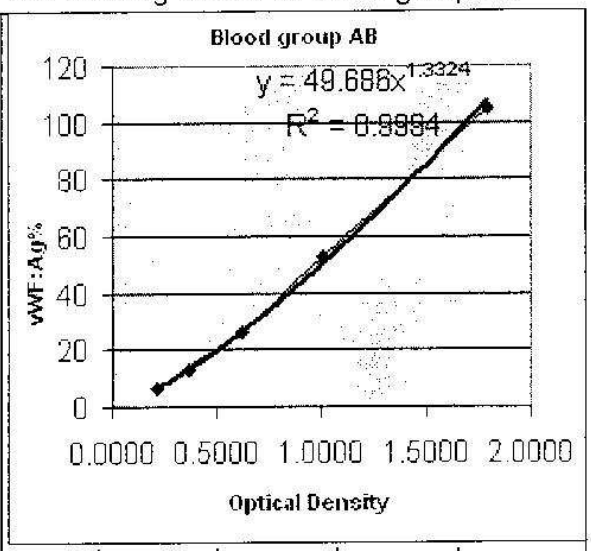
| *OD* | *Std. Dilution* | |
|---|---|---|
| 1.9375 | 105 | |
| 1.2550 | 52.5 | |
| 0.6880 | 26.25 | |
| 0.3740 | 13.125 | |
| 0.2245 | 6.5625 | |
| | | |
| **Group B** | **OD** | **vWF:Ag** |
| Control | 1.5990 | 77.2175 |
| 1 | 2.6595 | 145.9832 |
| 2 | 1.9465 | 98.7704 |
| 3 | 1.9030 | 96.0151 |
| 4 | 1.6090 | 77.8225 |
| 5 | 1.5515 | 74.3569 |
| 6 | 2.2480 | 118.2815 |
| 7 | 2.1090 | 109.1987 |
| 8 | 1.5230 | 72.6511 |
| 9 | 1.6590 | 80.8615 |
| 10 | 1.9150 | 96.7737 |
| 11 | 1.4100 | 65.9676 |
| 12 | 1.1435 | 50.7503 |
| 13 | 1.9665 | 100.0425 |
| 14 | 1.8545 | 92.9618 |
| 15 | 1.1970 | 53.7399 |
| 16 | 1.3290 | 61.2586 |
| 17 | 1.5010 | 71.3398 |
| 18 | 1.2505 | 56.7633 |
| 19 | 2.0980 | 108.4862 |
| 20 | 1.0775 | 47.1106 |
| 21 | 2.0875 | 107.8069 |
| 22 | 1.2735 | 58.0732 |
| 23 | 1.8780 | 94.4388 |
| 24 | 2.1205 | 109.9446 |
| 25 | 0.5845 | 21.9078 |
| 26 | 1.7595 | 87.0395 |
| 27 | 1.2065 | 54.2743 |
| 28 | 1.0300 | 44.5254 |
| 29 | 1.7275 | 85.0625 |
| 30 | 1.0005 | 42.9349 |
| 31 | 1.2760 | 58.2160 |
| 32 | 2.2245 | 116.7357 |
| 33 | 1.5085 | 71.7863 |
| 34 | 1.2650 | 57.5884 |
| 35 | 1.3485 | 62.3858 |
| 36 | 1.3950 | 65.0903 |
| 37 | 1.2875 | 58.8735 |
| 38 | 1.0715 | 46.7824 |
| 39 | 1.2050 | 54.1898 |
| 40 | 2.3220 | 123.1756 |
| 41 | 1.9405 | 98.3895 |



## Appendix 9

vWF Standard curve with the vWF OD and vWF:Ag values for blood group AB

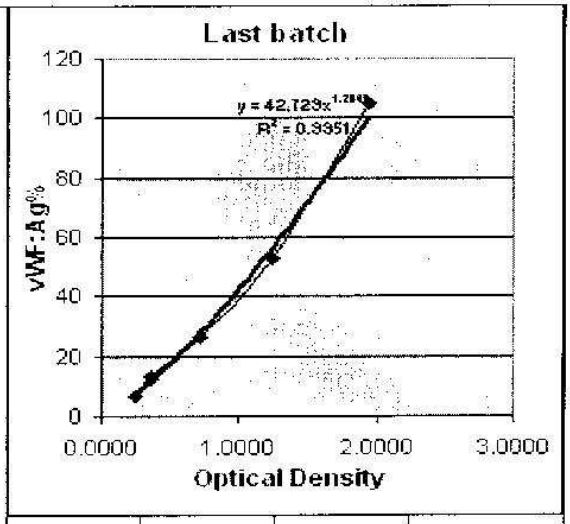
| *OD* | *Std. Dilution* | |
|---|---|---|
| 1.7905 | 105 | |
| 1.0070 | 52.5 | |
| 0.6230 | 26.25 | |
| 0.3700 | 13.125 | |
| 0.2195 | 6.5625 | |
| | | |
| **Group AB** | **OD** | **vWF:Ag** |
| Control | 1.4060 | 78.2365 |
| 1 | 0.7430 | 33.4456 |
| 2 | 1.3130 | 71.4184 |
| 3 | 1.0795 | 55.0174 |
| 4 | 1.8515 | 112.8971 |
| 5 | 2.1100 | 134.3718 |
| 6 | 1.6870 | 99.7338 |
| 7 | 0.8200 | 38.1417 |
| 8 | 1.7300 | 103.1351 |
| 9 | 1.2050 | 63.7003 |
| 10 | 1.2580 | 67.4603 |
| 11 | 1.3145 | 71.5271 |
| 12 | 1.2910 | 69.8284 |
| 13 | 1.7005 | 100.7986 |
| 14 | 2.2455 | 145.9902 |
| 15 | 0.5885 | 24.5157 |
| 16 | 1.3530 | 74.3319 |
| 17 | 1.0440 | 52.6200 |
| 18 | 0.8510 | 40.0749 |
| 19 | 1.7575 | 105.3253 |
| 20 | 1.0755 | 54.7459 |
| 21 | 0.8350 | 39.0741 |
| 22 | 1.2905 | 69.7924 |
| 23 | 1.3130 | 71.4184 |
| 24 | 0.7055 | 31.2156 |
| 25 | 1.1840 | 62.2254 |
| 26 | 1.9110 | 117.7567 |
| 27 | 1.3740 | 75.8731 |
| 28 | 1.0300 | 51.6819 |
| 29 | 1.7625 | 105.7247 |
| 30 | 1.5650 | 90.2412 |
| 31 | 1.2760 | 68.7495 |
| 32 | 1.0265 | 51.4480 |
| 33 | 1.3790 | 76.2412 |
| 34 | 1.4575 | 82.0779 |
| 35 | 0.9420 | 45.8838 |
| 36 | 0.7515 | 33.9564 |
| 37 | 1.4400 | 80.7674 |
| 38 | 1.6485 | 96.7127 |
| 39 | 1.8115 | 109.6590 |
| 40 | 1.3310 | 72.7259 |
| 41 | 1.1175 | 57.6128 |



## Appendix 10

vWF Standard curve with the vWF OD and vWF:Ag values for all the ABO blood groups

| *OD* | *Std. Dilution* | |
|---|---|---|
| 1.9370 | 105 | |
| 1.2310 | 52.5 | |
| 0.7095 | 26.25 | |
| 0.3660 | 13.125 | |
| 0.2425 | 6.5625 | |
| | **OD** | **vWF:Ag** |
| Control | 1.4405 | 68.2937 |
| **Group O** | | |
| 42 | 1.7955 | 90.636 |
| 43 | 1.4435 | 68.476 |
| 44 | 1.6100 | 78.787 |
| 45 | 1.6135 | 79.007 |
| 46 | 1.1800 | 52.854 |
| 47 | 1.2710 | 58.147 |
| 48 | 1.4760 | 70.464 |
| 49 | 1.5040 | 72.186 |
| 50 | 2.3775 | 130.006 |
| **Group A** | | |
| 42 | 1.6145 | 79.0697 |
| 43 | 2.2875 | 123.7171 |
| 44 | 1.4390 | 68.2023 |
| 45 | 1.3070 | 60.2717 |
| 46 | 2.2370 | 120.2191 |
| 47 | 2.0630 | 108.3406 |
| 48 | 1.3340 | 61.8761 |
| 49 | 1.1025 | 48.4363 |
| 50 | 2.1525 | 114.4164 |
| **Group B** | | |
| 42 | 1.7855 | 89.9878 |
| 43 | 1.8900 | 96.8101 |
| 44 | 2.1425 | 113.7339 |
| 45 | 1.3620 | 63.5497 |
| 46 | 1.0455 | 45.2429 |
| 47 | 1.0595 | 46.0227 |
| 48 | 1.3385 | 62.1444 |
| 49 | 1.5195 | 73.1428 |
| 50 | 1.5000 | 71.9391 |
| **Group AB** | | |
| 42 | 1.7870 | 90.0850 |
| 43 | 1.5215 | 73.2665 |
| 44 | 1.7560 | 88.0821 |
| 45 | 1.5630 | 75.8440 |
| 46 | 1.4895 | 71.2927 |
| 47 | 1.1630 | 51.8776 |
| 48 | 1.6690 | 82.5153 |
| 49 | 2.0935 | 110.4029 |
| 50 | 0.9875 | 42.0440 |



## Appendix 11

The vWF:RCo activity and plasma vWF assays results obtained from all the subjects of each ABO blood group

| | Group O | | Group A | | Group B | | Group AB | |
|---|---|---|---|---|---|---|---|---|
| Patient # | vWF:RCo% | vWF% | vWF:RCo% | vWF% | vWF:RCo% | vWF% | vWF:RCo% | vWF% |
| 1 | 68.5 | 45.7 | 51.0 | 83.2 | 45.6 | 146.0 | 80.5 | 33.4 |
| 2 | 39.6 | 45.9 | 65.3 | 76.7 | 82.1 | 98.8 | 62.2 | 71.4 |
| 3 | 66.8 | 67.6 | 62.1 | 91.8 | 86.6 | 96.0 | 120.5 | 55.0 |
| 4 | 53.4 | 18.9 | 66.9 | 51.9 | 69.9 | 77.8 | *50.8 | *112.9 |
| 5 | 35.0 | 49.6 | 72.1 | 104.5 | 73.7 | 74.4 | 240.6 | 134.4 |
| 6 | 100.3 | 45.8 | 53.6 | 88.1 | 84.3 | 118.3 | 156.2 | 99.7 |
| 7 | 124.5 | 113.6 | 104.4 | 89.9 | 66.3 | 109.2 | 60.4 | 38.1 |
| 8 | 99.5 | 77.8 | 77.6 | 53.5 | 91.3 | 72.7 | 185.7 | 103.1 |
| 9 | 38.6 | 48.0 | 41.9 | 53.3 | 86.6 | 80.9 | 44.0 | 63.7 |
| 10 | 72.0 | 68.3 | 124.1 | 24.1 | 82.1 | 96.8 | 175.3 | 67.5 |
| 11 | 34.1 | 131.5 | 107.0 | 126.3 | 43.2 | 66.0 | 113.8 | 71.5 |
| 12 | 62.0 | 25.8 | 184.2 | 99.9 | 61.2 | 50.8 | 76.0 | 69.8 |
| 13 | 99.5 | 54.9 | 57.7 | 54.8 | 104.4 | 100.0 | 191.1 | 100.8 |
| 14 | 121.5 | 58.2 | 136.3 | 121.1 | 82.1 | 93.0 | 127.7 | 146.0 |
| 15 | 49.6 | 45.4 | 39.8 | 102.7 | 49.4 | 53.7 | 57.0 | 24.5 |
| 16 | 60.5 | 76.6 | 60.6 | 78.4 | 88.9 | 61.3 | 62.2 | 74.3 |
| 17 | 39.6 | 90.9 | 198.4 | 108.8 | 66.3 | 71.3 | 113.8 | 52.6 |
| 18 | 59.0 | 48.2 | 83.6 | 86.3 | 107.2 | 56.8 | 50.8 | 40.1 |
| 19 | 46.0 | 60.4 | 130.4 | 81.0 | 86.6 | 108.5 | 131.4 | 105.3 |
| 20 | 65.2 | 64.3 | 198.4 | 106.2 | 66.3 | 47.1 | 80.5 | 54.7 |
| 21 | 50.8 | 51.6 | 124.1 | 72.6 | 101.6 | 107.8 | 38.1 | 39.1 |
| 22 | 49.6 | 71.1 | 130.4 | 47.7 | 53.5 | 58.1 | 45.3 | 69.8 |
| 23 | 77.6 | 51.8 | 130.4 | 52.0 | 62.8 | 94.4 | 41.5 | 71.4 |
| 24 | 35.0 | 50.7 | 118.1 | 93.0 | 107.2 | 109.9 | 45.3 | 31.2 |
| 25 | 40.6 | 45.8 | 79.5 | 67.8 | 30.6 | 21.9 | 73.9 | 62.2 |

The vWF:RCo activity and plasma vWF assays results obtained from all the subjects of each ABO blood group

| | Group O | | Group A | | Group B | | Group AB | |
|---|---|---|---|---|---|---|---|---|
| Patient # | vWF:RCo% | vWF% | vWF:RCo% | vWF% | vWF:RCo% | vWF% | vWF:RCo% | vWF% |
| 26 | 48.3 | 51.9 | 115.2 | 31.3 | 82.1 | 87.0 | 156.2 | 117.8 |
| 27 | 63.6 | 76.8 | 107.0 | 74.5 | 88.9 | 54.3 | 124.1 | 75.9 |
| 28 | 66.8 | 59.3 | 101.8 | 101.9 | 39.9 | 44.5 | 93.7 | 51.7 |
| 29 | 41.6 | 70.3 | 224.5 | 111.9 | 77.8 | 85.1 | *44.2 | *105.7 |
| 30 | 99.5 | 42.7 | 162.8 | 103.5 | 86.6 | 42.9 | 145.9 | 90.2 |
| 31 | 44.9 | 56.0 | 70.3 | 64.5 | 66.3 | 58.2 | 54.3 | 68.7 |
| 32 | 66.8 | 46.2 | 60.6 | 79.8 | 113.1 | 116.7 | 93.7 | 51.4 |
| 33 | 79.5 | 38.2 | 101.8 | 80.2 | 79.9 | 71.8 | 243.4 | 76.2 |
| 34 | 104.6 | 69.8 | 115.2 | 116.5 | 59.6 | 57.6 | 212.4 | 82.1 |
| 35 | 115.6 | 38.1 | 96.9 | 69.9 | 42.1 | 62.4 | 131.7 | 45.9 |
| 36 | *115.6 | *45.1 | 47.4 | 27.6 | 61.2 | 65.1 | 81.7 | 34.0 |
| 37 | *60.5 | *27.1 | 54.9 | 79.5 | 53.5 | 58.9 | 590.7 | 80.8 |
| 38 | 50.8 | 27.5 | 54.9 | 68.7 | 107.2 | 46.8 | 103.8 | 96.7 |
| 39 | 50.8 | 48.4 | 53.6 | 29.8 | 61.2 | 54.2 | 103.8 | 109.7 |
| 40 | 99.5 | 47.0 | 87.8 | 90.1 | 69.9 | 123.2 | 145.9 | 72.7 |
| 41 | 65.2 | 42.5 | 73.9 | 106.6 | 82.1 | 98.4 | 227.3 | 57.6 |
| 42 | 269.6 | 90.6 | 107.4 | 79.1 | 68.9 | 90.0 | 405.9 | 90.1 |
| 43 | 50.7 | 68.5 | 114.9 | 123.7 | 434.6 | 96.8 | 191.7 | 73.3 |
| 44 | 73.8 | 78.8 | 68.9 | 68.2 | 127.3 | 113.7 | 151.0 | 88.1 |
| 45 | 31.5 | 79.0 | 156.2 | 60.3 | 58.1 | 63.5 | 161.7 | 75.8 |
| 46 | 103.8 | 52.9 | 219.7 | 120.2 | 79.0 | 45.2 | 131.7 | 71.3 |
| 47 | 81.7 | 58.1 | 111.1 | 108.3 | 62.2 | 46.0 | 118.9 | 51.9 |
| 48 | 103.8 | 70.5 | 161.7 | 61.9 | 205.2 | 62.1 | 103.8 | 82.5 |
| 49 | 107.4 | 72.2 | 151.0 | 48.4 | 185.3 | 73.1 | 127.3 | 110.4 |
| 50 | 14.9 | 130.0 | 227.3 | 114.4 | 81.7 | 71.9 | 212.4 | 42.0 |