Comparison of ADAMTS13 and Von Willebrand factor antigen levels and activities and plasminogen levels, in the currently available plasma products for the treatment of thrombotic thrombocytopenic purpura in South Africa

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DECLARATION

I declare that this research report, *Comparison of ADAMTS13 and Von Willebrand factor antigen levels and activities and plasminogen levels, in the currently available plasma products for the treatment of thrombotic thrombocytopenic purpura in South Africa*, is my own original work. All sources used and/or quoted have been indicated and acknowledged by means of complete references. This work has not been submitted before for any other degree or at any other institution.

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Dr Anneke van Marle

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LIST OF ABBREVIATIONS AND ACRONYMS

ADAMTS13	A disintegrin and metalloprotease with thrombospondin Type I repeats, 13th member
ADAMTS13 Ag	ADAMTS13 antigen
ADAMTS13 Act	ADAMTS13 activity
BCSH	British Committee for Standards in Haematology
CD	Cluster of differentiation
CSP	Cryosupernatant
CUB	Complement C1r/C1s, Uefg (a sea urchin embryonic protein)
CV	Coefficient of variation
CV_A	Analytical coefficient of variation
CV_G	Between-subject coefficient of variation
CV_T	Total coefficient of variation
CV_W	Within-subject coefficient of variation
ELISA	Enzyme-linked immunosorbent assay
FDP	Freeze dried plasma
FFP	Fresh frozen plasma
GP	Glycoprotein
GST	Glutathione-S-transferase
HAART	Highly active antiretroviral therapy
HBsAg	Hepatitis B virus surface antigen
HIV	Human immunodeficiency virus
HMW	High molecular weight
IgG	Immunoglobulin gamma
ISBT	International Society of Blood Transfusion
L	Litre
NAT	Nucleic acid testing
NBI	National Bioproducts Institute

PEX	Plasma exchange
rADAMTS13	Recombinant ADAMTS13
SANBS	South African National Blood Service
SD	Standard deviation
SDP	Solvent/detergent-treated plasma
TMA	Thrombotic microangiopathy
TTI	Transfusion-transmitted infection
TTP	Thrombotic thrombocytopenic purpura
ULVWF	Ultra large Von Willebrand factor
uPAR	Urokinase plasminogen activator receptor
VWF	Von Willebrand factor
VWF:Ag	Von Willebrand factor antigen
VWF:CB	Von Willebrand factor collagen binding
VWF:RCo	Von Willebrand factor ristocetin cofactor
WPBTS	Western Province Blood Transfusion Service

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ABSTRACT

Thrombotic thrombocytopenic purpura results from a deficiency in the Von Willebrand factor cleaving protease, ADAMTS13. Treatment involves plasma exchange therapy with either fresh frozen plasma, cryosupernatant, or solvent/detergent-treated plasma (available locally as Bioplasma FDP®).

The research aimed to generate in vitro data on these products, and to explore possible differences between them that may offer treatment advantages. Twenty samples per product were analysed for levels and activities of ADAMTS13 and Von Willebrand factor, and plasminogen levels (a proposed physiological backup system for ADAMTS13). Fresh frozen plasma and cryosupernatant samples were subanalysed according to ABO blood group.

All samples had normal/high ADAMTS13 activity and plasminogen levels. Von Willebrand factor content was mostly normal for Bioplasma FDP®, typically deficient for cryosupernatant and mostly (unexpectedly) deficient for fresh frozen plasma. Depending on the parameter, Bioplasma FDP® was the most standardised (CV: 14.1% to 27.3%), whilst fresh frozen plasma showed great inter-individual variation (CV: 24.6% to 208.6%). Statistically significant differences were found across products (p values ≤ 0.0095) and ABO blood groups (p value: 0.0001).

In conclusion, all three products can be used for treatment of thrombotic thrombocytopenic purpura. Product choice depends on the need for additional viral safety, costs, product availability and the perceived impact of within-product variations.

Keywords: Thrombotic thrombocytopenic purpura, Von Willebrand factor, ADAMTS13, plasminogen, fresh frozen plasma, cryosupernatant, solvent/detergent-treated plasma

CHAPTER 1: INTRODUCTION

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening condition that is one of a diverse group of disorders, collectively known as thrombotic microangiopathy (TMA) syndromes.(1) Despite their diversity, these disorders are all characterised by widespread microvascular occlusion in several organs.(1)

Central to the pathophysiology of TTP is a severe deficiency of ADAMTS13 (a disintegrin and metalloprotease with thrombospondin Type I repeats, 13th member), the plasma protease that functions to cleave large Von Willebrand factor (VWF) multimers into smaller, less adhesive forms.(2) Unusually large VWF multimers accumulate and result in abnormal platelet aggregation and thrombus formation, with subsequent thrombocytopenia and an associated fragmentation haemolysis.(3)

Replacement of ADAMTS13 with daily plasma exchange (PEX) or, alternatively, large volume plasma infusion, forms the mainstay of therapy.(2, 4) Plasma products used for this purpose include fresh frozen plasma (FFP), cryosupernatant (CSP) and solvent/detergent-treated plasma (SDP).(2)

CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION: THROMBOTIC THROMBOCYTOPENIC PURPURA

As set out by Kremer Hovinga and Lämmle, Singer et al. were the first to recognise TTP as a unique entity in 1947, after reviewing the first clinical case (described by Moschcowitz in 1924), together with 11 subsequent, related cases.(5)

Case reports and literature reviews compiled by Amorosi et al. in 1966 recommend that TTP be considered as a pentad of clinical findings, which include thrombocytopenia, micro-angiopathic haemolytic anaemia, neurological signs, renal impairment and fever.(6) However, unlike the other primary TMA syndromes, TTP rarely causes acute renal injury. This is presumably because TTP primarily causes vascular thrombi and not direct injury to renal cells, as is seen with the other TMA syndromes.(1) The revised diagnostic criteria of Galbusera et al. (2006), as reviewed by Scully et al. (2012), now state that TTP should be considered in the presence of thrombocytopenia and micro-angiopathic haemolytic anaemia alone.(2)

TTP is considered a rare condition, with a reported incidence of 4-11 cases per million people per year in the United States,(7) and 6 cases per million per year in the United Kingdom.(2) Adults are more frequently affected than children – in children the estimated incidence is only 0.1 cases per million per year.(1) No published reports on the incidence of TTP in South Africa are available.

2.1.1 Von Willebrand factor

VWF is a large, multimeric glycoprotein, synthesised by endothelial cells and megakaryocytes and stored as ultra large VWF (ULVWF) multimers within Weibel-Pallade bodies (endothelial cell storage granules) and α-granules of platelets.(8)

Under physiological conditions, endothelial cells form the main source of plasma VWF, whilst platelets contribute only approximately 15% of the circulating protein. However, under pathological conditions associated with platelet activation, platelet-derived VWF may significantly increase plasma levels.(9, 10) Endothelial cell secretion of VWF follows

one of two routes. The vast majority of newly synthesised VWF is constitutively secreted. Secretion occurs through both luminal membranes into the bloodstream, as well as through subluminal membranes into the vascular subendothelium, where it acts as an extracellular matrix protein to bind platelets.(9, 10) Regulated secretion follows only after endothelial cell activation by several processes, including inflammation and thrombosis, and involves the release of ULVWF multimers from the Weibel-Pallade bodies.(9, 10) This form of secretion effectively switches endothelial cells, from an anti-inflammatory and anticoagulant to a proinflammatory and procoagulant surface.(11)

The essential haemostatic functions of VWF are to stabilise Factor VIII and promote platelet adhesion and aggregation at sites of vascular injury.(8) These functions are facilitated by the unique structure of VWF, which involves arrangement in multimers of increasing size, ranging from the smallest homodimers to large multimers – the larger, the more adhesive.(11)

Upon endothelial cell activation, VWF is released, with subsequent exposure to the hydrodynamic forces of blood flow. At sites of endothelial damage with vasoconstriction, the reduced vessel lumen significantly increases shear rates.(11) ULVWF multimers, whilst still temporarily bound to the endothelial cell surface, undergo transition from a globular to a stretched conformation, exposing several binding sites, including the VWF A1 domain, which is the binding site for platelet glycoprotein (GP) Ibα.(11) See Figure 2.1.

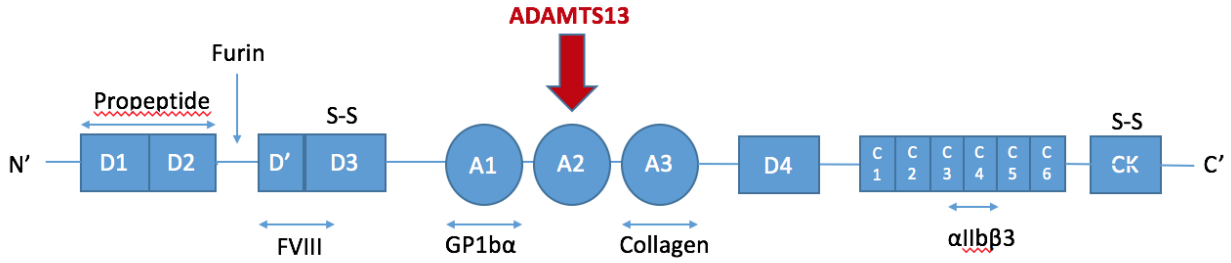


Figure 2.1: Von Willebrand factor structure. Adapted from Crawley JTB, Scully MA. Thrombotic thrombocytopenic purpura: basic pathophysiology and therapeutic strategies. ASH education book. 2013; 292-9 (ADAMTS13: A disintegrin and metalloprotease with thrombospondin Type I repeats, 13th member)

Under conditions of high shear stress, the VWF A1 domain GPIbα interaction has been shown to be the only effective initiator of adhesion of flowing platelets to thrombogenic surfaces.(12) VWF-mediated platelet adhesion at sites of vascular injury acts as the initiating step in thrombus formation.(9) In addition to various binding sites, high shear flow also causes exposure of the ADAMTS13 cleavage site on the elongated/stretched multimers. ADAMTS13 regulates VWF multimer length and binding capacity by constantly cleaving the multimers at the A2 domain into smaller, less thrombotic forms.(8) The first cleavage results in endothelial dissociation, followed by further degradation, rendering VWF functionally inactive.(11) The simultaneous shear-induced haemostatic activation of VWF, together with rapid ADAMTS13 cleavage and thus inactivation, forms a self-regulatory mechanism to prevent pathological thrombosis under physiological conditions.(13)

2.1.2 ADAMTS13

ADAMTS13, so called as it is the 13th member of the ADAMTS (a disintegrin and metalloprotease with thrombospondin Type I repeats) family of metalloproteases, is responsible for proteolysis of VWF.(2) Its discovery was prompted by Moake et al.'s findings, in 1982, of unusually large VWF multimers in patients with chronic relapsing TTP.(1) They concluded that the presence of these ultra large forms, not normally found in plasma, was the result of defective VWF processing following secretion.(14)

In 1996 Furlan et al. succeeded in purifying a protease from normal human plasma, which cleaved VWF multimers into low molecular weight forms, compatible with the physiological VWF fragments found in the circulation.(15) The protease responsible was eventually identified by Levy et al. in 2001. They performed genome-wide linkage analysis in four generations with congenital TTP, and succeeded in tracing the genetic locus involved to chromosome 9q34; they finally identified the enzyme in question as the 13th member of the ADAMTS family of metalloproteases. Their work confirmed ADAMTS13 deficiency as the molecular mechanism of TTP.(16)

2.1.3 Pathophysiology of thrombotic thrombocytopenic purpura

With ADAMTS13 deficiency, the elongated/stretched ULVWF multimers remain attached to the endothelial surface. Passing platelets adhere through interactions between their GPIbα receptors to the exposed A1 domains on the long multimers. Initial platelet adhesion is followed by activation and subsequent aggregation of many additional platelets by means of their GPIIb/IIIa complexes. This results in the formation of large, potentially occlusive, platelet thrombi.(3) Mechanical destruction (fragmentation) of red cells follows as they pass through these platelet thrombi in the microcirculation.(17)

2.1.4 A proposed backup mechanism for ADAMTS13

Patients with hereditary ADAMTS13 deficiency present with only intermittent episodes of TTP. In addition, patients with acquired ADAMTS13 deficiency can achieve remission of TTP without recovery of ADAMTS13 levels. These findings suggest the presence of other modifying factors that influence the pathophysiology of TMA.(8)

Plasmin, a plasma serine protease, is the major enzyme of the fibrinolytic system. Plasminogen, in turn, is the circulating precursor form, which is converted to plasmin by several plasminogen activators. Its primary substrates are fibrin and fibrinogen. Several in vitro studies have, however, demonstrated plasmin's ability to selectively cleave particular peptide bonds in other proteins, including Factor VIII/VWF complexes.(18)

Tersteeg et al. (2014) thoroughly investigated the role of plasmin as an ADAMTS13 backup mechanism for proteolytic cleavage of VWF multimers in the development of TTP.

Their study showed that plasminogen binds to the VWF A1 domain in a lysine-dependent manner. Plasminogen activation, which is mediated through a urokinase plasminogen activator receptor-dependent mechanism, results in rapid cleavage of platelet-VWF complexes on activated endothelial cells in the absence of ADAMTS13.(19) During acute TTP episodes, plasminogen-α2-antiplasmin complexes, a marker of plasminogen activation, are elevated in proportion to the degree of thrombocytopenia and, thus, the extent of the disease. In addition, hypoxia has been shown to augment endothelium-mediated plasminogen activation through upregulation of the urokinase plasminogen activator receptor. Tersteeg et al. hypothesised that the ischaemia following microvascular thrombosis prompts plasminogen activation, with subsequent plasmin-mediated degradation of platelet-VWF complexes.(20) They concluded that plasmin, independent of other enzymes, aids in the local clearance of microvascular obstruction, as seen in TTP.(8)

In light of these studies, the presence of plasminogen in the blood products used for plasma exchange in the treatment of TTP may provide yet another benefit. In addition to removing or diluting inhibitor antibodies and replacing the deficient ADAMTS13, the plasminogen present in these products may further assist in clearing large VWF multimers after subsequent in vivo conversion to plasmin.

2.1.5 Aetiology

Congenital TTP, known as Upshaw-Schulman syndrome, is a rare disorder caused by mutations of the ADAMTS13 gene on chromosome 9q34, which results in an inherited deficiency of the enzyme. More than a 140 different ADAMTS13 mutations have been identified.(5) Diagnosis requires proof of ADAMTS13 deficiency with no demonstrable autoantibody present.(1) In recent years molecular techniques have been used to confirm the diagnosis with the presence of either homozygous or compound heterozygous defects in the ADAMTS13 gene.(2)

Acute idiopathic TTP is the most common form of TTP. It is characterised by autoantibodies, usually IgG, directed against ADAMTS13.(2) Anti-ADAMTS13 antibodies may be inhibitory, directly neutralising the enzyme activity, or non-inhibitory, in which case

reduction in enzyme activity is thought to occur through opsonisation, amongst other unconfirmed mechanisms.(21) Antibody-mediated enzyme reduction should be considered an autoimmune manifestation.(21) The reason for acquiring this autoimmune disease remains unknown.

Much research has been done in an attempt to identify a trigger, the so-called “second hit”, that precipitates an acute attack of TTP.(20) Microbial agents have been suggested, and when considering three particular cases highlighted in a 2012 American Society of Hematology review on the role of ADAMTS13, it seems possible. These cases all had proven enterohaemorrhagic *Escherichia coli* infection together with severe acquired ADAMTS13 deficiency secondary to inhibitory IgG autoantibodies.(5) The presence of increased levels of DNA-histone complexes and neutrophil-derived components in the circulation during an acute TTP episode further connects infection to TTP. Whether these markers of infections are, however, a cause or an effect of TTP, still remains unclear.(20)

A genetic predisposition to developing ADAMTS13 autoantibodies is also suggested by the overrepresentation of the major histocompatibility complex Class II allele, human leukocyte antigen DRB1*11, in patients with acquired TTP in some western European countries.(5) Other subgroups of TTP have been identified and include pregnancy-associated TTP, drug-associated TTP, transplant-associated microangiopathy, malignancy-associated TMA, pancreatitis-associated TTP and the related condition, haemolytic uraemic syndrome.(2)

2.1.6 The effects of ABO blood group on the main determinants in the pathophysiology of thrombotic thrombocytopenic purpura

ABO blood group antigens are complex carbohydrate structures present on red blood cells as well as various other glycolipids and glycoproteins, including the N-linked oligosaccharide chains of circulating VWF, as explained by Jenkins and O’Donnell.(22)

A literature review by Franchini et al. elucidates the relationship between ABO blood group and VWF. Lower VWF levels and activities are a consistent finding in blood group O individuals; this finding is supported by an increased prevalence of inherited bleeding

disorders in Group O subjects, contrasted with increased risk of thromboembolic disease in non-O individuals.(23)

However, the mechanism by which ABO antigens influence plasma VWF levels, remains unclear. One hypothesis involves increased VWF cleavage by ADAMTS13.(23) Whether this is simply due to elevated ADAMTS13 activity levels in blood group O individuals, as found by Mannucci et al., or as a result of accelerated ADAMTS13-mediated proteolysis of blood group O VWF, as documented by Bowen et al., is still to be confirmed.(24, 25) The suggested increased susceptibility to ADAMTS13-mediated proteolysis is explained by the ABO antigenic structures present on the N-linked oligosaccharide chains of circulating VWF, which influence the VWF conformation to acquire a more vulnerable form for cleavage.(22)

Contrary to Mannucci et al., Al-Awadhi et al. found no differences in ADAMTS13 levels and activities between blood groups O and non-O healthy Arabs, but confirmed significant differences with increasing VWF levels in the individual blood groups: O<A<B<AB.(26) A study by Chion et al., that investigated the association between ADAMTS13 and VWF levels and the risk of myocardial infarction in men, also concluded that no relationship between ABO blood group and ADAMTS13 levels was evident.(27)

Zuberi et al. conducted a retrospective study to examine the relationship between ABO blood group and TTP. They reviewed electronic medical records of 74 patients diagnosed with idiopathic TTP at the Henry Ford Hospital in Detroit, Michigan, in the United States of America, between 1993 and 2008. The distribution of individual blood groups (A, B, AB and O) in these patients was assessed and compared with the expected frequencies of TTP, based on the known proportions of blood groups across the Detroit population. Although not statistically significant, the prevalence of blood group O in patients with TTP was lower than the expected frequency in the geographic population.(28)

These studies suggest that blood group O individuals with lower VWF levels and possibly higher ADAMTS13 activity should have a lower prevalence of TTP and/or less severe forms of TTP. Furthermore, consideration should be given to using blood-group-specific plasma products in the treatment of TTP.

A cross-sectional study conducted by Dolan et al. investigated the effects of age, sex and blood group on antithrombin, protein C and plasminogen levels in a cohort of healthy plasma donors. They found no obvious association between plasminogen concentration and ABO blood group.(29)

2.1.7 The effects of biological variation on the main determinants in the pathophysiology of thrombotic thrombocytopenic purpura

Correctly interpreting patient laboratory results requires an understanding of the biological variation of the analytes tested.(30) Major aspects to consider, as stipulated by Kilercik et al., include within-subject variability, explained as the result of the variability in the same subject over time, between-subject variability, which is dependent on the heterogeneity of physiological influences among subjects, and the index of individuality, which is defined as the ratio between within- and between-subject variation. These concepts are of particular importance when test results are used to confirm a clinical diagnosis. Diagnosis requires comparison of test results with population-based reference ranges, which can only be safely applied when the individuality for a given analyte is low. Biological variation is also useful for condition monitoring when significant changes in serial results are evaluated.(30, 31)

A study conducted by Kilercik et al. aimed to determine the biological variation of ADAMTS13 and VWF in human adults. Their results indicate high biological variation and individuality in VWF antigen levels and activities together with high biological variation in ADAMTS13 levels and activities, but with low individuality. They conclude that population-based reference ranges may be useful for monitoring ADAMTS13 levels and activities, but not for VWF.(30) Similarly, Shou et al. found high biological variation and individuality for VWF antigen levels, which exceed those recorded in the Westgard database. They did not, however, consider the different ABO blood groups, which may have led to greater between-subject variations being calculated.(31)

Published literature on the biological variation of haemostasis parameters, in general, and of plasminogen, in particular, is limited. Low biological variation for plasminogen is, however, reported in a review by Banfi et al.(32)

The importance of population-based reference ranges as opposed to standard reference intervals, as well as blood-group-specific reference ranges, specifically for VWF assays, is evident. However, when considering these studies, the high biological variation and individuality associated with VWF levels and activities, in particular, may hamper interpretation of test results.

2.2 HIV-ASSOCIATED THROMBOTIC THROMBOCYTOPENIC PURPURA

As reviewed by Meiring et al. (2012), human immune deficiency virus (HIV)-associated TTP was first described by Joakela et al. in 1987.(33) It differs from acquired TTP in that autoantibodies to ADAMTS13 are often not detected.(33) In fact, although it is severely decreased in some HIV-positive patients, ADAMTS13 activity has been found to be within the normal range in others.(21)

Opie stated that HIV is the most common virus precipitating TTP.(17) Gunther et al. supported this notion by reporting that HIV infection is by far the commonest cause of TTP in their hospitals (teaching hospitals associated with the University of the Witwatersrand, Johannesburg), accounting for more than 80% of all cases.(34) The reported prevalence of HIV-associated TMA in the pre-HAART (highly active antiretroviral therapy) era varied from 1.4% to 7%, depending on the stage of HIV disease.(35) A significant reduction, to 0.3%, can be attributed to the introduction of HAART.(21) Nevertheless, the incidence of TTP in HIV-positive individuals is still considered to be up to 40 times greater than that in the general population.(34) As a country with one of the world's highest HIV-prevalence rates, HIV-associated TTP is of particular importance in South Africa.(17) The estimated overall prevalence of HIV infection in South Africa is 11.2%, and the total number of people living with HIV was estimated to be 6.19 million in 2015. Approximately 16.6% of adults aged 15-49 years are HIV positive.(36)

Although the association between HIV and TTP has been evident since the 1980s, the exact pathogenesis still remains unclear, and it is considered to be a multifactorial process.(34) To complicate matters, concurrent comorbidities, including various opportunistic infections and malignancies as well as several drugs that are commonly prescribed in advanced disease, could all contribute to the development of TTP.(21, 35)

A study conducted by Gunther et al. correlated ADAMTS13 levels and activity with the presence of inhibitory autoantibodies in patients with HIV-associated TTP. They found that the majority of patients had reduced levels of ADAMTS13, however, only about 38% of patients had demonstrable inhibitory antibodies.(37) The clinical severity of HIV-associated TTP does not seem to correlate with the anti-ADAMTS13 IgG titre, suggesting that the autoimmune component may play a lesser role in the pathophysiology.(38)

HIV-associated TTP has a more diverse clinical picture compared to idiopathic TTP, which raises the question whether different subsets of HIV-associated TTP can be defined. Malak et al. explored this notion by studying the clinical and biological features of HIV-associated TTP patients according to ADAMTS13 activity. They classified patients into one of two groups, those with severe ADAMTS13 deficiency (<5%) and those with detectable activity (≥5%). In addition, these patients were then compared with patients with idiopathic TMA categorised into the two abovementioned groups. They found that, not only could two distinct subsets of patients be defined, but also that those patients with severe ADAMTS13 deficiency (<5%) had fewer AIDS-related complications, higher cluster of differentiation (CD) 4+ T-cell counts and a prognosis comparable to the idiopathic forms of TMA.(21) In 2007 Gunther et al. reported a case series of 20 HIV-associated TTP patients. Their findings also include an inverse relationship between ADAMTS13 activity and the CD4+ T-cell count.(37)

After reviewing the literature on HIV-associated TTP and conducting their own study, Meiring et al. summarise the current proposed mechanisms for the development of HIV-associated TTP. This includes an HIV-driven inflammatory process with elevated levels of proinflammatory cytokines, which stimulate excessive release of stored VWF from endothelial cells.(33) This increase in plasma VWF may reflect protein leakage from damaged endothelium as well as protein secretion from intact, perturbed cells.(9) Del Acro et al. reported on a case of a patient with HIV-associated TTP, in whom the HIV p24 antigen was detected in the patient's endothelial cells. This opens speculation as to whether the virus causes direct cell damage or, rather, functional impairment of endothelium.(39)

Another explanation involves alterations in endothelium regulation, which could either influence ADAMTS13 activity directly, or alter VWF susceptibility to proteolysis.(14) In vitro studies by Crawley et al. identify thrombin as a potential regulator of ADAMTS13 activity. One could therefore argue that thrombin, generated following viral-induced endothelial damage, may contribute to decreased ADAMTS13 activity.(40, 41) Gunther et al. speculate that this VWF-release may overwhelm ADAMTS13's cleaving capacity and result in a "consumptive deficiency".(37) In experiments where desmopressin acetate (which stimulates VWF release from endothelium)(10) was administered to healthy volunteers, a transient decrease in ADAMTS13 activity could be demonstrated.(41) ADAMTS13 consumption, as opposed to direct inhibition, may explain the more gradual onset of HIV-associated TTP compared with the acute onset of idiopathic TTP.(21)

HIV-associated micronutrient deficiencies may further contribute to decreased ADAMTS13 synthesis, whereas the presence of autoantibodies to ADAMTS13 may be ascribed to a weakened immune system with disturbances in CD4+ T-cell function.(21) Gunther et al. summarise the suspected pathogenesis of HIV-associated TTP to be a combination of endothelial cell damage with loss of its inherent anticoagulant properties, excessive VWF release, and reduction in ADAMTS13 activity, whether it be secondary to consumption, inhibition or proteolytic inactivation.(37)

TTP usually occurs in immunosuppressed patients with low CD4 counts, either as an initial presenting feature of HIV disease or following non-compliance with antiviral treatment.(2) The Collaborations in HIV Outcomes Research/US (CHORUS) study confirms that the risk of TTP increases with more advanced disease. It shows that, amongst HIV-positive patients, those diagnosed with TMA had markedly lower CD4+ T-cell counts and higher viral loads. It also demonstrates that HAART significantly decreases the incidence of TMA.(35) Brecher et al. summarise two similar cases, one reported by Gruszecki et al. and the other by Park et al., of patients with HIV-associated TTP who failed to respond to plasma therapy (plasma exchange and plasma infusion respectively), but who showed marked improvement of clinical features after initiation of HAART.(41) Remission may, therefore, be dependent upon improving a patient's immune status.(2)

2.3 DIAGNOSIS

Clinical history and examination of the patient in conjunction with the peripheral smear morphology forms the basis for diagnosing TTP. A case definition for TTP can be made in the presence of micro-angiopathic haemolytic anaemia with thrombocytopenia, even without overt renal failure, where disseminated intravascular coagulation or another plausible cause has been excluded.(5)

The addition of a reduced ADAMTS13 level (<10%) further supports the clinical diagnosis. However, this level, as summarised by George et al., lacks the sensitivity and specificity required to accurately diagnose all patients with TTP.(1) The 2012 British Committee for Standards in Haematology (BCSH) diagnostic guidelines stipulate more stringently that severely reduced ADAMTS13 activity (<5%), with/without a demonstrable autoantibody, confirms the diagnosis.(2)

ADAMTS13 assays available include those measuring antigen level, enzyme activity and anti-ADAMTS13 autoantibodies.(2) Measurement of both ADAMTS13 level and activity is recommended, as this allows assessment of whether the detected enzyme is fully active.(42) The ADAMTS13 antigen level may exceed the corresponding activity if the inhibitory antibody does not facilitate clearance.(5) Although ADAMTS13 deficiency is a distinctive feature of TTP, assays available to demonstrate this have restrictions. Serial dilutions of plasma, amongst other factors, may hamper detection of autoantibodies, and even allow dissociation of these inhibitors from ADAMTS13, thereby resulting in detection of false normal enzyme activities, in vitro.(5)

2.4 MANAGEMENT OF THROMBOTIC THROMBOCYTOPENIC PURPURA

2.4.1 Plasma therapy

Before PEX was introduced, only an estimated 10% of patients diagnosed with TTP survived.(4) Daily PEX has reduced mortality rates by more than 70%.(2) Proposed explanations for the efficacy of PEX include removal or dilution of inhibitor autoantibodies and ULVWF multimers, together with replacement of the deficient ADAMTS13.(43) Delay

in initiation of PEX leads to preventable early mortality, and current guidelines consequently recommend initiation of PEX within 24 hours of presentation.(44)

A less favourable alternative to PEX involves large-volume plasma infusions. This option should be reserved for cases in which PEX cannot be arranged promptly.(2) PEX remains the treatment of choice, as it offers the benefit of replacing the deficient ADAMTS13 whilst removing potential inhibitors without the risk of fluid overload.(44)

Rock et al. conducted a prospective randomised controlled trial comparing the two treatment options. Improved response and survival at the end of the first treatment cycle and after 6 months confirmed PEX to be superior.(45) However, one should take into account that the volume of plasma received in the infusion arm of this study was considerably lower than in the PEX arm. Novitzky et al. found no significant difference in response rate or long-term survival between the two treatment options when comparable plasma volumes were used.(46) The plasma infusion protocol followed by Rock et al. involved an initial infusion of 30 mL/kg of plasma over 24 hours, followed by 15 mL/kg daily thereafter.(45) The plasma volume infused in a study conducted by Novitzky et al. remained 30 mL/kg/d in three divided doses until recovery of the platelet count.(46) The TTP treatment protocol of the Universitas Academic Hospital Complex in Bloemfontein, South Africa, recommends a starting dose of 30 mL/kg/d in three divided doses, followed by a 5 mL/kg reduction in the infusion volume on alternate days, provided that the platelet count, lactate dehydrogenase and haemoglobin levels remain stable.(47)

PEX, although confirmed effective (and possibly superior to plasma infusion), still poses treatment questions. Large volumes of plasma are required and the optimal regimen has not been determined. The current recommendation in the 2012 BCSH guidelines is based on the Canadian Apheresis trial.(2) It states that PEX should be started with 1.5 plasma volume exchanges daily. The volume can subsequently be reduced to 1.0 plasma volume when the clinical condition and laboratory test results stabilise. Intensifying frequency and/or volume of PEX procedures should be considered in life-threatening cases. Daily PEX should be continued until the platelet count has been >150 x 10^9/L for at least two days and then stopped. Tapering is not required.(2)

Regarding HIV-associated TTP, Malak et al. found that HIV-positive patients with severe ADAMTS13 deficiency respond well to plasma therapy, and they recommend that a similar treatment protocol as for idiopathic TTP be followed. However, patients with associated AIDS-related complications showed poorer responses, raising questions about whether prolonged therapeutic PEX is likely to improve outcome.(21)

Novitzky et al. conducted a prospective study in the pre-HAART era to compare clinical and laboratory parameters at presentation as well as response to therapy in patients with HIV-associated TTP vs. idiopathic TTP. Response criteria included normalisation of haemoglobin level, platelet count and lactate dehydrogenase level. All patients enrolled in this study received cryosupernatant as mainstay of their treatment. They found that HIV-positive patients showed a significantly better and faster response to plasma infusion therapy than patients in the HIV-negative cohort, and none required apheresis.(46)

A study by Hart et al. reviewed 30 episodes of HIV-associated TTP in 24 patients from the South-East England Apheresis unit over a period of 10 years. They confirm that the swift and simultaneous initiation of both PEX therapy and HAART (with adequate HAART compliance), results in a more favourable outcome with a lower relapse rate than seen with idiopathic TTP. However, patients with higher viral loads (>500 000 copies/mL) required more PEX to achieve remission.(38)

2.4.2 Other treatment modalities

Immunosuppression: Autoimmune manifestations, such as the presence of anti-ADAMTS13 antibodies, justify the consideration of immunosuppressive treatments.(48) Daily PEX with adjuvant corticosteroid therapy is commonly prescribed. Although studies to confirm superiority of this combination therapy is lacking, the 2012 BCSH guidelines still recommend addition of intravenous methylprednisolone or high-dose oral prednisolone.(2) A review by Coppo et al. on treatment modalities for TTP reports no significant differences in outcomes in studies where PEX was used alone compared with studies where corticosteroids were added.(48) Successes have been documented with the use of other immunosuppressive drugs, including vincristine, cyclosporine A and

cyclophosphamide, however, given their associated adverse effects, they are not recommended as frontline therapies.(2, 4, 48)

Immunomodulation: Rituximab, an anti-CD20 monoclonal antibody, has shown promising results as a second-line therapy in patients with suboptimal responses to conventional therapy (daily PEX with corticosteroids).(2, 4, 48) Coppo et al. report on several studies that confirm the efficacy of rituximab in patients with TTP. Shorter times to remission, increased ADAMTS13 activities and reductions in anti-ADAMTS13 antibodies were almost consistently found. Whether rituximab should routinely be used as frontline therapy in conjunction with PEX and corticosteroids, however, remains debatable.(48) Rituximab therapy in the setting of HIV warrants caution, especially with very low CD4 counts, as increased infection risks have been reported.(49, 50)

Other: Splenectomy in the treatment of refractory or chronic relapsing TTP has largely been replaced by rituximab.(51)

Supportive therapy: The clinical benefit of antiplatelet agents, such as aspirin and dipyridamole, are unsubstantiated, however, with no significant increase in bleeding risk, low dose aspirin may be considered once the platelet count is above 50 x 10^9/L.(2, 51) The 2012 BCSH guidelines also recommend red cell transfusions when clinically indicated, folate supplementation and thromboprophylaxis with low molecular weight heparin when the platelet count exceeds 50 x 10^9/L.(2, 51)

Future targeted strategies: Recombinant ADAMTS13 (rADAMTS13) as treatment strategy for congenital TTP appears promising, however, the presence of anti-ADAMTS13 antibodies in acquired TTP may nullify its effect.(48) Strategies to overcome inhibition by these antibodies include higher doses of rADAMTS13 as well as amino acid sequence modifications to the rADAMTS13 structure to impart antibody resistance.(48, 51)

GBR600 is an inhibiting anti-VWF monoclonal antibody that inhibits VWF-GPIb interactions. Preclinical trials on baboon models demonstrate effective protection from, and treatment of, acquired TTP with administration of this compound. These promising results support the reasoning behind recommendation for clinical trials in future.(52)

Caplacizumab, an anti-VWF nanobody that prevents interaction between the VWF multimers and platelets, was recently evaluated in a Phase II trial in patients with acquired TTP.(53) The inhibition of VWF-mediated platelet aggregation resulted in faster resolution of the acute TTP episode and may therefore potentially limit the harmful sequelae of organ ischaemia following TTP-associated microvascular thrombosis. Although caplacizumab was associated with an increased propensity toward bleeding, the bleeding events were generally mild and did not necessitate active intervention.(53)

Degradation of soluble VWF multimers by N-acetylcysteine and resistant anti-ADAMTS13 antibodies by the proteasome inhibitor, bortezomib, as well as augmentation of plasminogen activation by thrombolytic agents, are interesting treatment proposals that require further evaluation.(20, 48)

2.5 PLASMA PRODUCTS AVAILABLE FOR TREATMENT

A number of plasma products is used for replacement in PEX or for plasma infusion therapy. Commonly used fluids include standard FFP, SDP and CSP, also known as cryoprecipitate-poor plasma.(44) Blood products used for the treatment of TTP in South Africa are FFP, Bioplasma FDP® (an SDP) and CSP.

2.5.1 Fresh frozen plasma

Plasma isolated from anticoagulated whole blood within 18 hours of donation is regarded as “fresh”;(54) this plasma is then rapidly frozen to below -18°C to produce FFP. FFP contains physiological levels of all the coagulation factors.(54, 55)

To minimise the risk of infections, the transfusion services of South Africa follow strict donor-selection protocols. All donors are obliged to complete a written questionnaire for any evidence of past or present infections that may be transmitted to recipients. All donated units are then screened for laboratory evidence of certain transfusion-transmitted infections (TTIs). Routine testing includes serology for hepatitis B surface antigen as well as antibodies to *Treponema pallidum* (causative organism of syphilis), hepatitis C virus and HIV 1 and 2.(54) To reduce the infection risk further, nucleic acid testing (NAT) for HIV 1, hepatitis B virus and hepatitis C virus have been implemented. NAT significantly

increases the sensitivity of viral detection by shortening the window period. Agarwal et al. found that NAT positively identified 121 virally infected blood samples that had gone undetected by enzyme-linked immunosorbent assays (ELISA) out of 73 898 samples.(56)

In addition, a donor plasma retest quarantine programme has been introduced in South Africa to minimise the risk of window-period infections further.(54) According to this programme, all plasma units are placed under quarantine. Only if a second donation is confirmed negative for TTIs, is the initial unit made available for transfusion.(57) No pathogen transmissions via FFP have been reported since the introduction of this programme.(54)

Nevertheless, TTIs remain a concern, especially in light of the current HIV pandemic. For patients likely to receive large quantities of plasma, pathogen inactivated plasma may be preferred.

2.5.2 Cryosupernatant

CSP or cryoprecipitate-poor plasma is FFP from which the cryoprecipitate fraction has been removed. The precipitable cryoproteins include Factor VIII, VWF, Factor XIII, fibronectin and fibrinogen. CSP is depleted of Factor VIII and fibrinogen, and deficient in high molecular weight (HMW) multimers of VWF, but shows preservation of ADAMTS13.(58) Accessible data confirms that local CSP contains Vitamin K-dependent coagulation factors at levels comparable to FFP.(54, 55) However, no published data on the ADAMTS13 or plasminogen content specifically are currently available. As a by-product after extraction of the cryoprecipitate from FFP, CSP is stored in small quantities (at not lower than -18˚C to -23˚C for up to 1 year) and mainly indicated for the treatment of TTP.(54, 55)

Many laboratory and clinical studies have compared and assessed the content and in vivo effectiveness of the different products internationally.(43-45, 59-66)

Considering that HMW multimers of VWF play a central role in the pathogenesis of TTP, CSP with VWF multimer deficiency may offer a theoretical advantage over FFP. A pilot study carried out by Rock et al. in 1996 supports this concept.(63) In addition, Owens et

al. conducted a retrospective analysis of all cases of TTP treated by PEX, with either FFP (1985-1989) or CSP (1989-1993) as exchange fluid, at the American Red Cross Apheresis Unit between the years 1985 and 1993. Their observations also suggest a survival advantage with the use of CSP. Unfortunately, several limitations, including small sample size, retrospective nature of the study, sequential rather than concurrent use of different exchange fluids, and dosage differences prevented definitive conclusions. Owens et al. deduced that a prospective, randomised, concurrent study was necessary.(62)

The North American TTP Group responded by initiating a randomised prospective study from 1993 to 1995, in which they compared the use of FFP to CSP for PEX in the initial treatment of patients with idiopathic TTP. Comparable results in the two treatment arms suggest both plasma products as acceptable exchange fluids for acute management.(64) These findings were reinforced by a similar study conducted by Rock et al.; the data from their randomised prospective trial, in contrast to results obtained from their earlier pilot project, failed to show any observable advantage from using CSP in TTP.(65)

2.5.3 Solvent/detergent-treated plasma

Solvent/detergent treatment, which solubilises lipid membranes, ensures viral safety against lipid-enveloped viruses, such as hepatitis B, C and HIV, but is of limited value against non-lipid-enveloped viruses, such as parvovirus B19 and hepatitis A virus.(59, 67) Several types of SDP exist. Differences relate predominantly to production methods and are country/region specific.(44) In South Africa, SDP is manufactured from pooled fresh human plasma by the National Bioproducts Institute (NBI).(54) Low titre plasma units of all ABO blood groups are pooled together in large quantities. Pathogen inactivation procedures, using Triton X-100 and tri-n-butyl phosphate, are then followed by lyophilisation into 50 mL and 200 mL bottles.(67, 68)

Solheim et al. reviewed the safety of pooled SDP. Clinical studies with Bioplasma FDP® as well as Uniplas® (a European product) confirmed solvent/detergent treatment to produce pathogen-reduced plasma products with preserved clotting factor content.(67) Safety against viruses is not solely dependent on the solvent/detergent treatment. Locally,

these plasma pools are also subjected to anti-HIV-1 & -2, hepatitis B virus surface antigen (HBsAg) and anti-hepatitis C virus serological testing, as well as to NAT for HIV, hepatitis A virus, hepatitis B virus and hepatitis C virus, to ensure early detection.(69) The pooling as such also ensures the presence of a wide variety of neutralising antibodies in the pool, for protection against non-lipid-enveloped viruses.(67) In addition, only pools containing $<10^4$ U/mL parvovirus B19 are used.(69) Another advantage of pooling is standardisation of the plasma protein content.(67)

When adhering to the BCSH treatment guidelines (2012), it is clear that large quantities of plasma are routinely used in the treatment of TTP. The study conducted by Rock et al. in 1991 compared PEX to plasma infusion for the treatment of TTP. For patients in the PEX group, a mean of 15.8 PEX treatments consisting of 1.0 to 1.5 blood volumes were required. This equates to high exposure rates to a variety of plasma donors.(45, 66)

Calculations done by Yarranton et al. indicate that a 60 kg patient with an estimated plasma volume of 40 mL/kg would require 2 400 mL of plasma per exchange. If one unit contains approximately 250 mL plasma, a patient may be exposed to as many as 10 donors per exchange. As mentioned before, if patients receive such large quantities of plasma, routine donor screening and unit quarantine and retesting may not be sufficient to ensure viral safety.(66)

Horowitz et al. compiled a report in which they described a process for preparing SDP. This process ensures lipid-enveloped virus inactivation whilst maintaining protein integrity. They conclude that replacement of FFP with SDP guarantees improved virus safety with no loss of product efficacy.(61)

Solvent/detergent treatment and methylene blue/light treatment are the two virus-inactivation processes for plasma currently available in Great Britain. Methylene blue was the first method used for this purpose; it was later modified by Lambrecht et al. by the introduction of visible light.(70) The process involves addition of methylene blue to the plasma units, which interpolates with nucleic acids. Upon exposure to different wavelengths of light, methylene blue is activated and it generates oxygen free radicals,

with subsequent breakage of nucleic-acid strands. Although not used in South Africa, methylene blue/light-treated plasma is widely utilised throughout Europe.(70)

Yarranton et al. investigated whether the viral inactivation processes altered plasma components, thereby reducing their efficacy. To assess this, they studied the levels and activities of, amongst others, ADAMTS13 and VWF antigen in methylene blue/light-treated plasma and SDP and compared it with levels in FFP and CSP. VWF multimer patterns were also analysed, specifically the ULVWF multimers, which are thought to be central to the pathophysiology of TTP.(43) Yarranton et al. demonstrated that viral-inactivation processes do not alter ADAMTS13 levels and activities, which were comparable to those in FFP and CSP. SDP, similar to CSP, showed the additional theoretical benefit of loss of the highest molecular weight multimers of VWF.(43)

These findings are emphasised in a review compiled by Liumbruno et al., who found 100% retention of ADAMTS13 and plasminogen levels, together with reduced VWF multimers in SDP.(71)

The general conclusion drawn from these comparative studies is that, in spite of documented differences among the different plasma products, no one product has been proven superior for treating TTP.(44)

The content of locally available plasma products is mainly tested for internal quality-control purposes. According to the package insert, Bioplasma FDP® contains a minimum of 0.4 IU/mL of each coagulation factor.(68) Average coagulation factor levels for FFP are available on the Western Province Blood Transfusion Service (WPBTS) website and are also published in the clinical guidelines for the use of blood products in South Africa, available from the South African National Blood Service (SANBS) and WPBTS.(54, 55) No other publications regarding composition, or any data on the ADAMTS13, VWF or plasminogen content of these products, are available. In addition, no clinical trials comparing the efficacy of these plasma products in the setting of HIV-associated TTP have been conducted.

CHAPTER 3: RESEARCH METHODOLOGY

3.1 INTRODUCTION

Plasma therapy forms the cornerstone of the management of TTP. In spite of extensive research and comparisons done internationally, no single plasma product has been proven superior in the treatment of TTP. South Africa has a different disease profile, with most of its TTP cases being associated with HIV. Considering the lack of empirical data on the composition of products available locally, an ideal treatment regimen is yet to be defined.

3.2 AIM AND OBJECTIVES

The aim of this study was to generate empirical in vitro data on the products available locally (FFP, Bioplasma FDP®, and CSP) to confirm their in vitro equivalence and utility for local use, and also to explore any possible differences in the products that may offer treatment advantages in the setting of HIV-associated TTP.

This study, a first in South Africa, specifically compared the levels and activities of the plasma proteins and enzymes relevant in the treatment of HIV-associated TTP, namely, ADAMTS13, VWF and plasminogen.

The objectives of the study were:

- To compare the antigen levels and activities of ADAMTS13 and VWF of the plasma products available locally;
- To compare the VWF multimer patterns of the plasma products available locally;
- To compare the antigen levels of plasminogen of the plasma products available locally; and
- To compare all the results obtained above for FFP and CSP for the different ABO blood groups.

3.3 ETHICAL CONSIDERATIONS

Ethics approval was obtained from the Ethics Committees of both the University of the Free State (ECUFS NR 191/2015) and SANBS (2015/25) – see Appendices A and B.

The study was executed in accordance with the World Medical Association Declaration of Helsinki (1964) as amended in Brazil (2013).

Samples of the plasma products used for analyses were provided by SANBS and NBI free of charge. No remuneration was received from either of the product providers. In addition, the abovementioned plasma product providers imposed no restrictions or conditions regarding publication and/or presentation of the study results based on data collected or generated by the researchers.

The FFP and CSP samples used for testing were obtained from the local SANBS blood bank at Universitas Academic Hospital. These testing segments had been appended to the individual units of FFP or CSP specifically for testing purposes. They had the same content as the primary bag, but could be removed for testing purposes without puncturing the primary product bag or jeopardising its quality.

No units could be traced back to the product donor or recipient. No data that could lead to patient, donor or physician identification were collected. Sample procurement did not influence product issuing or blood-bank workflow, nor incurred additional costs for SANBS.

Bioplasma FDP®, a product of NBI, is manufactured from pooled plasma donations from multiple donors and as such is classified as a medicine, and supplied with a package insert approved by the Medicines Regulatory Authority. No test result could be traced back to an individual donor.

The blood products were used solely for research purposes and were only used in vitro. Only those factors stipulated in the study were tested. Upon completion of testing, all blood products were disposed of as per relevant National Health Laboratory Service standard operating procedure. All manufacturer kit test strips were discarded after use in a container destined for incineration.

3.4 MATERIALS

Testing segments of the FFP and CSP units issued in December 2015 were collected (still frozen at -18°C) from the Universitas Academic Hospital blood bank and, whilst maintaining the cold-chain, subsequently stored at -80°C in the Department of Haematology and Cell Biology, until batch testing commenced in January 2016. The segments were subdivided into samples from the different ABO blood groups: 5 blood group A, 5 blood group B and 10 blood group O samples were included for each of the blood products, giving a total of 10 Group O and 10 Non-group O samples per product. Samples from Group AB products were not considered, due to cost constraints and limited availability.

The SDP tested in the study was Bioplasma FDP®, a product of NBI. NBI provided forty 200 mL bottles of Bioplasma FDP® from separate lots for analysis: two bottles per lot, from 20 different lots – see Table 3.1.

Table 3.1: Bioplasma FDP®: Lot numbers and expiry dates

Bioplasma FDP®		
Sample number	**Lot number**	**Expiry date**
1	STPM0792	31/03/2017
2	STPM0794	31/03/2017
3	STPM0791	31/03/2017
4	STMP0790	31/03/2017
5	STPM0793	14/07/2017
6	STPM0789	31/03/2017
7	STPM0796	28/07/2017
8	STPM0795	21/07/2017
9	STPM0776	28/02/2017
10	STPM0772	31/01/2017
11	STPM0782	28/02/2017
12	STPM0777	28/02/2017
13	STPM0775	31/01/2017
14	STPM0797	31/07/2017
15	STPM0778	28/02/2017
16	STPM0780	28/02/2017
17	STPM0781	28/02/2017
18	STPM0779	28/02/2017
19	STPM0787	31/03/2017
20	STPM0788	31/03/2017

3.5 METHODS

All laboratory preparation and analyses were performed by Prof SM Meiring, head of the Specialised Haemostasis Laboratory (Department of Haematology and Cell Biology, Faculty of Health Sciences, University of the Free State and NHLS) and her assistant, Ms C Conradie, with technical assistance from the researcher. The following methods were used.

3.5.1 ADAMTS13 antigen testing and activity assay

The ADAMTS13 antigen levels and activities were measured using the respective ELISA kits from Technoclone® according to the manufacturer's instructions.

The Technozym® ADAMTS13 Antigen ELISA kit is a chromogenic ELISA for the determination of ADAMTS13 antigen concentration in human plasma. The kit contains ELISA test strips that are coated with a monoclonal anti-ADAMTS13 antibody, directed against the CUB (complement C1r/C1s, Uefg, a sea urchin embryonic protein) and the bone morphogenetic protein 1 domain of the protein. Plasma incubation is followed by a conjugate reaction, which involves the addition of a polyclonal rabbit anti-ADAMTS13 antibody and subsequent tetramethylbenzidine colour reagent reaction.(72)

The Technozym® ADAMTS13 Activity ELISA kit is a chromogenic ELISA for the determination of ADAMTS13 activity in human plasma. The kit contains ELISA test strips that are coated with a monoclonal anti-glutathione-S-transferase (GST) antibody. VWF73, a recombinant VWF fragment that contains the ADAMTS13 cleavage site, is tagged with GST to form the GST-VWF73 substrate solution.(26) This GST-VWF73 substrate solution is added to the anti-GST-coated test strips and incubated. Subsequent plasma incubation allows cleavage of the VWF73 fragment by ADAMTS13 present in the sample. This step is followed again by the conjugation reaction, with horseradish-peroxidase-conjugated, monoclonal, anti-N10 antibody that now recognises only the cleaved VWF fragment. Tetramethylbenzidine colour reagent is used to detect the reaction.(73)

Measurements for both kits were performed using an ELISA reader with 450 nm filter, and concentrations were read from the calibration reference curve.(72, 73)

3.5.2 Von Willebrand factor testing

The following diagnostic tests were performed:

- VWF antigen level (VWF:Ag);
- Ristocetin cofactor activity (VWF:RCo);
- Collagen binding activity of VWF (VWF:CB); and
- Multimeric analysis of VWF.

Testing was performed according to the standard operating procedures, SHL-1, SHL-2, SHL-3 and SHL-5 of the Specialised Haemostasis Laboratory, University of the Free State, and NHLS.

Von Willebrand factor antigen (VWF:Ag): This is an ELISA-based assay that measures the amount of VWF in test plasma. An ELISA plate, pre-coated with specific rabbit anti-human VWF antibody, captures the VWF to be measured. This is followed by a conjugation reaction with a rabbit anti-VWF antibody coupled with peroxidase and subsequent substrate reaction to reveal the bound enzyme peroxidase activity. The colour intensity produced is directly proportional to the VWF concentration initially present in the test sample. The values are expressed as percentages.(74)

Ristocetin cofactor activity assay (VWF:RCo): The test measures platelet binding to VWF in test plasma. Formalin-fixed washed platelets do not agglutinate in the presence of ristocetin unless normal plasma, as a source of VWF, is added. Agglutination follows a dose-response curve that is dependent on the amount of plasma VWF added. The ristocetin cofactor assay was done on a Chronolog 560 CA whole-blood aggregometer using Aggrolink software (Chronolog, USA). A standard curve of calibrated human plasma was used as the standard against which the test plasma was measured. The values are expressed as percentages.(75)

Collagen binding assay of von Willebrand factor (VWF:CB): This is an ELISA-based functional assay that measures the ability of HMW multimers of VWF to bind to collagen.

Dilutions of test plasma are added to a collagen-coated ELISA plate and the amount of bound VWF is assessed using an anti-horseradish peroxidase-conjugated VWF antibody. The values are expressed as percentages.(76)

Von Willebrand factor multimer analysis: This procedure allows visualisation of the multimeric structure of VWF in plasma. The method involves electrophoretic separation of VWF multimers on an agarose gel, followed by the transfer (or "blotting") of the VWF multimers onto a polyvinylidene fluoride membrane. Localisation is dependent on binding of rabbit anti-human VWF/horseradish peroxidase, followed by luminographic visualisation with the aid of x-ray film and cassettes. VWF multimeric patterns from test plasma are then compared with normal plasma.(77)

3.5.3 Plasminogen levels

The Technozym® Glu-Plasminogen ELISA kit was used to measure the Glu-plasminogen levels in the different plasma products. Glu-plasminogen is the predominant native plasminogen that is converted to active plasmin through two consecutive cleavage actions by certain plasma proteases.(10) Plasmin-mediated modification of Glu-plasminogen exposes lysine binding sites to form Lys-plasminogen that, with its superior fibrin binding and activation capacity, constitutes a positive feedback mechanism for enhanced conversion of plasminogen to plasmin.(10) By testing Glu-plasminogen instead of Lys-plasminogen or plasmin, the total potential pool available for plasmin formation is therefore considered.

The Technozym® Glu-Plasminogen ELISA test is a solid phase enzyme immunoassay. The ELISA test strips, which are precoated with a monoclonal anti-plasminogen antibody, are incubated with the test samples and controls. A conjugated, polyclonal anti-plasminogen antibody (POX antibody) is added, followed by the tetramethylbenzidine substrate. Measurement was performed using an ELISA reader with 450 nm filter, and concentrations were read from the calibration reference curve.(78)

3.5.4 Test Schedule

All tests were performed in the first two weeks of January 2016. The FFP and CSP testing segments were thawed and one bottle Bioplasma FDP® per lot reconstituted on 5 January 2016, and the ADAMTS13 antigen and activity ELISAs, as well as the Glu-plasminogen ELISAs performed. The remaining FFP and CSP was aliquoted and frozen for the VWF functional assays and multimer analyses, which were performed on 7 and 12-13 January 2016 respectively. The second Bioplasma FDP® bottle per lot was prepared, aliquoted and frozen, for the VWF testing that took place in the same run as the FFP and CSP testing, namely on 7 and 12-13 January 2016.

Before the VWF multimer analyses, all specimens were essentially subjected to one additional freeze-thaw cycle. For a particular assay, all specimens from all three plasma products were assayed in the same run. The normal reference ranges for the different assays according to the ELISA kits' package inserts(72, 73, 78) and the relevant standard operating procedure(74-77) are summarised in Table 3.2.

Table 3.2: Normal reference ranges for assays

Diagnostic test	Normal range
ADAMTS13 Ag	0.60 – 1.60 µg/mL
ADAMTS13 Activity	40% – 130%
Glu-plasminogen	60 – 250 µg/mL
VWF:Ag	51% – 143%
VWF:RCo	50% – 150%
VWF:CB	49% – 157%
Multimers	Multimer pattern compared to normal plasma

3.6 BASIC DESCRIPTIVE STATISTICS

All results were documented on an Excel data capture sheet – see Appendix C.

Data were analysed by the Department of Biostatistics, University of the Free State. The results of the various tests were described using ranges, medians, means, standard deviation (SD), coefficients of variation (CVs) and analysis of variance (ANOVA).

For each plasma product, the various measurements – ADAMTS13 antigen, ADAMTS13 activity, Glu-plasminogen, VWF:Ag, VWF:RCo, VWF:CB and VWF multimers – were reported, first, as proportions below/within/above the recommended reference ranges.

These proportions of below/within/above range readings were then compared across the various plasma products by means of chi-square cross tabulation with Fisher's exact p-value. The CVs calculated were also compared with available biological variation database values.

CHAPTER 4: RESULTS

4.1 INTRODUCTION

The minimum and maximum values, medians, means, standard deviations and CVs for the levels and activities of ADAMTS13 and VWF, and Glu-plasminogen levels in the different plasma products, are summarised in Table 4.1 and Figure 4.1. Comparison of the proportions of below/within/above-range readings across the various plasma products for the different parameters tested, are summarised in Table 4.2 and the p-values of these comparisons summarised in Table 4.3.

4.2 COMPARISON OF THE DIFFERENT BLOOD PRODUCTS

4.2.1 ADAMTS13 antigen and activity

Of Bioplasma FDP® samples, 75% (15/20) had ADAMTS13 antigen levels exceeding the upper limit of normal, whilst 35% (7/20) of CSP and 80% (16/20) of FFP samples had ADAMTS13 antigen levels falling below the normal reference range. Statistically significant differences were present when the individual plasma products were compared with one another (CSP vs FFP, p-value: 0.0095; Bio vs CSP, p-value: <0.0001; Bio vs FFP, p-value: <0.0001).

None of the samples tested had ADAMTS13 activity levels falling below the normal reference range (40-130%). All 20 Bioplasma FDP® samples, and 18/20 CSP samples, were within normal limits, showing no statistically significant differences between these two products (Fisher's exact test p-value 0.4872). FFP, with 45% of samples (9/20) exceeding a 130% activity level, showed meaningful differences when compared with the other products (FFP vs CSP, p-value: 0.0310; FFP vs Bio, p-value: 0.0012).

4.2.2 Von Willebrand factor antigen

All 20 Bioplasma FDP® samples harboured normal levels, whilst all 20 CSP samples, in turn, had deficient levels of the VWF antigen (16/20 samples had undetectable levels). Nine of the FFP samples had levels below, and the remaining eleven FFP samples had

levels within, the reference range. Statistically significant differences were found across all three plasma products (Bio vs CSP, p-value: <0.0001; Bio vs FFP, p-value: <0.0012; FFP vs CSP, p-value: 0.0001).

4.2.3 Von Willebrand factor activity

VWF:RCo and VWF:CB activities were consistently below the reference range for CSP and below the reference range in 85% (VWF:RCo) and 80% (VWF:CB) of FFP samples. No significant differences between these two products were found (p-values: 0.2308 and 0.1060 for VWF:RCo and VWF:CB respectively). With only 10% and 20% of Bioplasma FDP® samples showing levels below the reference ranges for VWF:RCo and VWF:CB respectively, differences between Bioplasma FDP® and each of the other products were regarded significant (Bio vs CSP (VWF:RCo & VWF:CB), p-value <0.0001; Bio vs FFP (VWF:RCo), p-value: <0.0001; Bio vs FFP (VWF:CB), p-value: 0.0004).

4.2.4 Glu-plasminogen

All 60 samples tested had Glu-plasminogen levels falling within the reference range, with no significant differences between products (p-value: 0.1085).

4.2.5 Von Willebrand factor multimers

The VWF multimer patterns for the different blood products are summarised in Table 4.4 and visually represented in Figures 4.2, 4.3 and 4.4.

Of Bioplasma FDP® samples, 90% (18/20) showed VWF multimer patterns comparable to that of normal plasma controls, with absence of only the largest multimer forms. Further, 95% (19/20) of CSP samples and 85% (17/20) of FFP samples lacked the large VWF multimers as well. A similar pattern was seen for the intermediate and small multimer forms. Comparing the multimer patterns across all three plasma products yielded significant differences (p-value: <0.0001), in particular when Bioplasma FDP® was compared with each of the other two (Bio vs FFP, p-value: <0.0001; Bio vs CSP, p-value: <0.0001, CSP vs FFP, p-value: 0.2308).

4.3 COMPARISON OF BLOOD GROUPS (O AND NON-O) FOR CRYOSUPERNATANT AND FRESH FROZEN PLASMA

The minimum and maximum values, medians, means, standard deviations and CVs for the levels and activities of ADAMTS13 and VWF, and Glu-plasminogen levels, in CSP and FFP across the different ABO blood groups, are summarised in Tables 4.5 a and b.

Note that the reference ranges included for the different test parameters are not ABO blood group specific. To our knowledge, no blood-group-specific reference ranges are currently available in the published literature. The proportions of below/within/above range readings of the blood group O and non-O CSP and FFP samples for the various parameters tested, are summarised in Table 4.6 and the p-values of the blood group comparisons for the different parameters tested, are summarised in Table 4.7.

4.3.1 Cryosupernatant

ADAMTS13 antigen: Eight of the ten blood group O samples were within the normal reference range, with one of the remaining samples below and one above the range. Six of the non-O samples had abnormally low levels and the remaining four samples were normal. Differences appeared insignificant with a borderline p-value calculated at 0.0573.

ADAMTS13 activity: No difference was found, with 90% of both blood group O and non-O samples harbouring normal ADAMTS13 activity levels (p-value: 1.000).

VWF Ag, VWF:RCo, VW:CB: With all the CSP samples showing deficient levels or, in the majority of cases, complete absence of these parameters, no statistically significant differences were found between the group O and non-O cohorts.

Glu-plasminogen: Glu-plasminogen levels showed a similar distribution amongst the O and non-O blood group samples.

4.3.2 Fresh frozen plasma

ADAMTS13 antigen: All blood group O samples and 60% (6/10) of the non-O samples had deficient ADAMTS13 antigen levels, but differences were not significant (p-value: 0.0867).

ADAMTS13 activity: A significant difference (p-value: 0.0001) was found with 90% (9/10) of blood group O samples exceeding the upper limit of the reference range, whilst the activity levels of all non-O samples fell within the reference range – see Figure 4.5 a.

VWF Ag, VWF:RCo, VW:CB: VWF:Ag levels were comparable between the O and non-O blood groups with no significant difference detected (p-value: 1.000). All ten blood group O samples had undetectable VWF:RCo and VWF:CB activity. Six of the non-O blood group samples, similar to the O blood group samples, also showed complete absence of these parameters. Only four of the ten non-O blood group samples, all four of blood group B, showed normal VWF:RCo and VWF:CB activity (although one of these samples possibly had mildly decreased VWF:CB activity). Differences are not regarded significant, with p-values of 0.0867 and 0.2105 for VWF:RCo and VWF:CB respectively – see Figures 4.5 b and c.

Glu-plasminogen: Eighty percent of the non-O blood group samples had Glu-plasminogen levels bordering the lower limit of normal, whilst O blood group samples displayed a greater range, yet with all 20 sample results falling within the normal reference range, no statistically significant differences could be calculated.

Table 4.1: The minimum (min) and maximum (max) values, medians, means, lower and upper quartiles (LQ & UQ), SDs and CVs for the levels and activities of ADAMTS13 and VWF, and the antigen levels of Glu-plasminogen in the different plasma products

Variable	Normal range	Min	LQ	Median	UQ	Max	Mean	SD	CV%
Bioplasma FDP® (n = 20)									
ADAMTS13 Ag	0.6 – 1.6 µg/mLl	1.13	1.58	1.98	2.70	2.70	2.07	0.56	**27.34**
ADAMTS13 Act	40 – 130 %	62.00	82.50	94.00	104.00	127.00	92.95	15.67	**16.86**
VWF : Ag	51 – 143 %	54.00	76.50	87.50	95.50	122.00	86.15	16.21	**18.81**
VWF : RCo	50 – 150 %	39.00	54.50	58.00	68.00	81.00	61.00	10.21	**16.74**
VWF : CB	49 – 157 %	38.00	57.50	75.00	83.00	100.00	71.30	18.82	**26.39**
Glu-plasminogen	60 – 250 µg/mL	92.00	98.00	113.00	122.00	143.00	122.45	15.85	**14.10**
CSP (n = 20)									
ADAMTS13 Ag	0.6 – 1.6 µg/mL	0.20	0.31	0.84	1.33	1.73	0.86	0.51	**59.69**
ADAMTS13 Act	40 – 130 %	60.00	72.50	80.50	97.00	142.00	87.05	22.56	**25.92**
VWF : Ag	51 – 143 %	0.00	0.00	0.00	0.00	15.00	1.60	3.80	**237.69**
VWF : RCo	50 – 150 %	0.00	0.00	0.00	0.00	10.00	0.50	2.24	**447.21**
VWF : CB	49 – 157 %	0.00	0.00	0.00	0.00	17.00	1.00	3.80	**379.75**
Glu-plasminogen	60 – 250 µg/mL	71.00	91.50	100.50	138.50	194.00	113.35	32.34	**28.53**
FFP (n = 20)									
ADAMTS13 Ag	0.6 – 1.6 µg/mL	0.23	0.29	0.32	0.46	1.56	0.49	0.39	**78.96**
ADAMTS13 Act	40 – 130 %	63.00	100.50	122.00	160.00	160.00	124.70	33.29	**26.69**
VWF : Ag	51 – 143 %	22.00	45.00	55.50	70.00	95.00	57.05	20.59	**36.09**
VWF : RCo	50 – 150 %	0.00	0.00	0.00	0.00	75.00	12.85	26.80	**208.58**
VWF : CB	49 – 157 %	0.00	0.00	0.00	0.00	84.00	13.35	27.78	**208.09**
Glu-plasminogen	60 – 250 µg/mL	73.00	87.50	95.00	117.50	163.00	105.70	26.01	**24.61**

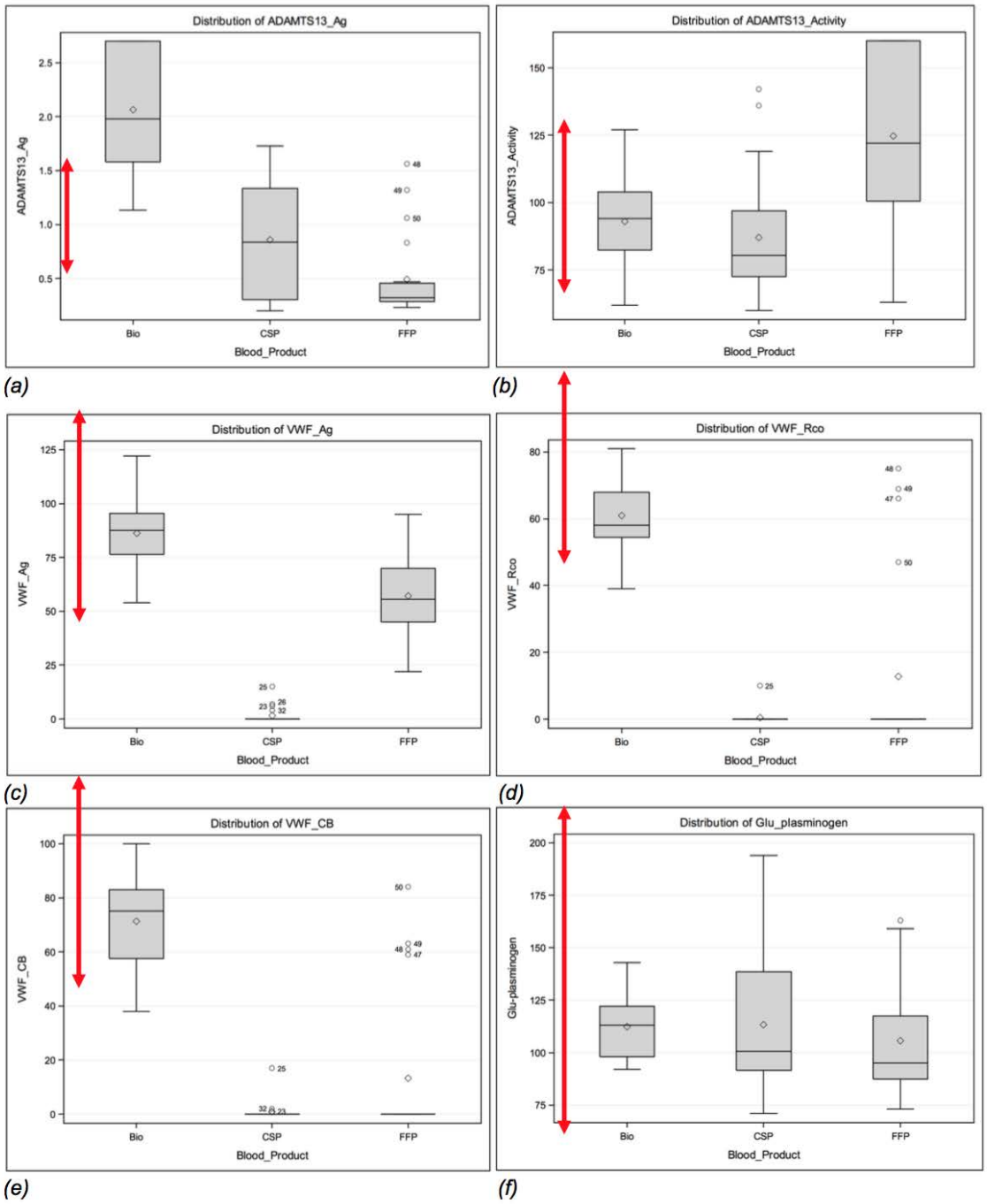


Figure 4.1: The ANOVA procedure illustrating the distribution of the different test parameters for each plasma product

In Figure 4.1 the red arrows highlight the normal reference ranges for each test. Note the deficiency in ADAMTS Ag levels (a) contrasted to the high ADAMTS13 Act levels (b) for FFP. As expected, CSP shows deficiency in VWF antigen and activity levels (c, d, e). Surprisingly though, FFP, which still contains the cryoprecipitate fraction with VWF, also shows deficiency of VWF activities (d, e).

Table 4.2: Number of below/within/above range readings of the different plasma products for the various parameters tested

Plasma product	Range reading	ADAMTS13 Ag	ADAMTS13 Act	VWF:Ag	VWF:RCo	VWF:CB	Glu-plasminogen
Bio (n = 20)	Below	0	0	0	2	4	0
	Within	5	20	20	18	16	20
	Above	15	0	0	0	0	0
CSP (n = 20)	Below	7	0	20	20	20	0
	Within	12	18	0	0	0	20
	Above	1	2	0	0	0	0
FFP (n = 20)	Below	16	0	9	17	16	0
	Within	4	11	11	3	4	20
	Above	0	9	0	0	0	0

From Table 4.2 it is clear that Bioplasma FDP® samples are almost consistently within the reference ranges for most of the parameters tested. CSP has predominantly normal ADAMTS13 levels and activities with absence of VWF levels and activities. FFP sample results show great variation. Note normal Glu-plasminogen levels in all three plasma products.

Table 4.3: Fisher's exact p-values for the comparisons across the different plasma products for the various parameters tested

Plasma products	ADAMTS 13 Ag	ADAMTS 13 Act	VWF:Ag	VWF:RCo	VWF:CB	Glu-plasminogen	VWF multimer patterns
Bio vs FFP	< 0.0001	0.0012	< 0.0012	< 0.0001	0.0004		<0.0001
Bio vs CSP	< 0.0001	0.4872	<0.0001	< 0.0001	<0.0001	0.1085	<0.0001
FFP vs CSP	0.0095	0.0310	0.0001	0.2308	0.1060		0.2308

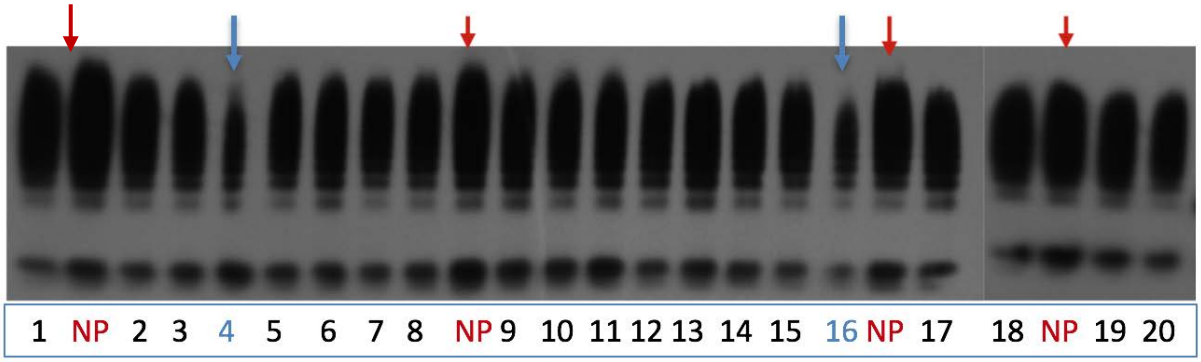
Table 4.3 reports statistically significant differences in at least one plasma product for almost every parameter tested, particularly when Bioplasma FDP® was compared with each of the other two plasma products.

Table 4.4: VWF multimer patterns for the different blood products

VON WILLEBRAND FACTOR MULTIMERS									
Plasma Product (n=20 each)	**LARGE**			**INTERMEDIATE**			**SMALL**		
	Absent	Decreased	Normal	Absent	Decreased	Normal	Absent	Decreased	Normal
BIO fr (%)	2 (10)	0 (0)	18 (90)	0 (0)	2 (10)	18 (90)	0 (0)	1 (5)	19 (95)
CSP fr (%)	19 (95)	1(5)	0 (0)	17 (85)	3 (15)	0 (0)	9 (45)	5 (25)	6 (30)
FFP fr (%)	17 (85)	0 (0)	3 (15)	16 (80)	1 (5)	3 (15)	0 (0)	0 (0)	20 (100)

fr: frequency

In Table 4.4, note the complete absence (in CSP) and marked deficiency (in FFP) of both the large and intermediate multimer forms.



NP: normal plasma

Figure 4.2: VWF multimer patterns of all 20 Bioplasma FDP® samples

The red arrows in Figure 4.2 indicate normal plasma controls. Except for two samples (indicated by blue arrows), all Bioplasma FDP® samples had VWF multimer patterns comparable to normal plasma controls.

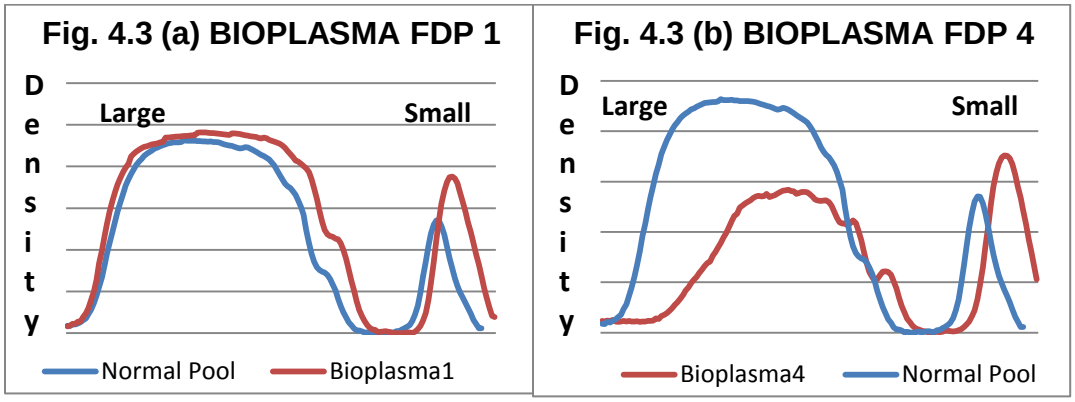
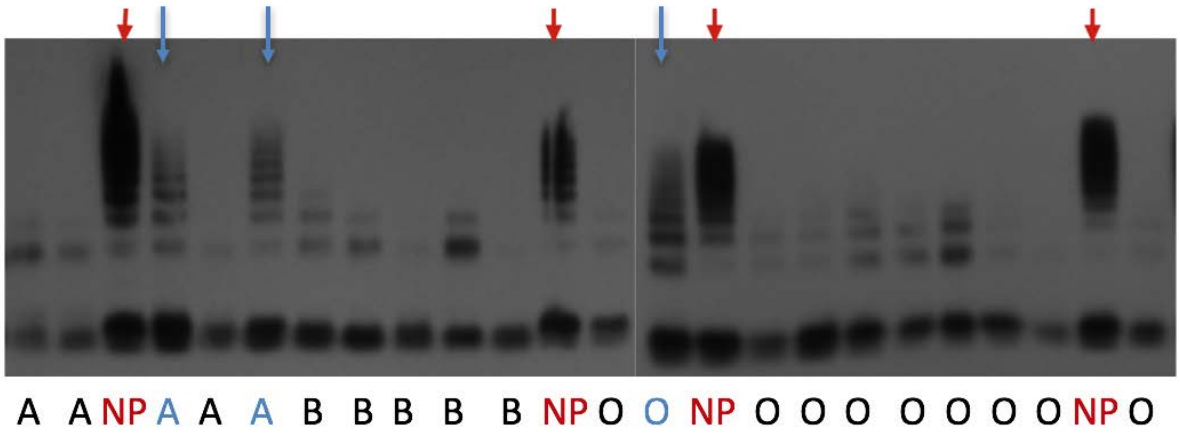


Figure 4.3 (a) & (b): Densitograms of the VWF multimer pattern of Bioplasma FDP® Sample 1, generally representative of most samples tested, and Sample 4 showing one of the two samples with an absence of the large multimer forms



A: blood group A, B: blood group B, O: blood group O, NP: normal plasma

Figure 4.4: VWF multimer patterns for CSP samples 1-20

The red arrows in Figure 4.4 indicate normal plasma controls. Only three samples (indicated with blue arrows) still contained intermediate multimers, the rest showed complete absence of the larger forms, with only small VWF multimers left.

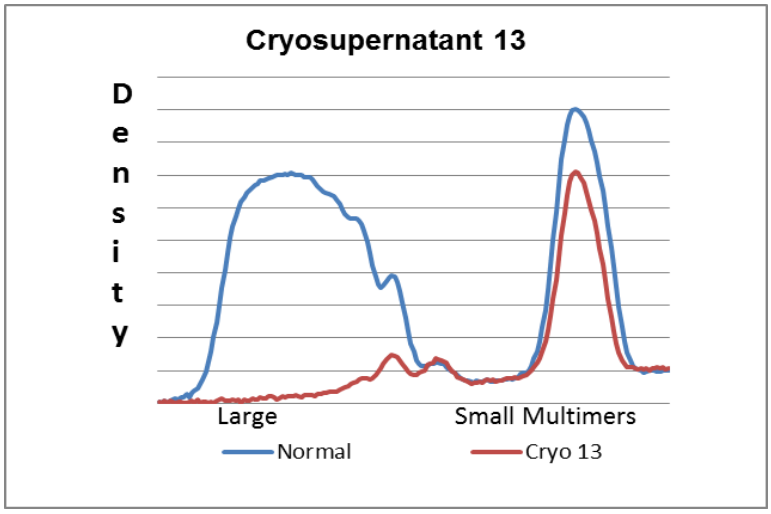
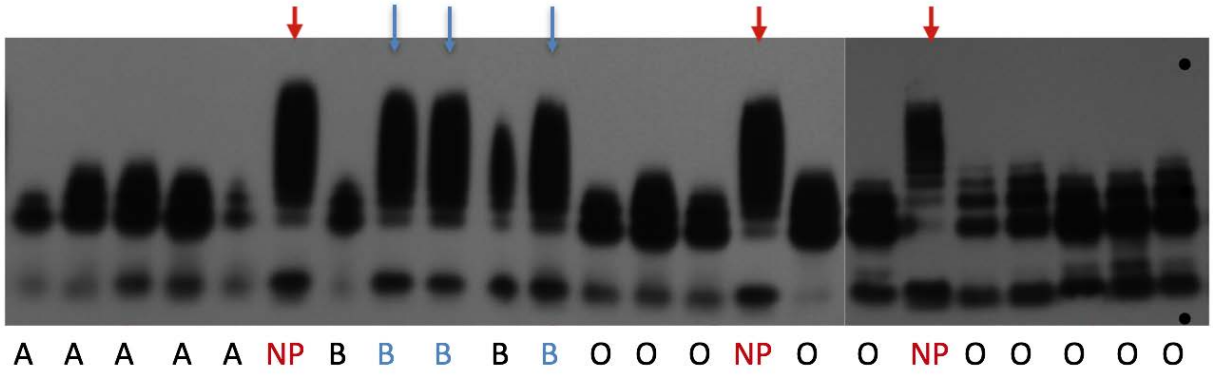


Figure 4.5: Densitogram of the VWF multimer pattern of CSP Sample 13, generally representative of most CSP samples tested



A: blood group A, B: blood group B, O: blood group O, NP: normal plasma

Figure 4.6: VWF multimer patterns of all 20 FFP samples

The red arrows in Figure 4.6 indicate normal plasma controls. Note the great inter-individual variation across the different samples with some patterns (all from ABO blood group B) comparable to that of the normal plasma controls showing absence of only the largest multimer forms (indicated by blue arrows), and others showing absence of the large and intermediate forms.

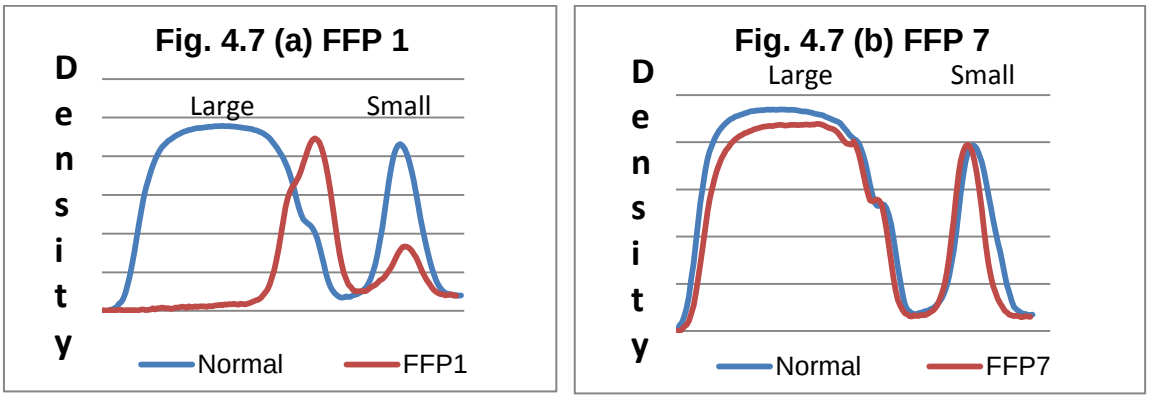


Figure 4.7: Densitograms of the VWF multimer patterns of FFP Samples 1 and 7, illustrating the two extremes in results obtained for this plasma product

Table 4.5: The minimum (min) and maximum (max) values, medians, means, SDs and CVs for the levels and activities of ADAMTS13, VWF and Glu-plasminogen in the different blood groups (O vs Non-O) for CSP

	Variable (normal range)	Min	LQ	Median	UQ	Max	Mean	SD	CV%
CSP O	**ADAMTS13 Ag** 0.6 – 1.6 µg/mL	0.320	0.910	1.310	1.350	1.730	1.156	0.401	34.727
	ADAMTS13 Act 40 - 130%	60.000	71.000	76.000	101.000	142.000	84.500	24.414	28.893
	VWF:Ag 51 - 143%	0	0	0	0	4.000	0.400	1.265	316.228
	VWF:RCo 50 - 150%	0	0	0	0	0	0	0	-
	VWF:CB 49 – 157%	0	0	0	0	2.000	0.200	0.632	316.228
	Glu-plasminogen 60 – 250 µg/mL	84.000	90.000	99.500	148.000	150.000	111.400	26.974	24.214
CSP Non-O	**ADAMTS13 Ag** 0.6 – 1.6 µg/mL	0.200	0.250	0.310	0.720	1.390	0.563	0.446	79.266
	ADAMTS13 Act 40 - 130%	65.000	79.000	82.500	93.000	136.000	89.600	21.547	24.048
	VWF:Ag 51 - 143%	0	0	0	6.000	15.000	2.800	5.073	181.172
	VWF:RCo 50 - 150%	0	0	0	0	10.000	1.000	3.162	316.228
	VWF:CB 49 – 157%	0	0	0	0	17.000	1.800	5.350	297.221
	Glu-plasminogen 60 – 250 µg/mL	71.000	93.000	103.000	129.000	194.000	115.300	38.358	33.268

Table 4.6: The minimum (min) and maximum (max) values, medians, means, lower and upper quartiles (LQ & UQ), SDs and CV for the levels and activities of ADAMTS13, VWF and Glu-plasminogen in the different blood groups (O vs Non-O) for FFP

	Variable (normal range)	Min	LQ	Median	UQ	Max	Mean	SD	CV%
FFP O	**ADAMTS13 Ag** 0.6 – 1.6 µg/mL	0.240	0.280	0.315	0.360	0.470	0.333	0.074	22.296
	ADAMTS13 Act 40 - 130%	125.000	155.000	160.000	160.000	160.000	153.100	12.538	8.190
	VWF:Ag 51 - 143%	22.000	44.000	52.500	63.000	75.000	51.900	15.452	29.773
	VWF:RCo 50 - 150%	0	0	0	0	0	0	0	-
	VWF:CB 49 – 157%	0	0	0	0	0	0	0	-
	Glu-plasminogen 60 – 250 µg/mL	87.000	92.000	117.500	135.000	163.000	120.600	27.427	22.743
FFP Non-O	**ADAMTS13 Ag** 0.6 – 1.6 µg/mL	0.230	0.290	0.320	1.060	1.560	0.646	0.505	78.235
	ADAMTS13 Act 40 - 130%	63.000	79.000	100.500	113.000	119.000	96.300	19.732	20.490
	VWF:Ag 51 - 143%	28.000	46.000	59.000	81.000	95.000	62.200	24.439	39.292
	VWF:RCo 50 - 150%	0	0	0	66.000	75.000	25.700	33.905	131.927
	VWF:CB 49 – 157%	0	0	0	61.000	84.000	26.700	35.116	131.520
	Glu-plasminogen 60 – 250 µg/mL	73.000	81.000	90.000	98.000	114.000	90.800	13.522	14.892

Table 4.7: Proportions of below/within/above range readings of the blood groups O and non-O CSP and FFP samples for the various parameters tested

Plasma product		Range reading	ADAMTS13 Ag	ADAMTS13 Act	VWF:Ag	VWF:RCo	VWF:CB	Glu-plasminogen
CSP	O	Below	1	0	10	10	10	0
		Within	8	9	0	0	0	10
		Above	1	1	0	0	0	0
	Non-O	Below	6	0	10	10	10	0
		Within	4	9	0	0	0	10
		Above	0	1	0	0	0	0
FFP	O	Below	10	0	10	10	10	0
		Within	0	1	0	0	0	10
		Above	0	9	0	0	0	0
	Non-O	Below	6	0	4	7	6	0
		Within	4	10	6	3	4	10
		Above	0	0	0	0	0	0

Table 4.8: Fisher's exact p-values for the comparisons between blood groups O and non-O FFP and CSP samples for the various parameters tested

Plasma product	ADAMTS13 Ag	ADAMTS13 Act	VWF:Ag	VWF:RCo	VWF:CB	Glu-plasminogen
CSP O vs Non-O	0.0573	1.000	-	-	-	-
FFP O vs Non-O	0.0867	0.0001	1.000	0.0867	0.2105	-

No statistics were calculated for the data in Table 4.8 if all sample results for a particular parameter fell within the same category (below/within/above reference range).

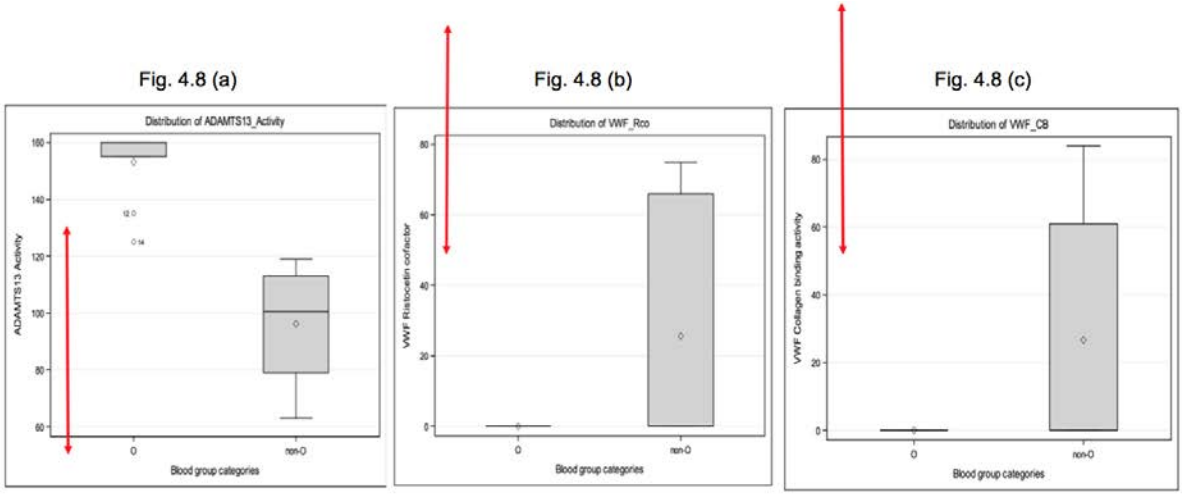


Figure 4.8: (a-c) The ANOVA procedure illustrating the distribution of ADAMTS13 activity, VWF:RCo and VWF:CB for blood groups O and non-O FFP samples.

The red arrows in Figure 4.8 highlight the normal reference ranges for each test. Note that 90% (9/10) of the blood group O samples had ADAMTS13 activity levels exceeding the upper limit of normal (a). Although the differences in VWF:RCo and VWF:CB levels between blood group O and non-O samples were not statistically significant, it is worth noting that the four non-O samples with normal values were all of blood group B (b, c).

CHAPTER 5: DISCUSSION

TTP, generally considered to be a rare condition,(2, 44) has a much greater incidence in HIV-positive populations, and is the commonest cause of TTP admissions in some South African hospitals.(34) Unpublished data from the Division of Clinical Haematology, Department of Internal Medicine, at Universitas Academic Hospital Complex in Bloemfontein, indicate that more than 80% of its TTP cases are associated with HIV.(79)

The rationale behind the use of plasma therapy, as the cornerstone of the management of TTP, includes removal or dilution of inhibitor autoantibodies and ULVWF multimers and replacement of the deficient ADAMTS13.(43) Although the exact pathogenesis of HIV-associated TTP is still not clear, excessive VWF release and reduction in ADAMTS13 activity, whether it be secondary to consumption, inhibition, or proteolytic inactivation, is evident.(37) In this light, the ideal plasma product for use in PEX/plasma-infusion therapy would be one with normal/high ADAMTS13 levels and activity and absent/low levels of the large and ULVWF multimers. In addition, adequate levels of plasminogen, the circulating zymogen of plasmin that is a proposed backup mechanism for ADAMTS13, can also be considered beneficial.

Daily PEX is considered the treatment of choice, while large-volume plasma-infusion therapy is the less favourable alternative, due to the risk of volume overload.(2) Novitzky et al. showed that patients with HIV-associated TTP respond significantly better to plasma-infusion therapy than their HIV-negative counterparts. Likewise, Visagie et al. confirm the benefits of rapid response to plasma infusion alone, and highlight its important cost-saving implications.(46, 79) This is of particular relevance in the South African setting, where apheresis is not always readily available and the majority of the TTP cases are HIV-associated.(46) Regardless of the treatment option used, patient exposure to large volumes of plasma is inevitable, hence, any possible treatment advantage of one product over the other cannot be ignored.

FFP, CSP and Bioplasma FDP®, the three locally available plasma products, are all used at institutions in South Africa for the treatment of TTP. Therapeutic PEX with either FFP or CSP as replacement fluid was used in a study conducted by Gunther et al. on HIV-

associated TTP patients who presented to tertiary teaching hospitals in Johannesburg.(37) Plasma infusion with CSP was the mainstay of treatment for HIV-associated TTP patients enrolled in a study conducted by Novitzky et al. at Groote Schuur Hospital in the Western Cape.(46) Visagie et al. described a case of myocardial injury in HIV-associated TTP at the Universitas Academic Hospital Complex in Bloemfontein, where treatment included plasma infusion with Bioplasma FDP®.(79) The TTP treatment protocol for the latter institution states that any one of the three plasma products can be used, however, personal communication with a consultant at the Division of Clinical Haematology, Department of Internal Medicine, confirmed that CSP is most frequently used.(47)

Several international comparative studies on the content and efficacy of FFP, CSP and SDP have failed to prove one product superior to the others.(32) Data on blood products in South Africa are, however, very limited. To our knowledge, no clinical trials comparing treatment response, or in vitro studies comparing composition of these products, have been conducted. The accessible data mainly involve information on the coagulation-factor levels for each product, with no direct comparisons drawn.

In this study, the levels and activities of ADAMTS13, VWF and plasminogen – all of which are central in the treatment of HIV-associated TTP – were compared in order to define the most ideal product. In doing so, the researchers attempted, firstly, to validate these products as fit-for-purpose and, secondly, to establish equivalence or identify significant differences that may translate into possible treatment advantages.

One of the major benefits of pooling plasma products is standardisation of the content.(67) Not surprisingly, Bioplasma FDP®, which is derived from pooled fresh human plasma, showed little variation for all parameters tested, with CVs ranging from 14.1% to 27.3% – see Table 4.1. Yarranton et al. also found less variation across different batches of SDP than other plasma products, and ascribe it to pooling.(43)

Several studies have confirmed that SDP retains normal in vitro levels and activities of selected plasma proteins, including plasminogen and ADAMTS13.(67, 68, 71, 80-82) This study's findings of adequate levels and activities of ADAMTS13 and normal levels of

plasminogen (Table 4.2) confirm that Bioplasma FDP® is on par with international SDP. One may therefore argue that Bioplasma FDP®, as replacement fluid, would at least guarantee consistent replacement of ADAMTS13. The VWF multimer patterns were equivalent to that of normal plasma controls, showing absence of only the largest forms (Table 4.4 and Figures 4.2 – 4.3). Studies on SDP done by Moake et al. in 1994 and Harrison et al. in 1996 describe a deficiency in the higher molecular weight VWF multimers, however, no distinction between ultra large and large forms are mentioned.(59) Considering that only the ultra large forms are implicated in the pathogenesis of TTP, presence of the large multimers would not be regarded as detrimental. In addition, similar to reports by Harrison et al., this study also found the VWF:Ag and VWF:RCo values to trend toward the lower limit of normal.(59)

The SANBS and WPBTS Lookback Programmes identify and follow up all recipients of previous blood units from donors who, on subsequent donations, have tested positive for a TTI.(83) According to the 2014 haemovigilance reports of these services, 863 donors seroconverted, of whom 65.8% were due to HIV, 29.9% due to hepatitis B virus, and 2.8% due to hepatitis C virus. Of the 1 152 836 components/blood products issued in 2014, only two confirmed TTIs, one of which due to HIV, were documented.(83) However small, the risk of contracting a debilitating virus still exists. When considering the large volume of plasma required for the treatment of an acute episode of TTP, the additional benefit of viral inactivation provided by the solvent/detergent process may be considered essential. In fact, both the BCSH guidelines for the use of FFP, cryoprecipitate and CSP, and South African clinical guidelines for the use of blood products, suggest the use of pathogen-inactivated plasma in patients likely to receive large or repeated doses of plasma.(54, 58)

Another important advantage of SDP is related to the effects of pooling, which, as explained by Solheim et al., results in dilution and neutralisation of antibodies, allergens and active substances, such as histamine. This significantly reduces the incidence of allergic/immunological reactions in the recipient.(67)

According to a review by Liumbruno et al. neutralisation of anti-human leucocyte antigen and anti-human neutrophil antigen antibodies by leucocytes, which are subsequently removed by the double filtration process associated with solvent/detergent-treatment,

explains the absence of transfusion-related acute lung injury with SDP administration.(71) Neutralisation of blood-group antigens by their respective antibodies eliminates the risk of potentially life-threatening ABO-incompatible transfusions further.(67) In clinical practice, however, ABO-incompatible plasma transfusions are frequently given if plasma from the same ABO blood group as the recipient is not available, provided that the anti-A and anti-B antibody titres are low.(54) Storage at room temperature (at or below 25°C) and at the point-of-care (e.g. theatre, casualty) without the need for thawing, also add to the convenience of SDP.

Some of the concerns associated with SDP include potential thromboembolic complications related to protein S deficiency, as well as possible transmission of non-lipid-coated viruses, such as hepatitis A virus and parvovirus B19.(58) Another potential predicament is associated with the reconstitution procedure, which requires strict adherence to instructions. Product quality and even utility could conceivably be jeopardised by a simple handling error.

CSP is FFP from which the precipitable cryoproteins (Factor VIII, VWF, Factor XIII, fibronectin and fibrinogen) have been removed.(58) As expected, in 17 of the 20 samples tested VWF multimer patterns reveal complete absence of large VWF multimers as well as intermediate forms (Table 4.4 and Figure 4.4). Yarranton et al. describe this “appropriate depletion” as “expected”.(43, 66) The BCSH guidelines for the use of FFP, cryoprecipitate and CSP confirm that these results can be anticipated, but state, however, that CSP should still contain ADAMTS13.(58) This conclusion is supported by several studies, confirming the preservation of ADAMTS13, not only in CSP, but also FFP and SDP.(43, 66, 80)

Seven of the CSP samples tested had deficient ADAMTS13 antigen levels, yet normal enzyme activity. This was also the case with 16 of the FFP samples tested. ADAMTS13 activity levels actually exceeded the upper limit of the kit’s detection range in 6 of these 16 FFP samples (Figure 4.1 a and b). A possible explanation could be that partial degradation of the enzyme may result in loss of the specific epitope required to bind to the anti-ADAMTS13 monoclonal antibody in the antigen assay without impairing enzyme function. The Technozym® ADAMTS13 Antigen ELISA kit relies on monoclonal anti-

ADAMTS13 antibodies, directed against the CUB domain of the protein. Test limitations included in the product package insert state that any mutation in the enzyme CUB domain may result in reduced binding to the capture antibody.(72)

The activity assay makes use of VWF73, a 73-amino acid substrate form D1596 to R1668 in the A2 domain of VWF. Studies by Kokame et al. first suggested VWF73 as the specific substrate for ADAMTS13. They showed that deletions in this region results in loss of ADAMTS13 cleavage, whilst plasma from known TTP patients, in turn, failed to cleave VWF73.(84)

Starke et al. investigated the clinical utility of the various ADAMTS13 assays available for the diagnosis and management of TTP. Retrospective studies were done on thawed aliquots of frozen plasma samples from patients with known or suspected TTP. They found that 70% of samples tested showed concordance between antigen level and activity, although antigen levels tended to be slightly higher. In total, 91% of the conflicting results showed low activity with a normal to high antigen level. These samples were also found to have elevated autoantibody levels, indicating that the antigen assay is less reliable when autoantibodies, with potential activity-neutralising effects, are present.(85) It was deduced that activity assays have more clinical significance that antigen assays.

Although our results showed a statistically significant difference in ADAMTS13 activity between the blood group O and non-O FFP samples (see Table 4.5), this may not translate into clinical significance, as all results were either within or above the normal range. However, when considering the rationale behind the use of rADAMTS13 as a therapeutic agent, supra-physiological levels of ADAMTS13, as seen in blood group O FFP samples, may be desirable.

An in vitro study conducted by Plaimauer et al. explored the effects of rADAMTS13 added to TTP patient plasma samples with confirmed anti-ADAMTS13 IgG antibodies. Their results showed a linear relationship between the inhibitor titre measured and the effective rADAMTS13 dose required for recovery of the VWF-cleaving activity in the presence of inhibitors.(86)

Higher ADAMTS13 activity in blood group O plasma had also been documented by Mannucci and co-workers, in 2004, and later affirmed by Scott et al. in 2007.(55) In contrast, lower VWF levels in blood group O individuals, compared with other ABO blood types, are well described.(10, 23) A review by Jenkins and O'Donnell cite several studies that report VWF levels in blood group O individuals of up to 30% lower than their non-O counterparts.(22)

Franchini et al. further iterated a study by Gill and colleagues that reported the latter's difficulties in differentiating patients with Type 1 Von Willebrand disease from individuals with blood group O-associated reduction in VWF.(23) It is, however, important to note that, although patients with Type 1 Von Willebrand disease have reduced total VWF protein, the VWF multimeric pattern is still normal.(10) With undetectable VWF:RCo and VWF:CB, as well as markedly abnormal multimer patterns in all 10 blood group O FFP samples, as well as in all five blood group A samples (Figures 5 a and. 6 b and c), our findings appear to suggest the presence of additional (if not entirely different) variables which influenced our results.

Repeated documentation of higher ADAMTS13 activity(24, 80) (including our results) associated with blood group O together with lower VWF levels(10, 22, 23), (even without considering our results), raises the question of whether blood group O plasma should preferentially be used in the treatment of TTP. Even though only four of the 10 non-O blood group samples showed normal to near-normal VWF activity and multimer patterns, it is worth noting that these samples were all of blood group B, a finding which further supports the effects of ABO blood group on VWF levels.

As reviewed by Jenkins and O'Donnell, Gill and colleagues reaffirm this relationship between ABO blood group and plasma VWF levels and demonstrate that, amongst non-O groups, VWF levels were highest in AB individuals followed by group B and then group A.(22) We did not consider blood group AB in our study due to limited availability and budget constraints. Nevertheless, these findings raise the converse question of whether non-O FFP should be favoured in the treatment of haemostatic disorders. To our knowledge, however, these questions have not been explored further in the published

literature. The BCSH guidelines still recommend blood-group-specific plasma transfusions, reserving the use of blood group O FFPs for O recipients.(58)

McRoyan et al. tested a number of plasma proteins, including plasminogen, in donor blood and plasma components. Their results suggest plasminogen to be a partially cryoprecipitable plasma protein, and infer that CSP should be modestly plasminogen deficient. However, the difference between CSP and FFP plasminogen levels in our study was of no statistical significance.(87) Our finding of normal plasminogen levels across all three products tested also suggest any cryoprecipitation to be negligible.

No difference between blood group O and non-O samples was evident in CSP, possibly due to the small numbers of units tested. Regardless of blood group, adequate ADAMTS13 activity and plasminogen levels together with deficient VWF levels and activities across all CSP samples make CSP an attractive option in the treatment of TTP. It is a by-product after the removal of cryoprecipitate from FFP, and is only indicated for the treatment of TTP – if not used for this purpose it would be discarded.(54, 55) Utilising CSP therefore promotes responsible blood product use.

Although donated blood is not specifically subjected to viral inactivation procedures, SANBS and WPBTS have implemented several measures to improve viral safety of all blood products, including strict donor selection protocols, screening of all units for TTIs by means of routine serology testing, as well as ID-NAT and donor retest quarantine programmes.(54, 55) Unfortunately CSP is kept in small quantities only, which limits access, and (like FFP), it is stored at -18°C (54), and therefore requires thawing before administration.

When analysing all 20 FFP samples as a single cohort, significant variation was found across the different FFP samples with regard to all the parameters tested. These variations may, in part, be explained by the different ABO blood groups, however, with inter-individual variations evident even amongst samples of the same blood group category (O vs non-O), additional factors should be considered.

Beeck et al. analysed the in vitro characterisation of SDP and FFP, and likewise, found much greater batch-to-batch variations in FFP than in SDP for most proteins tested.(82)

This higher inter-individual variation is not completely unexpected, considering that FFP is prepared from single-donor whole-blood units following application of physical separation methods.(54) Variations across the different FFP samples could therefore reflect biological variations in the donors. Shou et al. explored the biological variations of some thrombophilia screening tests, including VWF:Ag, in healthy adult volunteers. Their results showed a between-subject variation (CV_G) of 31.71% for VWF:Ag, compared with 27.30% reported in the 8th edition of the Westgard database 2014 (Table 5.1). They did not differentiate between different blood groups and postulated this to be the reason for an increased CV_G.(31)

A similar study by Kilercik et al., specifically investigating the biological variations of ADAMTS13 and VWF, calculated CV_G of 18.5%, 22.59%, 9.63% and 6.28% for VWF activity, VWF:Ag, ADAMTS13 activity and ADAMTS13 antigen levels respectively (Table 5.1).(30) The median CV_G for plasminogen, described by Banfi et al., is 10.5% (Table 5.1).(32) Even when taking ABO blood groups into account, the CV_G for almost all the parameters tested by this study still exceeded that reported in the literature – see Table 5.1.

Table 5.1: CV_G of the different parameters tested in FFP samples, compared with those found in the published literature

Between-subject biological variation (CV_G (%))							
Test	**FFP**			**Shou et al. (2016)**	**Westgard database 8th ed (2014)**	**Kilercik et al. (2014)**	**Banfi et al. (2008)**
	All (n=20)	**Non-O (n=10)**	**O (n=10)**				
ADAMTS13 Act	26.69	8.19	20.50	-	-	9.63 **(CV_{TG}: 9.63)**	-
ADAMTS13 Ag	78.96	22.30	7.82	-	-	6.28 **(CV_{TG}: 10.70)**	-
VWF:Ag	36.09	29.77	39.29	31.71	27.30	22.59 **(CV_{TG}: 22.87)**	-
VWF:RCo	208.58	131.93	-	-	-	18.5 **(CV_{TG}: 19.83)**	-
VWF:CB	208.09	131.52	-	-	-	-	-
Glu-plasminogen	24.61	22.74	14.89	-	-	-	10.5

CV_{TG}: between-subject total variation (biological and analytical)

Shou et al. reviewed research on the various sources of variability that may affect laboratory tests, and summarised that biological variations, together with pre-analytical, analytical and post-analytical variations, are major contributors.(31) In addition to biological variations, Kilercik et al. also used commercially available quality-control samples to evaluate the analytical variation of the ADAMTS13 and VWF levels and activities in order to calculate the total variation (CV_{TG} - biological and analytical) for every test (Table 5.1).(30) The highest CV_{TG} calculated for every parameter was still lower than the CV_G of this study.

We speculate that these variations may, in part, be attributable to, amongst others, pre-analytical variables, including handling, transport and storage of blood products after

donation, or specimen handling, transport or storage. Information regarding the date and place of blood donation of the individual units, as well as the time taken for those units to reach the local processing facility, and the conditions in which the units reached the facility and were subsequently stored at, may expose variables that could explain the observed variations. These data are unfortunately not available, as product processing variation was not initially a research parameter.

According to the International Society of Blood Transfusion (ISBT) Science Series, donated blood units should be placed in controlled-temperature environments as soon as possible after collection (ideally within 30 minutes) to prevent deterioration of haemostatic factors.(88) They recommend that whole blood be cooled down rapidly (in less than 4 hours) after collection to +22˚C ± 2˚C and maintained at this temperature for a maximum transit time of 24 hours. Alternatively, fresh donated units can be stored at refrigerator temperatures (+4˚C ± 2˚C), which would necessitate cooling of blood to below +10˚C in no more than 6 – 8 hours.(89) Validated transport containers and coolant packs are required for this purpose.(89, 90)

Freezing of plasma must follow within 24 hours of donation to preserve labile clotting factors, most notably Factor VIII.(91) Whole-blood units stored as whole blood for more than 24 hours before freezing of the separated plasma are deemed unsuitable for treatment of bleeding disorders.(91) Specifications for the preparation of FFP, in particular, involve freezing of the plasma separated from whole blood within 18 hours of collection.(91) It is conceivable that, depending on a particular blood bank processing facility's coverage area, some donated units will have a longer in-transit storage time than others. It would be interesting to determine whether different in-transit storage times, even if all still within the acceptable 24-hour time frame, already lead to differences in the haemostatic protein content of the donated fresh-blood units.

Another critical quality control point is mixing of the donated blood with the anticoagulant in the collection bag. Improper mixing, overfilling of the bag (which results in an insufficient anticoagulant-to-blood ratio) or prolonged donation (>15 minutes) may all result in activation of the coagulation cascade, with consumption of platelets and clotting factors.(88)

Unlike with CSP, the cryoprecipitate fraction, rich in HMW VWF multimers, is still included in the units of FFP. Yarranton et al. confirm that the whole spectrum of VWF multimers of all weights are present in FFP.(43) This study's most surprising result, therefore, is the absence of high, and even intermediate, molecular weight multimers found in 85% (17/20) of all FFP samples tested.

One could postulate that this may, in part, be attributable to the storage of FFPs in the blood bank at -18˚C to -23˚C, or other pre-analytical factors. Meiring et al. compared VWF levels, activity and multimer patterns of plasma samples stored at -20˚C and -70˚C. Whilst activity assays and multimer patterns remained normal after storage at -70˚C, a reduction in activity and loss of HMW multimers were found in the majority of samples stored at a temperature of -20˚C.(92)

Favaloro et al. also investigated the effects of prolonged ambient-temperature plasma storage on assay results and found a small, though statistically significant, reduction in the collagen-binding activity of VWF.(93) The BCSH guidelines for the use of FFP, cryoprecipitate and CSP, however, consider -30˚C to be the recommended plasma-storage temperature.(58) The ISBT recommends that FFP units are best stored at temperatures consistently below -25˚C. When stored at temperatures between -18˚C and -25˚C, the product shelf life decreases from up to 3 years to only 3 months.(91)

When taking into account that it may take up to 24 hours to process donated whole-blood units, the effects of pre-analytical storage conditions of whole blood (rather than plasma) on VWF levels and activities should be considered. Bohm et al. explored this proposition by testing the VWF levels and activities of whole-blood and plasma samples, both stored at room temperature and on crushed ice, at two different time intervals (3 hours and 6 hours). Whilst storage of plasma caused only minor changes in VWF levels, a significant time-dependent decrease in VWF levels and activities was observed in whole-blood samples after 6 hours of being stored on crushed ice. They postulate that this cold-induced loss of VWF may be attributable to the platelets in whole blood. Upon cooling, platelet GP1b receptors cluster together, which promote platelet-VWF binding.(94) The ISBT recommends that, if a 24-hour hold between blood receipt and processing is used, fresh blood must be stored at +22˚C ± 2˚C in a temperature-controlled environment.(89)

One can speculate that possible differences in the time period and conditions under which donated whole-blood units were stored/transported before processing may, to some extent, have contributed to variations noted in this study's FFP results.

Another culprit that may be implicated in the loss of higher VWF multimers is exposure to repeated freeze-thaw cycles. During the validation of a sandwich ELISA for quantification of VWF, the stability of test and control samples (rabbit and purified human VWF respectively) were tested. Samples were subjected to 1 – 3 freeze-thaw cycles and then assayed in 6 replicates. Significant reductions in VWF concentrations suggest decreasing protein stability with every cycle.(95)

In a study conducted by Hillarp et al. to assess the performance of the modified automated VWF:RCo assay, samples of VWF-deficient plasma spiked with the 1st International Standard for VWF concentrate were used. These samples were subjected to repeated freeze-thaw cycles to assess stability. They demonstrated a maintained stability with consistent VWF:RCo activity, even after 4 freeze-thaw cycles.(96) Whether the International Standard VWF concentrate can be compared to the VWF in human plasma samples is, however, debatable. Repeated freeze-thaw cycles, possibly resulting in decreased protein stability, as the only explanation for the great inter-individual variation found in this study's FFP samples, is probably unlikely when considering that all samples were subjected to the same number of freeze-thaw cycles, yet some FFP samples harboured normal VWF levels and activities.

Although the absence of HMW multimers and reduced VWF functional activity may be considered beneficial in the treatment of TTP, when considering the other indications for the use of FFPs, such as the replacement of inherited single or multiple-factor deficiencies as seen with haemorrhage, liver disease or disseminated intravascular coagulation, (54, 55, 58) this absence may impair efficacy and delay treatment response.

The great inter-individual variation poses a potential problem. One could postulate that treatment response is not dependent only on patient factors, such as immune status, but also on the particular FFP units received, and could therefore be unpredictable.

Conversely, it can be argued that the large number of FFP units required during PEX/plasma-infusion therapy is likely to balance out any inter-individual unit variations.

Finally, choice of blood product is also dependent on cost. Table 5.2 summarises the cost per unit and PEX of the different blood products.

Table 5.2: Prices per unit and average price calculated per exchange for the different plasma products according to the 2016 state-sector price lists(97, 98)

Product	Unit volume	2016 Price including value-added tax	Average estimated price per PEX
Bioplasma FDP®	200 mL	R 1 081.21	R 12 974.52
FFP	240 – 300 mL	R 1 337.45	R 13 374.50
CSP	190 – 310 mL	R 1 078.97	R 10 789.70

Yarranton et al. calculated that a 60 kg patient with an estimated plasma volume of 40 mL/kg would require 2 400 mL of plasma per exchange.(66) Based on this calculation, a patient may need up to 12 bottles of Bioplasma FDP® for a single PEX. The volume of a single adult unit of FFP ranges from 240 mL to 300 mL and CSP 190 mL to 310 mL. (54, 55) This equates to roughly 10 units of FFP or CSP per exchange. Even with fewer units required, on average, FFP is still less economical at R 13 374.50 per exchange, compared to R 12 974.52 and R 10 789.70 on average per exchange for Bioplasma FDP® and CSP respectively. All things considered, the wide availability and accessibility of FFP possibly still outweighs its perceived drawbacks.

CHAPTER 6: CONCLUSION

6.1 LIMITATIONS OF THE STUDY

It is recommended that, when values fall outside the assay detection range, as was seen with the ADAMTS13 antigen levels in some of the Bioplasma FDP® samples, which exceeded the highest point on the curve, sample dilutions should be made to improve accuracy. However, due to budget constraints, only one 96-well plate was available for each of the ELISA tests (ADAMTS13 antigen, ADAMTS13 activity and Glu-plasminogen), allowing these tests to be performed only once.

The small sample sizes and, in particular, the small numbers representing the different ABO blood groups, also reduce the likelihood of statistically significant results and necessitate cautious interpretation. Neither were Group AB plasma products evaluated – a significant study limitation. In addition, each sample was only tested once for every parameter, increasing the possibility that random errors were included in the results.

The additional freeze-thaw cycle may have negatively affected the VWF multimer patterns and functions, and may explain the unexpected results obtained for the FFP samples, although all FFP samples would conceivably have been affected equally had the effect of freeze-thawing been significant. ADAMTS13 activity and VWF:RCo have, however, been shown to remain stable for at least 2 and 4 freeze-thaw cycles respectively.(80, 96)

FFP and CSP samples were also obtained from different donors. Comparisons between these two plasma products may therefore not reflect solely the differences in the manufacturing processes, but also the inter-individual variation of the various donors.

6.2 FUTURE STUDIES

The results of this study need to be confirmed by a larger study with better representation of the different ABO blood groups. Should the current results prove to be accurate, a randomised, controlled clinical trial, comparing the in vivo effectiveness of the various plasma products in the treatment of HIV-associated TTP, is desirable. This will ultimately

determine whether the theoretical advantages established in this study actually translate into treatment benefits for the patient.

The unexpected VWF results for FFP samples warrant further investigation. To ascertain whether our speculations of pre-analytical variables as a possible culprit are accurate, a future study, specifically investigating the effects of blood collection, storage and transport (before reaching a processing facility), on VWF, may be justified.

6.3 SUMMARY

All 60 samples had normal/high ADAMTS13 activity and normal Glu-plasminogen levels.

With a few exceptions, Bioplasma FDP® sample results were consistently within the normal reference ranges for all the parameters tested. As expected, CSP had deficient/absent VWF levels and activities, with absence of the large and intermediate multimer forms. No significant difference between blood group O and non-O CSP samples were found.

Surprisingly, the majority of FFP samples also showed reduced/absent VWF activities with abnormal multimer patterns. Those FFP samples with near-normal VWF activities and multimer patterns were all of blood group B, whilst the FFP samples with above-normal ADAMTS13 activities were all representative of blood group O. These findings suggest that non-O FFP may be more beneficial in the treatment of bleeding diatheses, whereas group O FFP may be preferable in the treatment of TTP – a hypothesis that requires further investigation.

Theoretically, all three plasma products meet the minimum criteria for a replacement fluid, i.e., it contributes to improved VWF cleavage, be it secondary to replacement of ADAMTS13 or its purported backup mechanism, plasminogen, without adding additional pathogenic ULVWF multimers. Choice of product would depend on factors such as the need for additional viral safety, costs, product availability and the perceived practical impact of within-product variations.

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APPENDICES

A. Letter of approval: Health Sciences Research Ethics Committee, University of the Free State

B. Letter of approval: Human Research Ethics Committee, SANBS

C. Data capture sheet

APPENDIX A

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

IRB nr 00006240
REC Reference nr 230408-011
IORG0005187
FWA00012784

19 October 2015

DR AC VAN MARLE
DEPARTMENT OF HAEMATOLOGY AND CELL BIOLOGY
FACULTY OF HEALTH SCIENCES
UFS

Dear Dr Van Marle

ECUFS NR 191/2015
DR AC MARLE
DEPARTMENT OF HAEMATOLOGY AND CELL BIOLOGY
PROJECT TITLE: COMPARISON OF THE ANTIGEN LEVELS AND ACTIVITIES OF ADAMTS13, VON WILLEBRAND FACTOR AND PLASMINOGEN IN THE DIFFERENT BLOOD PRODUCTS CURRENTLY AVAILABLE IN SOUTH AFRICA FOR THE TREATMENT OF THROMBOTIC THROMBOCYTOPENIC PURPURA

1. You are hereby kindly informed that, at the meeting held on 15 October 2015, the Ethics Committee approved the above project.

2. Any amendment, extension or other modifications to the protocol must be submitted to the Ethics Committee for approval.

3. A progress report should be submitted within one year of approval of long term studies and a final report at completion of both short term and long term studies.

4. Kindly use the ECUFS NR as reference in correspondence to the Ethics Committee Secretariat.

5. The Ethics Committee functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the Ethics Committee of the Faculty of Health Sciences.

Yours faithfully

.....
DR SM LE GRANGE
CHAIR: ETHICS COMMITTEE

Cc: Dr J Joubert

Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | F: +27 (0)51 444 4359 | E: ethicsfhs@ufs.ac.za
Block D, Dean's Division, Room D104 | P.O. Box/Posbus 339 (Internal Post Box G40) | Bloemfontein 9300 | South Africa
www.ufs.ac.za

SOUTH AFRICAN NATIONAL BLOOD SERVICE NPC

Human Research Ethics Committee

OHRP Number : IORG0006278
FWA Registration Number : IRB00007553
SA NHREC Registration Number : REC-270606-013

SANBS
South African National Blood Service

Secretariat: Tel: 011 761 9135 | Fax: 011 761 9137 | Cell: 082 523 8523 | thandiwe.matsoso@sanbs.org.za

To: Dr Anneke van Marle
E-mail:annekevanmarle@gmail.com

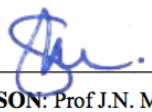
Dear Dr van Marle

DATE OF COMMITTEE MEETING:	**8 September 2015**
PROJECT TITLE:	**Comparison o f the antigen levels and activities of ADAMTS13, von Willebrand factor and plasminogen in the different blood products currently available in South Africa for the treatment of thrombotic thrombocytopenic purpura**
DECISION OF THE COMMITTEE:	**Approved**
CLEARANCE CERTIFICATE NO:	**2015/25**

- Execution of the study must be compliant with applicable guidelines and policies.
- Any amendment, extension or other modifications to the protocol must be submitted to this Ethics Committee for approval prior to implementation.
- The Committee must be informed of any serious adverse event, planned and unplanned termination of the study.
- A progress report should be submitted yearly for long-term studies and a final report at completion of both short term and long term studies.
- Kindly refer to the SANBS HREC clearance certificate number on all future correspondence on this study to the HREC secretariat.
- This approval is valid for 5 years from the date stated above.

COMMITTEE GUIDANCE DOCUMENTS:

- International Conference on Harmonization (ICH) Good Clinical Practices (GCP) Guideline (ICH, 1996), Ethics in Health Research: Principles, Structures and Procedures (SA Department of Health, 2004); Guidelines for Good Practice in the Conduct of Clinical Trials in Human Participants in South Africa (SA Department of Health, 2006); Ethical Principles for Medical Research Involving Human: Declaration of Helsinki (World Medical Association, 2013); Reviewing Clinical trials: A Guide For Ethics Committees (Karlberg and Speers, 2010)



17 September 2015

CHAIRPERSON: Prof J.N. Mahlangu **DATE**

Data capture sheet

APPENDIX C

	Product:				Results:																
								Von Willebrand factor testing:													
Sample nr:	FFP / CSP/ Bioplasma:	Blood group (A/B/0)	Expiry date:	LOT Nr:	Test date (ADAMTS13 Ag & Activity)	ADAMTS13 Ag Normal: 0.6-1.6 µg/ml	ADAMTS13 Activity: Normal: 40-130%	Test date (VWF:Ag/ VWF:Rco/ VWF:CB)	VWF:Ag Normal: 51-143%	VWF:Rco Normal: 50-150%	VWF:Rco/ VWF:Ag ratio Normal: >0.7	VWF:CB Normal: 49-157%	VWF:CB / VWF:Ag ratio Normal: 0.6-1.6	Test date VWF multimers	Large VWF Multimers: present / decreased /absent	Intermediate VWF multimers present: / decreased /absent	Small VWF multimers: present / decreased / absent	VWF multimer pattern: normal/abnormal	Glu-plasminogen test date	Glu-plasminogen Normal: 60-250 µg/ml
1	Bio	N/A	31/3/2017	STPM0792	5/1/2016	2,05	106	7/1/2016	122	72	0,6	95	0,8	2016/12/01	present	present	present	normal	6/1/2016	141
2	Bio	N/A	31/3/2017	STPM0794	5/1/2016	1,81	70	7/1/2016	105	81	0,8	100	1	2016/12/01	present	present	present	normal	6/1/2016	121
3	Bio	N/A	31/3/2017	STPM0791	5/1/2016	1,91	90	7/1/2016	80	69	0,9	96	1,2	2016/12/01	present	present	present	normal	6/1/2016	106
4	Bio	N/A	31/3/2017	STPM0790	5/1/2016	2,27	85	7/1/2016	101	73	0.7 (0.72)	82	0,8	2016/12/01	absent	decreased	present	abnormal	6/1/2016	112
5	Bio	N/A	14/7/2017	STPM0793	5/1/2016	>2.6	97	7/1/2016	98	67	0.7 (0.68)	78	0,8	2016/12/01	present	present	present	normal	6/1/2016	143
6	Bio	N/A	31/3/2017	STPM0789	5/1/2016	2,49	85	7/1/2016	102	64	0,6	72	0,7	2016/12/01	present	present	present	normal	6/1/2016	105
7	Bio	N/A	28/7/2017	STPM0796	5/1/2016	2,6	108	7/1/2016	84	39	0,5	76	0,9	2016/12/01	present	present	present	normal	6/1/2016	117
8	Bio	N/A	21/7/2017	STPM0795	5/1/2016	>2.6	114	7/1/2016	90	54	0,6	83	0,9	2016/12/01	present	present	present	normal	6/1/2016	131
9	Bio	N/A	28/7/2017	STPM0776	5/1/2016	1,74	105	7/1/2016	89	56	0,6	74	0,8	2016/12/01	present	present	present	normal	6/1/2016	116
10	Bio	N/A	31/3/2017	STPM0772	5/1/2016	1,28	86	7/1/2016	86	55	0,6	83	1	2016/12/01	present	present	present	normal	6/1/2016	102
11	Bio	N/A	28/7/2017	STPM0782	5/1/2016	>2.6	92	7/1/2016	93	75	0,8	85	0,9	2016/12/01	present	present	present	normal	6/1/2016	97
12	Bio	N/A	28/7/2017	STPM0777	5/1/2016	1,8	96	7/1/2016	91	55	0,6	77	0,8	2016/12/01	present	present	present	normal	6/1/2016	92
13	Bio	N/A	31/3/2017	STPM0775	5/1/2016	1,8	103	7/1/2016	86	49	0,6	55	0,6	2016/12/01	present	present	present	normal	6/1/2016	94
14	Bio	N/A	31/3/2017	STPM0797	5/1/2016	1,4	62	7/1/2016	89	67	0,8	73	0,8	2016/12/01	present	present	present	normal	6/1/2016	116
15	Bio	N/A	28/7/2017	STPM0778	5/1/2016	>2.6	98	7/1/2016	73	54	0.7 (0.74)	60	0,8	2016/12/01	present	present	present	normal	6/1/2016	114
16	Bio	N/A	28/7/2017	STPM0780	5/1/2016	>2.6	127	7/1/2016	86	53	0,6	72	0,8	2016/12/01	absent	decreased	decreased	abnormal	6/1/2016	123
17	Bio	N/A	28/7/2017	STPM0781	5/1/2016	1,42	100	7/1/2016	64	55	0,9	39	0,6	2016/12/01	present	present	present	normal	6/1/2016	95
18	Bio	N/A	28/7/2017	STPM0779	5/1/2016	1,13	80	7/1/2016	64	60	0,9	38	0,6	2016/12/01	present	present	present	normal	6/1/2016	99
19	Bio	N/A	31/3/2017	STPM0787	5/1/2016	>2.6	79	7/1/2016	66	66	1	46	0,7	2016/12/01	present	present	present	normal	6/1/2016	132
20	Bio	N/A	31/3/2017	STPM0788	5/1/2016	1,4	76	7/1/2016	54	56	1	42	0,8	2016/12/01	present	present	present	normal	6/1/2016	93
21	FFP	A			5/1/2016	0,29	119	7/1/2016	31	0	0	0	0	2016/12/01	absent	absent	present	abnormal	6/1/2016	75
22	FFP	A			5/1/2016	0,23	118	7/1/2016	49	0	0	0	0	2016/12/01	absent	absent	present	abnormal	6/1/2016	114
23	FFP	A			5/1/2016	0,29	113	7/1/2016	46	0	0	0	0	2016/12/01	absent	absent	present	abnormal	6/1/2016	73
24	FFP	A			5/1/2016	0,32	99	7/1/2016	62	0	0	0	0	2016/12/01	absent	absent	present	abnormal	6/1/2016	91
25	FFP	A			5/1/2016	0,32	102	7/1/2016	28	0	0	0	0	2016/12/01	absent	absent	present	abnormal	6/1/2016	86
26	FFP	B			5/1/2016	0,24	106	7/1/2016	56	0	0	0	0	2016/12/01	absent	absent	present	abnormal	6/1/2016	110
27	FFP	B			5/1/2016	0,83	63	7/1/2016	81	66	0,8	59	0,7	2016/12/01	present	present	present	normal	6/1/2016	90
28	FFP	B			5/1/2016	1,56	70	7/1/2016	94	75	0,8	61	0,6	2016/12/01	present	present	present	normal	6/1/2016	90
29	FFP	B			5/1/2016	1,32	79	7/1/2016	95	69	0.7 (0.72)	63	0,7	2016/12/01	absent	decreased	present	abnormal	6/1/2016	81
30	FFP	B			5/1/2016	1,06	94	7/1/2016	80	47	0,6	84	1,1	2016/12/01	present	present	present	normal	6/1/2016	98
31	FFP	O			5/1/2016	0,36	>160	7/1/2016	48	0	0	0	0	2016/12/01	absent	absent	present	abnormal	6/1/2016	87
32	FFP	O			5/1/2016	0,44	135	7/1/2016	50	0	0	0	0	2016/12/01	absent	absent	present	abnormal	6/1/2016	113
33	FFP	O			5/1/2016	0,28	>160	7/1/2016	44	0	0	0	0	2016/12/01	absent	absent	present	abnormal	6/1/2016	114
34	FFP	O			5/1/2016	0,35	125	7/1/2016	63	0	0	0	0	2016/12/01	absent	absent	present	abnormal	6/1/2016	92
35	FFP	O			5/1/2016	0,27	>160	7/1/2016	65	0	0	0	0	13/1/2016	absent	absent	present	abnormal	6/1/2016	135
36	FFP	O			5/1/2016	0,47	156	7/1/2016	55	0	0	0	0	13/1/2016	absent	absent	present	abnormal	6/1/2016	88
37	FFP	O			5/1/2016	0,3	>160	7/1/2016	61	0	0	0	0	13/1/2016	absent	absent	present	abnormal	6/1/2016	121
38	FFP	O			5/1/2016	0,29	>160	7/1/2016	75	0	0	0	0	13/1/2016	absent	absent	present	abnormal	6/1/2016	134
39	FFP	O			5/1/2016	0,33	>160	7/1/2016	22	0	0	0	0	13/1/2016	absent	absent	present	abnormal	6/1/2016	159
40	FFP	O			5/1/2016	0,24	155	7/1/2016	36	0	0	0	0	13/1/2016	absent	absent	present	abnormal	6/1/2016	163
41	CSP	A			5/1/2016	0,29	119	7/1/2016	0	0	0	0	0	13/1/2016	absent	absent	present	abnormal	6/1/2016	129
42	CSP	A			5/1/2016	0,29	83	7/1/2016	0	0	0	0	0	13/1/2016	absent	absent	decreased	abnormal	6/1/2016	95
43	CSP	A			5/1/2016	1,39	75	7/1/2016	6	0	0	1	0,2	13/1/2016	absent	decreased	decreased	abnormal	6/1/2016	166
44	CSP	A			5/1/2016	0,33	82	7/1/2016	0	0	0	0	0	13/1/2016	absent	absent	absent	abnormal	6/1/2016	83
45	CSP	A			5/1/2016	1,3	93	7/1/2016	15	10	0.7 (0.66)	17	1,1	13/1/2016	absent	decreased	decreased	abnormal	6/1/2016	93
46	CSP	B			5/1/2016	0,72	79	7/1/2016	7	0	0	0	0	13/1/2016	absent	absent	decreased	abnormal	6/1/2016	194
47	CSP	B			5/1/2016	0,25	83	7/1/2016	0	0	0	0	0	13/1/2016	absent	absent	decreased	abnormal	6/1/2016	97
48	CSP	B			5/1/2016	0,62	136	7/1/2016	0	0	0	0	0	13/1/2016	absent	absent	absent	abnormal	6/1/2016	109
49	CSP	B			5/1/2016	0,2	81	7/1/2016	0	0	0	0	0	13/1/2016	absent	absent	present	abnormal	6/1/2016	116
50	CSP	B			5/1/2016	0,24	65	7/1/2016	0	0	0	0	0	13/1/2016	absent	absent	absent	abnormal	6/1/2016	71
51	CSP	O			5/1/2016	1,29	71	7/1/2016	0	0	0	0	0	13/1/2016	absent	absent	absent	abnormal	6/1/2016	84
52	CSP	O			5/1/2016	1,44	80	7/1/2016	4	0	0	2	0,5	13/1/2016	decreased	decreased	present	abnormal	6/1/2016	148
53	CSP	O			5/1/2016	0,76	60	7/1/2016	0	0	0	0	0	13/1/2016	absent	absent	absent	abnormal	6/1/2016	110
54	CSP	O			5/1/2016	1,73	71	7/1/2016	0	0	0	0	0	13/1/2016	absent	absent	absent	abnormal	6/1/2016	149
55	CSP	O			5/1/2016	1,34	66	7/1/2016	0	0	0	0	0	13/1/2016	absent	absent	present	abnormal	6/1/2016	150
56	CSP	O			5/1/2016	1,33	142	7/1/2016	0	0	0	0	0	13/1/2016	absent	absent	present	abnormal	6/1/2016	90
57	CSP	O			5/1/2016	1,35	101	7/1/2016	0	0	0	0	0	13/1/2016	absent	absent	present	abnormal	6/1/2016	104
58	CSP	O			5/1/2016	0,91	74	7/1/2016	0	0	0	0	0	13/1/2016	absent	absent	absent	abnormal	6/1/2016	89
59	CSP	O			5/1/2016	0,32	102	7/1/2016	0	0	0	0	0	13/1/2016	absent	absent	absent	abnormal	6/1/2016	95
60	CSP	O			5/1/2016	1,09	78	7/1/2016	0	0	0	0	0	13/1/2016	absent	absent	absent	abnormal	6/1/2016	95