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VON WILLEBRAND FACTOR CLEAVING PROTEASE LEVELS IN PATIENTS WITH HIV RELATED THROMBOCYTOPENIA



Dominique Gilda Garizio

A research report submitted to the faculty of Health Sciences, University of the Witwatersrand, in the partial fulfilment of the requirements for the degree

of

Master of Medicine (Haematology)

Johannesburg, 2007

Declaration

I, Dominique Gilda Garizio, hereby declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Medicine (Haematology) in the University of the Witwatersrand. It has not been previously submitted for any other degree at any other university.

This 5th day of September 2007.

.....

Dominique Gilda Garizio

Ethics Approval

University approval was obtained from the University of the Witwatersrand Medical School, and ethical approval was given by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (Protocol number M030311 and M070740).

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Dedication

To my family

Publications

Publications resulting from this MMED research report:

1. Gunther, K., Garizio, D. & Dhlamini, B. 2006. The pathogenesis of HIV-related thrombotic thrombocytopenic purpura – is it different? *ISBT Science Series,* vol. 1, pp. 246-250.
2. Gunther, K., Garizio, D & Nesara, P. 2007. ADAMTS13 activity and the presence of acquired inhibitors in HIV-related TTP. *Transfusion,* vol. 47 (9), pp.1710-1716.

Abstract

Background: Deficiency of Von Willebrand Factor Cleaving Protease (VWFCP) has been implicated as the cause of Thrombotic Thrombocytopenic Purpura (TTP). TTP is a life-threatening disease characterised by microangiopathic thrombosis due to accumulation of Ultralarge Von Willebrand Factor (ULVWF) multimers. The clinical features of TTP include microangiopathic haemolysis and thrombocytopenia. TTP is being seen with increased frequency in the context of HIV. However, in the context of HIV infection, cytopenias are often multifactorial in nature and levels of VWFCP in HIV-related thrombocytopenia have not specifically been assessed.

This study assessed VWFCP activity in the setting of patients with HIV and thrombocytopenia in the absence of TTP, in order to determine the utility of a VWFCP assay in the diagnosis of HIV-related TTP. Acquired VWFCP deficiency is generally assumed to be due to the presence of autoantibody inhibitors to the enzyme, but limited data are available regarding VWFCP activity in HIV positive TTP patients. There is also currently no assay available for measuring VWFCP activity in our laboratory.

Aim of Study: To establish a practical assay for VWFCP activity for routine use in our laboratory. The rapid collagen binding assay, based on the ELISA method of Rick, et al., 2002, was chosen. This was initially used to measure VWFCP activity in patients with HIV with and without thrombocytopenia (of any cause except TTP), in order to ascertain whether assessment of VWFCP activity is likely to be of value in facilitating early diagnosis of HIV related TTP.

The ELISA assay was performed to establish cut-off values for VWFCP in HIV negative controls and two HIV positive groups (HIV thrombocytopenia / low platelets and HIV normal platelets). Depending on the outcome of this, the assay could then be performed to assess VWFCP activity in HIV positive patients with TTP.

Methods: The rapid collagen binding assay for VWFCP activity was established and optimised for routine use in our laboratory. The cut-off values for percentage Residual Collagen Binding Activity (RCBA) in both HIV negative and HIV positive groups were identified. The assay could then be used to assess VWFCP activity in 20 HIV positive patients with TTP at the time of presentation. In patients with reduced VWFCP activity, patient plasma was mixed with normal pool plasma in a 50:50 mix, to assess for the presence of inhibitors. Correlation of VWFCP activity, inhibitors and other laboratory and clinical parameters were performed.

Results: The cut-off values for percentage RCBA in both HIV negative (<37.12%) and HIV positive (<51.51%) patients were established. The % RCBA for the HIV negative control group was statistically significantly different from the HIV positive group with normal platelets (p=0.0001) and from the HIV positive group with low platelets (p=0.0006). The cut-off value in the two HIV positive patient groups was higher than for HIV negative control patients, indicating mildly reduced VWFCP enzyme activity in HIV positive patients (regardless of the platelet count), in the absence of TTP. However, no significant difference in the cut-off value was noted between HIV positive patients with low platelet counts versus HIV positive patients with normal platelet counts (p=0.7783). The assay could therefore be used in HIV positive patients with TTP.

VWFCP activity was assessed in twenty HIV positive patients with TTP. Two groups of HIV positive patients with TTP were identified based on VWFCP activity. Six patients (30%) had normal (one borderline) VWFCP activity (RCBA <51.51%), while the remaining 14 patients had severely reduced VWFCP levels (RCBA >90%). Of the patients with reduced VWFCP activity, only 5 patients had a detectable inhibitor, while an inhibitor was not detected in the remaining 8 patients.

Conclusion: The rapid collagen binding ELISA assay is a cost effective semi-quantitative assay for the assessment of VWFCP activity. VWFCP activity in HIV positive patients appears to be slightly lower, however is not related to the platelet count. This suggests a slight

baseline deficiency of VWFCP in the setting of HIV. The baseline VWFCP cut-off value in HIV allowed assessment of HIV positive patients with TTP. The results suggest heterogeneity of VWFCP activity in HIV-related TTP. A negative result (normal VWFCP activity) does not exclude TTP in patients with HIV-related TTP and other pathogenic factors may therefore be involved.

Key Words: *Von Willebrand Factor Cleaving Protease (VWFCP); Thrombotic Thrombocytopenic Purpura (TTP); Human Immunodeficiency Virus (HIV); Von Willebrand Factor (VWF); Residual Collagen Binding Activity (RCBA).*

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List of abbreviations

RCBA	Residual Collagen Binding Activity
VWFCP	Von Willebrand Factor Cleaving Protease
ADAMTS13	A Disintegrin And Metalloprotease with ThromboSpondin motifs.
TTP	Thrombotic Thrombocytopenic Purpura
VWF	Von Willebrand Factor
HIV	Human Immunodeficiency Virus
AIDS	Acquired ImmunoDeficiency Syndrome
HAART	Highly Active Anti-Retroviral Therapy
DIC	Disseminated Intravascular Coagulopathy
HELLP	Haemolysis Elevated Liver Enzymes and Low Platelets
HUS	Haemolytic Uraemic Syndrome
LDH	Lactate Dehyrogenase
TMA	Thrombotic Microangiopathy
FFP	Fresh Frozen Plasma

1. Introduction

1.1 Background

In the context of HIV infection there are numerous causes for anaemia and thrombocytopenia (Blazes & Decker, 2004; Cines, Konkle & Furlan, 2000; Karpatkin, Nardi & Green, 2002). Thrombotic Thrombocytopenic Purpura (TTP) is one of the causes which constitutes a haematological emergency. TTP is being seen with increasing frequency in association with HIV infection (Cines, et al 2000; Karpatkin, Nardi & Green, 2002). Early diagnosis and aggressive therapy with plasmapheresis is essential for survival in this condition which used to carry a mortality of greater than 90% (Cines. et al., 2000; Rock, 2000). However, the diagnosis of TTP can be particularly difficult in the context of HIV, where numerous other causes for the cytopenias may be present.

TTP is a disease characterized by microvascular platelet aggregation and thrombus formation in selected tissue beds resulting in a pentad of: thrombocytopenia, microangiopathic haemolytic anaemia, renal failure, neurological symptoms and fever (Amorosi & Ultman, 1966; Cines, Konkle & Furlan, 2000; Moake, 2002; Rock, 2000). The differential diagnosis for TTP includes other thrombotic microangiopathies such as: Disseminated Intravascular Coagulation (DIC), Haemolysis Elevated Liver enzymes and Low Platelets (HELLP syndrome), Haemolytic Uraemic Syndrome (HUS) and Pre-Eclamptic Toxaemia (PET).

Distinction between these possibilities depends on clinical correlation and background laboratory investigations including: a full blood count, a DIC screen, LDH (lactate dehydrogenase) level, haptoglobin level, liver function tests, renal function tests (Urea and Electrolytes) and examination of the peripheral blood smear to detect red cell fragmentation suggestive of microangiopathic haemolysis.

One cannot wait for the full pentad of symptoms of TTP to develop, as early diagnosis and treatment are imperative to decrease the high mortality of this disease. However, as the therapy for TTP is very costly (approximately R10 000 per episode of therapeutic plasmapheresis), improved diagnostic tools would be of great value to confirm the diagnosis

of TTP and avoid the use of costly therapy in conditions which cause similar features to TTP, but require different management.

A possible key role for Von Willebrand Factor (VWF) in the pathogenesis of TTP was first suggested by Moake and co-workers in 1982, who postulated a deficiency of a VWFCP as the cause for the presence of Ultralarge VWF (ULVWF) multimers found in the plasma of four patients with chronic relapsing TTP (Moake, et al., 1982). The interest in TTP is likely associated to its striking presentation in previously healthy individuals, its rapid progression and often fatal clinical course and to the fact that early diagnosis can be life saving (Levy, Motto & Ginsburg, 2005).

In 2001, several groups identified ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1 motif 13) , a novel member of the ADAMTS family of metalloproteases, as the Von Willebrand Factor Cleaving Protease (VWFCP) (Furlan, Robles & Lämmle, 1996; Tsai, 1996) and deficiency of ADAMTS13 was implicated in the pathogenesis of TTP (Furlan, et al., 1998). Measuring VWFCP activity in the setting of TTP as well as other pathological conditions is a focus of substantial research (Levy, Motto & Ginsburg, 2005). A deficiency of VWFCP (either hereditary or acquired due to the presence of an autoantibody inhibitor) is present in many cases of TTP (Cines, Konkle & Furlan, 2000; Levy, et al., 2001; Ruggenenti, Noris & Remuzzi, 2001). The decreased activity of VWFCP, results in accumulation of ULVWF, which contribute to the pathogenesis of TTP by binding to platelets, causing platelet aggregates and intravascular thrombosis. This leads to tissue ischaemia and haemolysis (Levy, et al., 2001; Ruggenenti, Noris & Remuzzi, 2001).

Congenital ADAMTS13 deficiency has been shown to be on the basis of mutations in the encoding gene (Levy, et al., 2001) whilst acquired deficiency may be caused by autoantibodies (Furlan, Robles & Lämmle, 1996; Tsai, 1996). These autoantibodies may be idiopathic or arise secondary to various conditions including autoimmune disease, pregnancy, drugs and infections (Moake, 2002). The sensitivity of VWFCP deficiency for the diagnosis of TTP is somewhat controversial ranging from 33-100% in studies with similar data for

specificity (Furlan, et al., 1998; Tsai, 2003; Raife, et al., 2002). Furthermore acquired deficiency may occur in the absence of detectable antibodies (Peyvandi, et al., 2004; Zheng, et al., 2004) suggesting other factors may be important in the pathogenesis.

The occurrence of TTP in association with HIV infection is now well recognized (Hymes & Karpatkin, 1997; Blazes & Decker, 2004). The pathophysiology of TTP in this setting is not definitively known and there is limited data available regarding VWFCP activity or the presence of autoantibodies in these patients. The majority of large series assessing these parameters either do not include (Peyvandi, et al., 2004; Vesely, et al., 2003; Zheng, et al., 2004) or specifically exclude (Coppo, et al., 2005) HIV positive patients. In addition, it has been shown that levels of VWFCP may be altered in other settings in the absence of TTP (Reiter, et al., 2003; Mannucci, et al., 2001), and levels of VWFCP in patients with HIV related thrombocytopenia have not specifically been assessed.

The activity of VWFCP has been shown to be normal in many patients with a similar condition, HUS, thus indicating that evaluation of VWFCP levels may be helpful in the differential diagnosis (Levy, et al., 2001; Ruggenenti, Noris & Remuzzi, 2001). Conventional assays for VWFCP activity are expensive, cumbersome and not practical for routine use (Cines, Konkle & Furlan, 2000; Rick, et al., 2002; Aronson, Krizek & Rick, 2001; Furlan, et al., 1998; Tsai & Lian, 1998). For example, one other assay for measuring VWFCP activity, requires preparation of substrate Von Willebrand Factor which is impractical in a routine laboratory setting (Krizek & Rick 2000; Gerritsen, et al., 1999). Initial methods for assessment of the VWFCP took 24 to 48 hours and involved electrophoresis and specialized reagents (Furlan, et al., 1998; Tsai & Lian, 1998). There is currently no assay available for measuring VWFCP activity in our laboratory.

Recently, a less complicated “shorter” assay has been described, which appears to be more applicable for routine use (Aronson, Krizek & Rick, 2001; Rick, et al., 2002). This method is based on a rapid collagen binding assay for VWFCP (Aronson, Krizek & Rick, 2001; Rick, et al., 2002). This assay has been validated against a previous VWFCP assay (Aronson, Krizek

& Rick, 2001; Rick, et al., 2002) and by analysis of VWF multimer patterns. This assay yields the same diagnostic information as others, at less cost and time, without the need for a prepared substrate (Aronson, Krizek & Rick, 2001; Rick, et al., 2002).

1.2 Summary of Research Hypothesis

- To establish a practical assay for VWFCP activity for routine use in our laboratory, based on the method of Rick and co-workers (Rick, et al., 2002).

- To measure VWFCP activity in patients with HIV infection with low platelet counts (other than TTP) and normal platelet counts to determine cut-off levels for normal VWFCP activity in HIV and to assess for any significant differences from HIV negative individuals or between groups.

If the outcome of initial studies revealed:

- the cut-off value in the HIV positive patients to be similar to the HIV negative patients and not significantly different in HIV positive patients with low platelet counts (due to causes other than TTP) then this assay would be expected to be useful as a diagnostic tool in the setting of HIV positive patients with TTP. VWFCP activity could then be assessed in patients HIV-related TTP as an additional component of this MMED project.

1.3 Clinical manifestations of TTP

TTP is associated with microvascular platelet thrombi which causes microngiopathic haemolytic anaemia (with red cell fragmentation on the peripheral smear) and thrombocytopenia (Allford, et al., 2003; Wyrick-Glatzel, 2004). A shift toward earlier diagnosis has occurred and the full pentad of clinical manifestations of TTP, comprising microangiopathic haemolytic anaemia, thrombocytopenia, with associated fever, neurological symptoms and renal dysfunction may not be present (Sadler, 2006). Early recognition and treatment of TTP is critical to outcome (Wyrick-Glatzel, 2004). At presentation up to 35% of

patients with TTP may not have neurological signs or symptoms (Rock, et al., 1991). Fever and renal impairment are seen in a minority of cases (Rock, et al., 1991; Rock, et al., 1998).

A diagnosis of TTP may be made in the presence of microangiopathic haemolytic anaemia and thrombocytopenia in the absence of any other identifiable cause (Allford, et al., 2003). Neurological symptoms include headache, transient sensorimotor deficits, seizure, altered mental status, visual impairment and coma (Allford, et al., 2003; Wyrick-Glatzel, 2004). Micro-occlusive or micro-haemorrhagic vascular changes are suggested as causes for the latter symptoms (Wyrick-Glatzel, 2004). Presence of coma at presentation is considered a poor prognostic indicator (Pereira, et al., 1995; Sarode, et al., 1997). Other complications that may occur include gastrointestinal ischaemia and serous retinal detachment (Allford, et al., 2003).

Red cell haemolysis results from passage of red cells in microvasculature occluded by platelet thrombi, and is not immune in nature (Wyrick-Glatzel, 2004). Degree of haemolysis may be confirmed with elevation of LDH level, unconjugated hyperbilirubinaemia and raised reticulocyte production index. The coagulation studies are usually normal in TTP as compared to DIC (Wyrick-Glatzel, 2004). Raised D-Dimers may be found in association with TTP, in particular HIV-associated TTP (Gunther & Dhlamini, 2007).

1.4 Pathogenesis of TTP

1.4.1 Historical perspective and the role of VWFCP/ ADAMTS13

In 1924, Dr Eli Moschcowitz described a 16 year old girl who had developed acute onset of haemolytic anaemia, fever, petechiae which was soon followed by paralysis, coma and death (Moschcowitz, 1924). Most of the organs of this patient demonstrated occlusion of terminal arterioles and capillaries by hyaline thrombi without inflammation (Lian, 2005). Subsequently intraluminal thrombi were shown to comprise predominantly platelets (Gore, 1950; Feldman, et al., 1966; Asada, et al., 1985). Red cells fragment as blood flows through microvessels which are occluded by platelet thrombi, and microangiopathic haemolysis with raised LDH levels then occurs (Lian, 2005).

TTP usually occurs in adults (Lian, 2005), a paediatric form of the disease from early childhood with repeated episodes of anaemia and thrombocytopenia was reported by Schulman in 1960 and by Upshaw in 1978 and is termed Upshaw-Schulman syndrome (Schulman, et al., 1960; Upshaw, 1978; Lian, 2005). Patients with this syndrome were noted to respond to plasma infusion and Upshaw thought the disease was due to a deficiency of a plasma factor that may promote red cell and platelet survival (Upshaw, 1978).

Four patients with chronic relapsing TTP (two were brothers) with plasma deficiency of VWFCP, were described in 1997 (Furlan, et al., 1997). Subsequently it was reported that deficiency in VWFCP was due to autoantibodies in acquired idiopathic acute TTP (Furlan, et al., 1998; Tsai & Lian, 1998). A multicentre retrospective study on the prevalence of VWFCP deficiency in patients with familial and acquired TTP, confirmed an association of TTP with a hereditary as well as an acquired VWFCP deficiency (Furlan, et al., 1998). A marked decrease in VWFCP activity (<5% of normal plasma) was noted in 26 of 30 patients during an acute event (6 familial and 24 non-familial cases) and an inhibitor of VWFCP was detected in 20 of 24 patients with non-familial TTP but in none of the patients with familial TTP (Furlan, et al., 1998).

Tsai and co-workers independently confirmed a deficiency of VWFCP in TTP patients and found no activity in the plasma samples of 37 patients during the acute episode (Tsai & Lian, 1998). Immunoglobulin G antibodies with inhibitory activity against VWFCP were detected in two-thirds of samples collected during the acute event (Tsai & Lian, 1998). Inhibitors were not detected in 16 samples obtained during the remission of TTP or in plasma samples of 74 normal subjects or patients with haemolysis, thrombocytopenia or thrombosis from other causes (Tsai & Lian, 1998).

In 2001, A Disintegrin-like And Metalloprotease with ThromboSpondin type 1 motif 13 (ADAMTS13) gene was identified to code for a VWF protease protein, through the use of genetic linkage studies of affected family members (Levy, et al., 2001) or peptide sequence analysis of purified VWFCP *(*Fujikawa, et al., 2001; Gerritsen, et al., 2001; Zheng, et al.,

2001). These findings firmly establish the importance of the role of VWF and VWFCP in the pathogenesis of TTP (Lian, 2005).

As the identity of the ADAMTS13 gene was confirmed as the VWFCP (Fujikawa, et al., 2001; Gerritsen, et al., 2001), the terms VWFCP and ADAMTS13 are used interchangeably in the write-up of my MMED research report. ULVWF multimers were considered to be pathogenic in patients with chronic relapsing TTP (Moake, et al., 1982). ULVWF multimers were reported in the plasma of four patients with chronic relapsing TTP (Moake, et al., 1982) and some cases of acute TTP (Moake, et al., 1986). The investigators suggested that the accumulation of ULVWF multimers might be due to the excessive release of VWF from the endothelial cells and/or impaired degradation of the highly multimeric forms of VWF by an enzyme and as a result TTP developed (Moake, et al., 1982; Moake, et al., 1986). In the microvessels of TTP patients, an abundance of VWF with little fibrin, in platelet thrombi was described, further supporting a role for VWF in TTP (Asada, et al., 1985).

The ADAMTS13 mediated cleaving process results in the production of smaller VWF multimers that circulate after cleavage and do not induce platelet adhesion or aggregation during normal blood flow (Wyrick-Glatzel, 2004). The binding affinity of the large VWF multimers is ten times higher than that of the smaller molecular forms of VWF (Federici, et al., 1989). The ULVWF multimers are extremely adhesive due to the increased number of binding sites for platelets compared with the smaller forms (Wyrick-Glatzel, 2004).

ULVWF multimers released from endothelial cells are even more effective in inducing platelet agglutination under conditions of high shear stress than the largest forms of VWF circulating in normal plasma (Moake, et al., 1986). In the presence of normal physiology, plasma is devoid of ULVWF multimers due to cleavage by ADAMTS13 as they emerge from the endothelial cell surface (Wyrick-Glatzel, 2004). In the absence of ADAMTS13, ULVWF multimers accumulate unchecked in the circulation resulting in VWF-platelet binding and microvascular platelet thrombosis (Tsai, 2003).

Conditions that cause increased VWF secretion and decreased ADAMTS13 activity may favour the clinical entity of TTP. Deficiency of ADAMTS13 is a very strong risk factor for TTP, however the development of acute TTP requires a trigger that possibly causes activation or apoptosis of the microvascular endothelial cells (Furlan & Lämmle, 2001). ULVWF multimers are secreted and ADAMTS13 deficiency in this setting may trigger the sequence of events resulting in thrombotic microangiopathy (Furlan & Lämmle, 2001). Studies have documented that <5% normal ADAMTS13 activity is found in most patients with acute acquired TTP (Bianchi, et al., 2002). In ADAMTS13 deficiency, there is no cleavage of the ULVWF multimers as they emerge from the endothelial cells and the ULVWF multimers remain anchored to the endothelial cells forming 'beads on a string' structures (Wyrick-Glatzel, 2004).

P-Selectin molecules with transmembrane domains appear to partially mediate this anchor activity of ULVWF multimers to the endothelial cells. P-Selectin is synthesised by cells that also produce VWF and is stored in the same endothelial cell and platelet alpha granules as VWF. P-Selectin is considered to play an essential role in anchoring VWF to endothelial cells as well as in inducing platelet string detachment from the endothelial cell surface (Wyrick-Glatzel, 2004).

Circulating platelets initially adhere via the interaction of VWF with the glycoprotein Ib membrane receptor and additional platelets aggregation during blood flow via activated IIb/IIIa complexes, forming potentially occlusive microvascular platelet thrombi (Wyrick-Glatzel, 2004).

Platelet adhesion and aggregation occurs in vitro in the presence of conditions of high shear stress (Arya, et al., 2002). Many of the platelet strings may be as long as three millimetres and in the absence of ADAMTS13 activity, these platelet strings may detach from the endothelial cell and 'embolise' to blood vessels thereby causing organ ischaemia (Wyrick-Glatzel, 2004).

Refer to Figure 1 for an illustration of the pathogenesis of idiopathic TTP caused by ADAMTS13 deficiency.

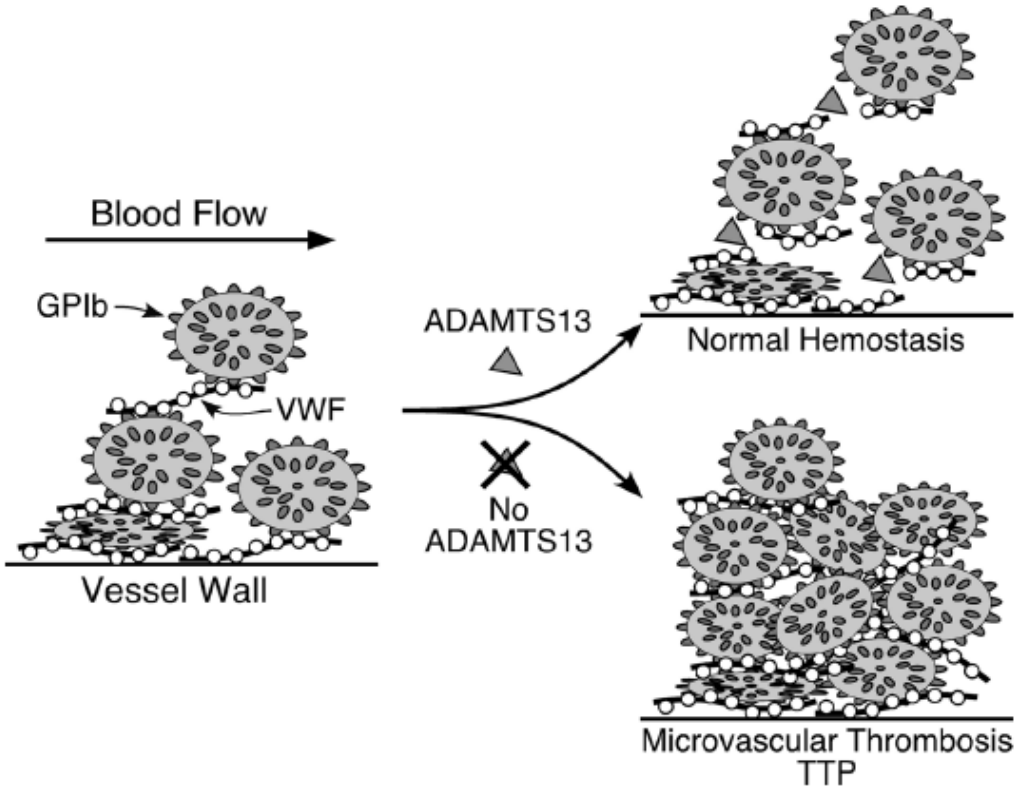

Figure 1. Schematic depiction of the pathogenesis of idiopathic TTP caused by ADAMTS13 deficiency. ULVWF multimers adhere to exposed connective tissue in vessel wall or to endothelial cells. Thereafter platelets adhere to VWF through platelet glycoprotein Ib (GPIb). In the presence of flowing blood, the VWF within the platelet rich thrombus is stretched and cleaved by ADAMTS13 and thrombus growth is limited. If there is a deficiency or absence of ADAMTS13, VWF-dependent platelet aggregation continues and eventually microvascular thrombosis with the development of TTP occurs. Diagram from: Sadler, 2006. TTP a moving target. *American Society of Haematology Education Program Book*, pp. 416.

1.4.2 Antibodies against ADAMTS13

In the literature, reported autoantibodies against ADAMTS13 in patients with acquired idiopathic TTP, ranged from 30% to 83% (Furlan, et al., 1998; Tsai & Lian, 1998; Veyradier, et al., 2001; Vesely, et al., 2003; Peyvandi, et al., 2004a; Matsumoto, et al., 2004). The autoantibodies may be IgM or IgG in nature (Furlan, et al., 1998; Rieger, et al., 2005). The autoantibodies may be transient in nature and may disappear after plasmapheresis has been performed for several days, whereas in some cases autoantibodies may persist for long

periods (Lian, 2005). In three patients with TTP, the major epitopes of antibodies were found to reside within the cysteine-rich/spacer domains (Soejima, et al., 2003).

ADAMTS13 epitope mapping of autoantibodies from 25 patients with acute TTP (severe or borderline to severe ADAMTS13 deficiency and protease inhibiting antibodies), by Klaus and co-workers, showed that all 25 patients had antibodies reacting with ADAMTS13 Cys-rich/spacer domain, 16 of the 25 plasmas reacted with the two CUB domains, 14 of the 25 plasmas with the catalytic/disintegrin/thrombospondin type 1 domains, 14 of the 25 plasmas with the first thrombospondin type 1 domain, 7 of 25 with the thrombospondin type 2 to 8 domains and 5 of the 25 plasmas recognised the propeptide (Klaus, et al., 2004).

This demonstrates that in acute TTP, antibodies to different antigenic regions of ADAMTS13 are present (Lämmle, Kremer Hovinga & Alberio, 2005). Antibodies directed against the Cys-rich/spacer domain are likely to account for inhibition of ADAMTS13 activity in static in vitro assays. Antibodies to other antigenic sites may impair ADAMTS13 interaction with endothelial cell anchored ULVWF in vivo, with such antibodies leading to the clinical entity of TTP (Lämmle, Kremer Hovinga & Alberio, 2005).

Other pathological mechanisms that do not involve the ADAMTS13-VWF interaction may also lead to a syndrome that is clinically indistinguishable from that of acquired ADAMTS13 deficiency (Lämmle, Kremer Hovinga & Alberio, 2005).

Non-neutralising IgG and IgM antibodies have been reported in one patient (Scheiflinger, et al., 2003) and are considered to influence the half-life of ADAMTS13 and cause in vivo ADAMTS13 deficiency in some TTP patients (Lian, 2005). Autoantibodies may occur due to the interaction of a susceptible host to endogenously produced triggers such as denatured proteins and cytokines from inflammation and tissue injury or to environmental factors including non-infectious and infectious agents (Lian, 2005). ADAMTS13 autoantibodies have been reported in association with clopidogrel and ticlopidine (Tsai, et al., 2000; Bennett, et al., 2000).

1.4.3 Inhibitory effect of Haemoglobin on ADAMTS13

Studt and co-workers showed that in a pre-mortem serum sample of a paediatric patient with TTP, a severe ADAMTS13 deficiency was documented and sequencing of the ADAMTS13 gene revealed a homozygous mutation confirming hereditary ADAMTS13 deficiency. However the patient's serum had a profound ADAMTS13 inhibitor effect that was not attributable to purified antibody but caused by haemolysis products present in the sample. Subsequent investigation revealed that haemoglobin had an inhibitory effect on ADAMTS13 (Studt, et al., 2005). Inhibition of ADAMTS13 activity occurred at haemoglobin concentrations that would normally be associated with strong intravascular haemolysis as found in incompatible red cell transfusion or immune-mediated haemolytic anaemia (Studt, et al., 2005). These conditions are not usually associated with TTP and it is not certain whether short-lived increase of free haemoglobin in plasma will lead to clinically relevant ADAMTS13 inhibition (Studt, et al., 2005). In this case, the serum haemoglobin concentration of 17g/l was probably due to in vitro haemolysis in stored native whole blood (Studt, et al., 2005).

Nonetheless, marked haemolysis and the presence of haemoglobin affect most current ADAMTS13 assays, lowering of values for ADAMTS13 activity should be expected under these circumstances and inhibitor screening may lead to the incorrect assumption of ADAMTS13 inhibitory antibodies (Studt, et al., 2005). However whether the capacity of haemoglobin to inhibit ADAMTS13 is of any physiological or pathophysiological relevance is still to be ascertained (Studt, et al., 2005).

1.4.4 Pathogenic mechanisms beyond deficiency of ADAMTS13 and autoantibodies

A severe deficiency of ADAMTS13 is certainly a predisposing risk factor for the development of TTP (Lian, 2005). Even in patients with hereditary TTP with a life-long severe ADAMTS13 deficiency, clinical symptoms of TTP may only develop in the second or third decade and the episodes of clinical symptoms are often associated with certain triggers such as pregnancy or surgery (Lian, 2005). In between clinical relapses of TTP in patients with chronic relapsing TTP, ULVWF multimers persist despite no other signs of disease activity, thereby suggesting that ULVWF multimers cannot be the only risk factor for microvascular thrombosis

(Galbusera, et al., 1999b). Initially Furlan and co-workers and Tsai and co-workers, reported that 83% (20 of 24) and 100% (37 of 37) of patients with acute Idiopathic TTP had severe ADAMTS13 deficiency of less than 5% (Furlan, et al., 1998; Tsai & Lian, 1998). However later studies in patients with idiopathic TTP demonstrated severe ADAMTS13 deficiency in 33% (16 of 48), 48% (48 of 100), 53% (56 of 108), 60% (56 of 93), 80% (16 of 20) and 91% (21 of 23) as reported by Vesely and co-workers, Peyvandi and co-workers , Matsumoto and co-workers, Kremer Hovinga and co-workers, Zheng and co-workers and Böhm and co-workers, respectively (Vesely, et al., 2003; Peyvandi, et al., 2004a; Matsumoto, et al., 2004; Kremer Hovinga, et al., 2004; Zheng, et al., 2004; Böhm, et al., 2005). The above observations suggest that in approximately 40% of patients with acquired idiopathic TTP, ADAMTS13 deficiency may not be the cause of microvascular platelet thrombi (Lian, 2005).

Autoantibody response could be triggered by infection, inflammation, tissue injury or drugs (Lian, 2005). The formation of microvascular platelet thrombi in patients with acquired TTP without ADAMTS13 deficiency may be caused by endothelial injury and/or platelet aggregation triggered by various stimuli that may be related to inflammation, infectious agents, immune response or chemicals (Lian, 2005). Some triggering agents may cause vascular injury/platelet aggregation and result in TTP without ADAMTS13 autoantibody formation (Lian, 2005). Lämmle and co-workers suggest that not all patients diagnosed with idiopathic TTP may have severe ADAMTS13 deficiency as measured with static protease activity assays (Lämmle, Kremer Hovinga & Alberio, 2005) as opposed to suggesting other different aetiological causes for idiopathic TTP aside from severe ADAMTS13 deficiency, as suggested by Lian (Lian, 2005).

- **Intravascular platelet aggregation secondary to substances other than VWF**

A platelet agglutinating protein was isolated from the plasma of a patient with typical manifestations of TTP, with a molecular weight of 37,000 (p37) (Siddiqui & Lian, 1986). Independent of VWF, p37 induces agglutination of homologous and autologous platelets and binds specifically to platelet CD36. Hirudin, heparin (in the presence of antithrombin) or aspirin do not inhibit platelet aggregation caused by p37 (Lian, 2005). Platelet aggregation

induced by p37 is inhibited by IgG from normal adults and from the same patient after recovery (Lian, 2005).

In two, of another four TTP plasmas possessing platelet-aggregating activity, p37 was present and it was not documented in normal controls, patients with disseminated intravascular coagulopathy or idiopathic thrombocytopenic purpura (Lian, 2005). The presence of p37 is therefore suggested in a subset of patients with TTP (Lian, 2005). Platelet aggregating factors of different molecular mass such as 900 (Lian, 1980) and 58 (Chen, et al., 1989) have been documented and the platelet aggregating factors in TTP plasma are therefore considered heterogeneous (Lian, 2005).

- **Antibodies against platelets and endothelial cells**

Several studies have investigated the mechanism of endothelial damage in TTP. In the plasma of patients with TTP and HUS, lytic anti-endothelial antibodies have been identified (Burns & Zucker-Franklin, 1982; Leung, et al., 1988; Wight, et al., 1999). In vitro, endothelial cell-reactive plasmas were found to induce human platelet aggregation (Burns & Zucker-Franklin, 1982), thereby suggesting that TTP plasma may cause both endothelial cells damage and direct thrombotic vascular occlusion resulting in TTP (Furlan & Lämmle, 2001).

Studies have assessed the presence of anti-CD36 antibodies in the setting of TTP (Tandon, Rock & Jamieson, 1994; Schultz, et al., 1998; Wight, et al., 1999). CD36, also known as platelet glycoprotein IV, is an integral membrane protein expressed by platelets and some other cells including endothelial cells (Furlan & Lämmle, 2001). Of interest is that endothelial cell CD36 expression is limited to capillary endothelial cells (Swerlick, et al., 1992). CD36 is the receptor of a previously recognised platelet-aggregating factor (the platelet-agglutinating 37kDa protein) present in the plasma of TTP patients (Lian, et al., 1991; Siddiqui & Lian, 1986). It is not known whether anti-endothelial and anti-platelet antibodies induce endothelial cell damage and apoptosis with subsequent release of ULVWF multimers or whether they are directly involved in the formation of platelet microvascular aggregates (Furlan & Lämmle, 2001).

In addition, antibodies against endothelial cells and platelets may arise secondary to immune responses to inflammatory events in TTP patients, which may lead to the exposure of crypto-antigens or the expression of cellular neo-antigens (Rock, 2000). Endothelial cell antibodies have also been documented in patients with disorders which are unrelated to TTP (Rock, et al., 1994). Another study suggested that in the setting of TTP, endothelial cell or platelet reactive antibodies were not prevalent and more than a third of reactive antibodies were human leucocyte antigen alloantibodies (Raife, et al., 1999). Of note is that complement is not depleted in the setting of acute TTP (Ucar, et al., 1994). Depleted complement levels would be expected if directly toxic endothelial cell antibodies were involved in TTP pathogenesis (Furlan & Lämmle, 2001). Therefore the contribution of endothelial cell injury mediated by antibodies as a primary aetiological factor in the setting of TTP is controversial (Furlan & Lämmle, 2001).

- **Inflammatory cytokines and apoptosis**

By electron microscopy, ultrastructural alterations in microvascular endothelial cells have been noted in TTP patients, showing swollen mitochondria and cytoplasmic vacuoles, reflecting early apoptotic lesions (Furlan & Lämmle, 2001). Even in the absence of platelet microthrombi, these lesions have been documented, suggesting that these changes are not merely a consequence of vascular occlusion (Furlan & Lämmle, 2001).

Apoptosis has been induced in human endothelial cells of dermal microvascular origin by the plasma of patients with idiopathic TTP or HIV associated microangiopathy (Laurence, et al., 1996). Detachment of endothelial cells from the renal microvasculature and detection of these cells in the circulation supports the presence of an apoptotic process (Lefevre, et al., 1993).

Plasma from patients with TTP and sporadic HUS induced apoptosis and expression of the apoptosis associated protein Fas (CD95) in microvascular endothelial cells of cerebral, renal and dermal origin but not in those of hepatic or pulmonary origin and this difference parallels the in vivo pathology of TTP/HUS whereby the pulmonary and hepatic vasculature are spared (Mitra, et al., 1997).

The primary apoptosis-inducing factors have not been identified, although a possible consideration is that impaired endothelial cell survival may be linked to loss of extracellular matrix proteins (Dang, et al., 1999). Wu and co-workers demonstrated that one of twenty TTP plasmas induced microvascular endothelial cell apoptosis in addition to platelet aggregation (Wu, et al., 1999). Jimenez and co-workers reported an increase in the number of microparticles in the blood from TTP patients and showed that TTP plasma causes endothelial cell activation instead of apoptosis (Jimenez, et al., 2003).

Cytokines may have a role in the pathogenesis and upon exposure to TNF-α, endothelial cells upregulate Fas expression (Laurence, et al., 1993). The upregulation of Fas is accompanied by induction of cell cycle protein Cdc2 in dermal microvascular endothelial cells, however not in large vessel endothelial cells (Laurence & Mitra, 1997). However, the induction of Fas by TTP and HUS plasma may be irrelevant to apoptosis and merely reflect an activated cell phenotype (Laurence & Mitra, 1997). Induction of endothelial cell apoptosis by TTP plasma was confirmed by Wu and co-workers, however Fas did not appear to be implicated (Wu, et al., 1999). Karpman and co-workers reported renal cortical cell apoptosis in children with post-enterohepatic (D+) HUS (Karpman, et al., 1998).

Interleukin-6 was documented to be increased in the serum of 33 out of 35 children with HUS and 2 out of 2 children with recurrent TTP (Karpman, et al., 1995). Karpman and co-workers also documented raised TNF-α in the serum of 7 out of 35 HUS patients and in both children with TTP (Karpman, et al., 1995).

In another study of children with HUS secondary to *E. coli* infection, Interleukin-6 (pro-inflammatory) levels were increased and the concentration of Interleukin-10 (anti-inflammatory) was also elevated to a lesser extent, and this suggests that an imbalance between pro- and anti-inflammatory cytokines may be involved in the pathogenesis of verotoxin-induced HUS (Litalien, et al., 1999). Normal human platelets were activated in vitro by Interleukin-6 (Oleksowicz, et al., 1995). Wolf and co-workers reported that calpain is involved in apoptosis-like events in washed human platelets, and although platelets contain

pro-apoptotic caspases 3 and 9, calpain and not the caspases was found to promote apoptosis-like events in platelets during platelet activation (Wolf, et al., 1999).

- **Calpain and TTP**

Calpain II comprise approximately 2% of total platelet protein and represents a major protein component of platelets. Approximately 50% of platelet calpain is present in the cytosol and approximately 50% is membrane associated (Schmaier, et al., 1990). In the presence of calcium ions, calpain becomes membrane-associated and platelet activation by thrombin results in presentation of calpain on the external platelet surface (Schmaier, et al., 1990). The calcium ion concentration in resting platelets is not sufficient for calpain activation, however the binding of an adhesive ligand to glycoprotein IIb/IIIa causes a rise in the calcium ion concentration sufficient for calpain activation (Fox, et al., 1993).

An intracellular calpain inhibitor, Calpastat ®, inhibits thrombin-induced platelet alpha-granule secretion and platelet aggregation (Croce, et al., 1999). In the sera of 15 patients with TTP, during the acute phase and not remission, a calcium-dependent cysteine protease was documented which was able to induce platelet aggregation (Murphy, Moore & Kelton, 1987). This protease cleaved glycoprotein Ib (platelet receptor for VWF), and interfered with ristocetin-induced platelet aggregation, and this was a previously reported finding for calpain purified from human platelets (Yoshida, Weksler & Nachman, 1983).

Moore and colleagues documented that calpain from human platelets degraded VWF into 205kDa and 85kDa fragments with complete loss of large VWF multimers (Moore, Murphy & Kelton, 1990). The calpain degraded VWF fragments were found to bind to glycoproteins IIb/IIIa on the surface of activated platelets and cause platelet aggregation (Moore, Murphy & Kelton, 1990).

Active calpain is associated with platelet microparticles in the plasma of TTP patients, which protects calpain from plasma inhibitors such as α-2 macroglobulin and high molecular weight kininogen (Kelton, et al., 1992). Plasma from normal subjects readily inhibits purified calpain,

however microparticle-bound calpain from TTP patients is not inhibited by normal plasma (Kelton, et al., 1992). The serum from normal control subjects lacks calpain-containing microparticles (Kelton, et al., 1992).

Calpain activity was detected in bone marrow transplant-associated TTP, with 13% of grade 1 (subclinical), 38% of grade 2 (mild), 56% of grade 3 (moderate) and 100% of grade 4 (severe) cases (Zeigler, et al., 1999). Whether calpain is associated with triggering the acute episode of TTP or whether it reflects platelet aggregation by ULVWF multimers leading to release of calpain, is still to be assessed (Furlan & Lämmle, 2001).

- **Fibrinolysis**

In TTP, at the site of microthrombi, the fibrinolytic activity of the vessel wall was shown to be depressed (Kwaan, et al., 1979). A diminished tissue plasminogen activator activity and protein C level and raised tissue plasminogen activator inhibitor with decreased fibrinolysis has been demonstrated in TTP (Glas-Greenwalt, et al., 1985). Digestion of VWF has been noted by various proteases such as calpain (Kunicki, Montgomery & Schullek, 1985), cathepsin (Jelenska, et al., 1991) and plasmin (Lian, Nunez & Harkness, 1976; Hamilton, et al., 1985). Fibrinolysis impairment and proteolysis of VWF independent of ADAMTS13 may thus increase platelet aggregation and the stability of platelet-fibrin-VWF thrombi thereby adding to the clinical picture and delay the recovery (Lian, 2005).

1. 5 Other associations with TTP

1. 5.1 Drugs

Many drugs have been associated with the development of TTP/HUS syndromes including penicillin, quinine, mitomycin C, oral contraceptives and antiplatelet drugs such as ticlopidine and clopidogrel (Wyrick-Glatzel, 2004). TTP may develop in this setting due to either an acute immune-mediated response with drug dependent antibody formation or an insidious dose-dependent toxic outcome (Wyrick-Glatzel, 2004).

A severe reduction or complete absence of VWFCP was described in seven patients with Ticlopidine associated TTP (Tsai, et al., 2000). Within 2 to 7 weeks of initiation of Ticlopidine therapy, an inhibitor of VWFCP was documented in six of these patients. The VWFCP deficiency resolved after Ticlopidine therapy had been discontinued and after plasmapheresis had been commenced (Tsai, et al., 2000). VWFCP levels were undetectable in 2 patients with clopidogrel-associated TTP during acute episodes and IgG inhibitors of VWFCP were present (Bennet, et al., 2000).

1. 5.2 Post-bone marrow transplantation

Few studies have clarified the aetiology of post bone marrow transplantation and TTP (Wyrick-Glatzel, 2004). In patients with bone marrow transplantation-associated TTP, normal VWFCP activity was found (van der Plas, et al., 1999). Schriber & Herzig, reviewed cases of post bone marrow transplant-associated TTP and revealed that fatal outcomes were observed when: TTP developed within 120 days post-transplant, patients received cyclosporine for graft-versus-host-disease, received an allogeneic transplant, had renal or neurological abnormalities (Schriber & Herzig, 1997). A cytotoxic effect on endothelial cells and an increase in VWF release is documented with the use of Cyclosporine (Wyrick-Glatzel, 2004). However many patients on clinical regimens that include immunosuppressive agents post bone marrow transplant, do not develop TTP and other factors are likely to be contributing to the development of TTP (Wyrick-Glatzel, 2004).

1. 5.3 Malignancies

Various malignancies are associated with coagulation disturbances and adenocarcinomas are well associated with TTP/HUS, in particular gastric adenocarcinoma (Wyrick-Glatzel, 2004). It is difficult to define the predisposing mechanism to TTP in malignancy especially since various chemotherapeutic agents predispose to the development of TTP (Wyrick-Glatzel, 2004). Evaluating the degree of anaemia and thrombocytopenia relative to the particular malignancy may assist in identifying and diagnosing TTP in this setting (Wyrick-Glatzel, 2004). Four patients with microangiopathic haemolytic anaemia secondary to metastasizing neoplasia were found to have a normal or subnormal VWFCP activity, as opposed to patients

with classical TTP where complete absence of VWFCP activity is noted (Furlan & Lämmle, 2001).

1.5.4 Autoimmune disease

Patients with autoimmune disease such as systemic lupus erythematosis, antiphospholipid syndrome, polyarteritis nodosa and scleroderma, may develop TTP (Wyrick-Glatzel, 2004). As patients with autoimmune disease may have multiorgan dysfunction, the pathological features may be difficult to distinguish from that of TTP, thereby complicating the diagnosis and initiation of effective treatment (Wyrick-Glatzel, 2004).

1. 6 Hereditary TTP

A number of mutations have been documented in the ADAMTS13 gene that affect the synthesis or function of ADAMTS13 resulting in a congenital form of TTP (Furlan, et al., 1997; Levy, et al., 2001). By contrast, VWF mutations have been identified that cause increased susceptibility of VWF to ADAMTS13 proteolytic cleavage, which causes loss of high molecular weight VWF multimers in plasma and defective binding of platelets. This is known as type 2A Von Willebrand disease (Tsai, et al., 1997; Haberichter & Montgomery, 2006).

Levy and co-workers identified 12 different mutations in the ADAMTS13 gene which accounted for 14 of 15 different disease alleles in families with hereditary TTP, and this study convincingly related the ADAMTS13 gene to hereditary TTP (Levy, et al., 2001). As of January 2005, 75 candidate mutations have been described in patients with hereditary TTP (Levy, et al., 2001; Kokame, et al., 2002; Antoine, et al., 2003; Schneppenheim, et al., 2003; Studt, et al., 2005; Motto, et al., 2002; Assink, et al., 2003; Savasan, et al., 2003; Bestetti, et al., 2003; Veyradier, et al., 2004; Uchida, et al., 2004; Matsumoto, et al., 2004; Pimanda, et al., 2004; Peyvandi, et al., 2004b; Kremer Hovinga, et al., 2004; Licht, et al., 2004; Snider, et al., 2004; Tao, et al., 2004; Lämmle, Kremer Hovinga & Alberio, 2005). The majority of the ADAMTS13 mutations are missense mutations, and this is followed by splice site, silent, nonsense and frameshift mutations (Levy, et al., 2001; Zheng, Majerus & Sadler, 2002; Kokame, et al., 2002). Most ADAMTS13 mutations have been identified in patients with

familial TTP. However, it is uncertain whether these mutations contribute to acquired TTP, which represent the majority of TTP cases (Shelat, Ai & Long Zheng, 2005).

ADAMTS13 mutations exhibit a heterogeneous phenotype as based on in vitro expression studies, this depends on the effect on ADAMTS13 biosynthesis, secretion, and protease bioactivity (Shelat, Ai & Long Zheng, 2005). The latter reflects the clinical heterogeneity seen in patients with hereditary TTP, most of whom have compound heterozygous ADAMTS13 mutations (Shelat, Ai & Long Zheng, 2005). Affected patients are either homozygous carriers or double heterozygotes of mutated alleles and the heterozygous parents of affected individuals are found to be consistently asymptomatic (Lämmle, Kremer Hovinga & Alberio, 2005). Only approximately a third of described mutations have been expressed in mammalian cells and most of the mutated proteins are not secreted (Kokame, et al., 2002; Uchida, et al., 2004; Matsumoto, et al., 2004; Pimanda, et al., 2004; Schneppenheim, et al., 2004). Some of the mutated proteins are secreted and these demonstrate deficient VWFCP activity (Uchida, et al., 2004; Pimanda, et al., 2004).

Half of the patients with familial TTP experience acute episodes during childhood and half during adulthood (Furlan & Lämmle, 2001). Patients presenting in adulthood may have acute episodes associated with certain triggers such as pregnancy or infection (Furlan & Lämmle, 2001). Two pairs of sisters from two families with hereditary TTP, suffered their first episode of TTP during pregnancy, whereas their brothers who were protease deficient remained asymptomatic at greater than 35 years of age (Furlan & Lämmle, 2001).

Pregnancy is a known risk factor for TTP (Fuchs, et al., 1976; George, et al., 2003) and increased VWF during pregnancy may be hypothesised as a triggering factor (Lämmle, Kremer Hovinga & Alberio, 2005).

Hereditary TTP is assumed to be generally very rare. Nonetheless, this disease may have been greatly misdiagnosed and underestimated (Lämmle, Kremer Hovinga & Alberio, 2005).

In the Japanese population, a single nucleotide change in the ADAMTS13 gene that leads to a Pro475Ser substitution, has an allele frequency of approximately 5% (Kokame, et al., 2002). Normal secretion of a dysfunctional protease was shown with expression of this mutant and the activity of the protease in a static assay was about 5% compared with wild-type ADAMTS13 (Kokame, et al., 2002). It has however not been reported as to whether homozygous carriers of the latter mutation are at risk for TTP or whether the presumably fairly low ADAMTS13 activity is completely asymptomatic (Lämmle, Kremer Hovinga & Alberio, 2005).

A single nucleotide insertion in exon 29 (4143insA), is a disease-associated mutation reported in two brothers from Sweden (Assink, et al., 2003), in 4 unrelated patients from Germany (Schneppenheim, et al., 2003) and in one patient from Australia (Pimanda, et al., 2004). An additional 7 patients with this mutation have been detected and a common founder mutation was suggested on preliminary haplotype analysis, with the possibility of originating in an ancestor near the Baltic sea region (Schneppenheim, et al., 2005).

As several patients with homozygous 4143insA mutation have been documented (apparently non consanguinous), there may be many as yet undiagnosed homozygous individuals with undiagnosed hereditary TTP and the medical profession should be aware of hereditary TTP so as to institute timeous appropriate therapy and prophylaxis in these patients (Lämmle, Kremer Hovinga & Alberio, 2005).

1.7 Differential diagnosis of TTP and related syndromes

Various different diseases may have similar features at presentation to TTP, such as HUS, Thrombotic microangiopathy in pregnancy (including Pre-eclampsia / eclampsia and HELLP syndrome: Haemolysis Elevated Liver enzymes and Low Platelets), autoimmune disorders or DIC (Allford, et al., 2003; Wyrick-Glatzel, 2004).

Different clinical variants of TTP have been documented including congenital (ADAMTS13 mutations) or acquired. Acquired forms of TTP include acute idiopathic TTP (no identifiable

precipitant), secondary TTP (to drugs, post bone marrow transplantation, in Systemic lupus erythematosis, pregnancy, malignancy or infection including HIV) or intermittent TTP with recurrent episodes of TTP at unpredictable time intervals (Allford, et al., 2003; Wyrick-Glatzel, 2004; Yarranton & Machin, 2002; Furlan & Lämmle, 2001).

Drugs associated with TTP include the oral contraceptive pill, ticlopidine, clopidogrel, cyclosporine and mitomycin C (Allford, et al., 2003; Tsai, et al., 2000; Bennet, et al., 2000; Yarranton & Machin, 2002).

Congenital TTP has been associated with ADAMTS13 mutations and may present in infancy or childhood (Upshaw-Schulman Syndrome) or adulthood (Congenital / familial chronic relapsing TTP) (Wyrick–Glatzel, 2004). Acquired TTP may be associated with ADAMTS13 deficiencies due to autoantibodies (acquired transient or recurrent / intermittent TTP), secondary to underlying diseases or idiopathic (Wyrick–Glatzel, 2004).

Clinical features and laboratory investigations are required to distinguish between the different types of microangiopathic haemolysis. These conditions exhibit a spectrum of clinical and laboratory parameters with respect to: neurological symptoms / signs, renal impairment, fever, liver impairment, hypertension, haemolysis, thrombocytopenia and coagulopathy (Allford, et al., 2003). Although overlap of clinical and laboratory parameters occurs and many conditions are associated with a multitude of the above-mentioned features, some conditions are associated with an emphasis of certain features such as renal dysfunction in HUS, liver dysfunction in HELLP syndrome, coagulopathy in DIC and haemolysis and neurological changes in TTP (Allford, et al., 2003; Wyrick-Glatzel, 2004).

Clinical and laboratory correlation is required to make the diagnosis of TTP. Although there is no ‘absolute’ criteria for the diagnosis of TTP, severe ADAMTS13 deficiency with activity of less than 5% together with severe thrombocytopenia, microangiopathic haemolysis, normal INR and PTT and correlation with clinical and other laboratory parameters, defines a diagnosis of TTP (Wyrick-Glatzel, 2004).

As per recommendation by Allford and co-workers, TTP may be diagnosed and treatment commenced if a patient present with microangiopathic haemolytic anaemia and thrombocytopenia, in the absence of any other identifiable cause clinically (Allford, et al., 2003). Routine clinical investigations recommended at presentation include: full blood count, peripheral blood smear, DIC screen, LDH level, Direct Coomb's test, urea and electrolytes, liver function tests, and urine dipstick for protein. An underlying precipitant of TTP should be considered and HIV and Hepatitis serology are recommended at presentation (Allford, et al., 2003).

1.7.1 Thrombotic microangiopathy in pregnancy

The differential diagnosis of thrombotic microangiopathy (TMA) in pregnancy includes: TTP, HUS, Pre-eclampsia / eclampsia / HELLP syndrome, DIC, acute fatty liver of pregnancy or Systemic lupus erythematosis/antiphospholipid syndrome (Allford, et al., 2003).
Pre-eclampsia may produce a TMA with a variety of clinical pictures which may be similar to TTP / HUS and the pentad of features of TTP can occur in pregnant women with pre-eclampsia (Allford, et al., 2003). HELLP syndrome is usually diagnosed in the clinical setting of pre-eclampsia with microangiopathic haemolytic anaemia and severe thrombocytopenia and HELLP syndrome usually resolves within days following delivery (Wyrick-Glatzel, 2004). Distinction between the various TMA in pregnancy remains based on history and physical examination as well as routine laboratory investigations (Allford, et al., 2003).
The management of the different TMA in pregnancy is different and distinction between the varying syndromes is essential to minimise maternal and foetal mortality (Weiner, 1987).

The clinical use of ADAMTS13 assays may bring clarity to the diagnosis and management of TMA in pregnancy (Allford, et al., 2003). However this may be complicated by the knowledge that ADAMTS13 levels are reduced in the last two trimesters of pregnancy (Mannucci, et al., 2001) with a concomitant increase in VWF (George, Vesely & Terrell, 2004).

1.7.2 Acute Idiopathic TTP

Acute Idiopathic TTP may be difficult to diagnose clinically and relapse is considered common and frequent in these cases (Wyrick-Glatzel, 2004). Many cases are due to autoantibody formation which mediates a severe ADAMTS13 deficiency (Wyrick-Glatzel, 2004).
These cases often demonstrate remission which is associated with absence of the autoantibody and normal ADAMTS13 levels and reappearance of the autoantibody often precedes clinical relapse with subsequent severe ADAMTS13 deficiency and ULVWF multimers are documented in some cases (Wyrick-Glatzel, 2004). Standard plasma exchange with replacement of fresh frozen plasma (FFP) is efficacious in these patients and this is thought to be due to this therapy removing the inhibitory autoantibody (plasma exchange) and supplying the ADAMTS13 protease (FFP) (Wyrick-Glatzel, 2004).

1.7.3 Haemolytic Uraemic Syndrome

Haemolytic Uraemic Syndrome (HUS) is characterised by the presence of microangiopathic haemolytic anaemia with thrombocytopenia and renal failure (Allford, et al., 2003). HUS may be associated with more extensive multiorgan disease, including neurological complications, liver dysfunction, enterocolitis, pancreatic and cardiac problems (Siegler, 1994). There is clinical overlap with TTP in such patients, especially when there are associated neurological problems (Siegler, 1994). It is important to distinguish between TTP and HUS, where possible, as the most appropriate clinical management can then be ascertained (Allford, et al., 2003).

HUS is a primarily renal microangiopathy which is associated with glomerular microvascular platelet aggregation and formation of fibrin polymers (Nolasco, et al., 2005). The consequences include microangiopathic haemolytic anaemia, thrombocytopenia, acute renal failure and sometimes dysfunction of other organs may occur (Moake, 2002; Nolasco, et al., 2005; Tarr, Gordon & Chandler, 2005).

HUS often occurs after an episode of haemorrhagic colitis or diarrhoea (Nolasco, et al., 2005). Microbes involved include *Shigella dysenteriae* serotype 1 or more often, specific

enterohaemorrhagic serotypes of *Escherichia coli* (O157:H7) (Cleary, 1988; Karmali, et al., 1985; Banatvala, et al., 2001; Brett, et al., 2003; Misselwitz, et al., 2003). The exotoxins produced by these bacteria include the Shiga toxin (Stx: produced by *S. dysenteriae*) or Stx-1 and Stx-2 (produced by enterohaemorrhagic *E. coli* subtypes) (Brett, et al., 2003; Misselwitz, et al., 2003; Matussek, et al., 2003; Eklund, Leino & Siitonen, 2002).

Various clinical subtypes of HUS are present including the Epidemic form (D^+) associated with a prodromal illness of diarrhoea (bloody) and the Sporadic form (D^-) (Allford, et al., 2003). In D^+ HUS, verotoxin producing organisms are identified in 90% and in the D^- HUS, verotoxin producing organisms are identified in only 2-7% and other non-verotoxin infections may be present such as HIV, *Streptococcus pneumoniae, Aeromonas hydrophilia, Campylobacter upsaliensis, Capnocytophaga canimorsus* and Cytomegalovirus (Allford, et al., 2003).
A reduced level of the third component of the complement system (C3) has been documented in the serum of patients with familial and atypical HUS (Ohali, et al., 1998; Noris, et al., 1999). The decreased serum concentration of C3 may be related to a functional abnormality or deficiency of complement factor H, an important plasma regulator of the alternative complement activation pathway (Pichette, et al., 1994; Warwicker, et al., 1998; Rougier, et al., 1998; Ying, et al., 1999). Therefore complement-mediated endothelial cell activation and subsequent release of ULVWF multimers may be hypothesised as a cause for familial and sporadic HUS (Furlan & Lämmle, 2001).

Remuzzi and co-workers, suggest that ADAMTS13 deficiency does not distinguish TTP (at least the recurrent and familial forms) from HUS and low values of ADAMTS13 may not be specific for TTP (Remuzzi, et al., 2002). Remuzzi and co-workers challenge the statement by Furlan and co-workers, that a single laboratory test for ADAMTS13 activity may be able to distinguish TTP from HUS (Remuzzi, et al., 2002; Furlan, et al., 1998; Moake, 1998) and that the protease defect should not be narrowed to specific subtypes of thrombotic microangiopathies (Remuzzi, et al., 2002). The issue of whether ADAMTS13 activity may enable clinicians to distinguish TTP from HUS remains controversial (Remuzzi, et al., 2002).

It has been proposed that TTP and HUS are merely different expressions of the same disease process (Furlan & Lämmle, 2001; Remuzzi & Garella, 1987; Ruggenenti, Remuzzi & Rossi, 1991).Furlan and co-workers recommend that a new classification should replace the inappropriate clinical discrimination between TTP and HUS (Furlan & Lämmle, 2001). TTP/HUS can be viewed as a collection of clinical and laboratory findings which occur due to a variety of unrelated causes (Furlan & Lämmle, 2001). In all forms of TMA, the primary event appears to involve the injury, death or activation of microvascular endothelial cells and the term TTP is preferred in adults with predominant neurological abnormality and cases with predominant glomerular dysfunction, occurring mainly in childhood, termed as HUS (Furlan & Lämmle, 2001).

Drummond, suggested a new classification for HUS, based on insights accumulated in the early 1980's: classic form representing cases of D^+ HUS in children, post-infectious form (following certain bacteria or viruses), immune-associated form (activation of alternative complement pathway), forms associated with other illnesses or precipitants or forms associated with pregnancy or the oral contraceptive (Drummond, 1985).

Remuzzi & Garella, proposed a modified version of this scheme: Infantile and childhood HUS/TTP (usually characterised by a diarrhoeal prodrome but not associated with a recognised infection), Hereditary and recurrent HUS/TTP (in children and adults), post-infectious HUS/TTP (occurs at any age, caused by *E. coli*, Shigella, Salmonella or Streptococcus infection), HUS/TTP accompanying systemic disease, HUS/TTP associated with pregnancy, contraceptives, cyclosporin A or antineoplastic drugs (Remuzzi & Garella, 1987).

Furlan & Lämmle, propose that a new classification should be established based on further advances and this should recognise the differences between congenital and acquired (due to autoantibodies) deficiencies of ADAMTS13 and secondary precipitating causes of TTP (such as infection, pregnancy, drugs, autoimmune disease) (Furlan & Lämmle, 2001).

1.8 Treatment of TTP

Preceding the use of plasma exchange, the mortality of patients with TTP was greater than 90% (Wyrick-Glatzel, 2004). In 1977, Byrnes & Khurana, reported that recurrence of TTP could be prevented by infusion of Fresh Frozen Plasma (FFP) or cryosupernatant without the associated use of plasma exchange (Byrnes & Khurana, 1977; Stefano, et al., 2004).

Normal ADAMTS13 activity was documented in FFP, cryosupernatant (which lacks the larger VWF multimers) and solvent/detergent-treated plasma (SD-FFP) and these components were shown to be effective in the treatment of congenital relapsing TTP (Moake, 2004). Although TTP patients have been managed by plasma infusion with or without plasma exchange, the Canadian Apheresis group demonstrated that plasma exchange was more effective than plasma infusion in the therapeutic management of TTP (Rock, et al., 1991).
Guidelines for the management of thrombotic microangiopathy have been proposed (Allford, et al., 2003).

Many studies have noted that FFP, cryosupernatant and SD-FFP contain biologically active ADAMTS13 (Wyrick-Glatzel, 2004). Plasma exchange is considered efficacious in the treatment of TTP with a significant decrease in the mortality rate to between 10% and 20% (Wyrick-Glatzel, 2004). The therapeutic effect of plasma exchange in the treatment of TTP is attributed to removing ADAMTS-13 neutralising autoantibodies and restoration of ADAMTS13 activity by replacing the enzyme (Böhm, et al., 2005).

Corticosteroids have also frequently been used in addition (Bell, et al., 1991). In conjunction with plasma exchange, corticosteroids may be used if a response is not seen with normal plasma exchange (Wyrick-Glatzel, 2004). As many patients may have auto-antibody induced ADAMTS13 deficiency, plasma exchange may remove autoantibodies, FFP replace the deficient enzyme and corticosteroids may suppress autoantibody formation (Lämmle, Kremer Hovinga & Alberio, 2005). Cryosupernatant which lacks the larger multimers of VWF, has been used instead of plasma exchange as a replacement fluid and seemed to be more effective if compared to an historical control group in which FFP was used (Rock, et al.,

1996). Although subsequent randomised studies have not substantiated the latter finding (Zeigler, et al., 2001; Lämmle, Kremer Hovinga & Alberio, 2005).

Splenectomy performed in remission, after relapse may be effective and may relate to the elimination of B-cells which are autoantibody producing (Lämmle, Kremer Hovinga & Alberio, 2005). Several patients with autoimmune-mediated ADAMTS13 deficiency and relapsing TTP, have been treated with a monoclonal anti-CD20 antibody, Rituximab ^{TM}, and successful short-term results have been reported (Chemnitz, et al., 2002; Gutterman, Kloster & Tsai, 2002; Tsai, et al., 2003; Zheng, et al., 2003b; Ahmad, et al., 2004; Sallah, et al., 2004). Prospective trials are required to assess whether Rituximab ^{TM} has a long-term advantage over the use of corticosteroids, other immunosuppressive therapy or splenectomy (Lämmle, Kremer Hovinga & Alberio, 2005).

Hereditary TTP due to double heterozygous or homozygous ADAMTS13 gene defects may be treated with FFP infusion (Furlan & Lämmle, 2001). Relapses in congenital TTP patients may be prevented by regular infusion of FFP every 2 to 3 weeks (Barbot, et al., 2001). Severe thrombocytopenia with a platelet count below 20x10^9/l together with microangiopathic haemolytic anaemia in the absence of another underlying cause, is considered sufficient for the diagnosis of TTP and to commence plasma exchange as standard therapy (Wyrick-Glatzel, 2004). Plasma exchange should ideally be commenced within the first 24 hours of presentation and therapy should continue for a few days after a normal platelet count, stable haemoglobin level, normal LDH level and normal neurologic findings have been documented, until remission has been achieved (Wyrick-Glatzel, 2004). When plasma exchange cannot be performed then the use of FFP is recommended to bridge the gap (Wyrick-Glatzel, 2004). The use of platelet infusion is contraindicated in the setting of TTP, numerous studies suggest higher morbidity and mortality rates when platelet concentrates are administered in TTP (Wyrick-Glatzel, 2004). A cohort of patients who received platelet concentrates during the course of therapy for TTP, post-mortem revealed extensive cerebral nervous system platelet aggregates (Nabban, et al., 2003).

Although plasma exchange is considered generally safe, approximately 10% of patients receiving plasma exchange may experience adverse reactions such as central venous catheter thrombosis, allergic reactions, pneumothorax, haemorrhage, infection or hypotension (Wyrick-Glatzel, 2004). Infusion of plasma containing products may result in Transfusion-related acute lung injury (TRALI) which is estimated to have an incidence of 0.02% to 0.03% per transfused plasma containing unit (Stefano, et al., 2004). When evaluating the therapeutic management of patients with thrombotic microangiopathy, these risks should be taken into consideration (Wyrick-Glatzel, 2004).

Novitsky and co-workers report that in patients with HIV, TTP is highly responsive to plasma infusion (Novitsky, et al., 2005). In HIV positive patients with TTP (n=21), Novitsky and co-workers, report that the disease presentation parameters reflect a more advanced disease stage compared to an HIV negative cohort (n=23), however the HIV positive patients had a significantly better and faster response to plasma infusion (Novitsky, et al., 2005). Novitsky and co-workers recommend, that where apheresis is not available, adequate FFP infusion should be commenced without delay and, that future prospective trials should assess whether apheresis is superior to FFP infusion in individuals with HIV-related TTP (Novitsky, et al., 2005).

At least for all patients with idiopathic TTP, plasma exchange therapy with FFP replacement remains crucial, even in the absence of a severe ADAMTS13 deficiency (Lämmle, Kremer Hovinga & Alberio, 2005). TTP patients who are plasma refractory or relapsing patients may be treated with more rigorous plasma exchange regimens, such as twice daily (Lämmle, Kremer Hovinga & Alberio, 2005).

1.9 Von Willebrand Factor Cleaving Protease / ADAMTS13

Levy and co-workers performed a genome-wide linkage analysis in patients with congenital TTP and the responsible genetic locus was mapped to chromosome 9q34 (Levy, et al., 2001). ADAMTS13 was identified as a new member of the ADAMTS family of zinc metalloproteases and deficiency of ADAMTS13 (required for proteolysis of VWF) was documented as the

molecular mechanism responsible for the development of TTP (Levy, et al., 2001). The data from a number of studies suggests that ADAMTS13 is the VWFCP itself (Levy, et al., 2001; Moake, et al., 1982; Tsai, 1996; Furlan, Robles & Lämmle, 1996; Tsai & Lian, 1998; Furlan, et al., 1998). Reported purification and sequence analysis of the amino-terminal amino acids of human VWFCP confirmed the identity of ADAMTS13 as the VWFCP (Fujikawa, et al., 2001; Gerritsen, et al., 2001).

1.9.1 Molecular biology of ADAMTS13

ADAMTS13 is a zinc-binding metalloproteinase with 1427 amino acids which forms a multidomain structure (Fujimura, 2005). The main site of synthesis of ADAMTS13 is in the liver (by hepatic stellate cells) and it is also synthesised in vascular endothelial cells (Shelat, Ai & Long Zheng, 2005). The cleavage of VWF into 140kDa and 176kDa fragments in normal plasma, not caused by plasmin or cathepsin, was first described by Berkowitz and co-workers in 1987 (Berkowitz, et al., 1987). The cleavage was shown to be at the tyrosine 1605-methionine 1606 bond (Dent, et al., 1990).

In 1996, a plasma metalloprotease was described that cleaved VWF at the same bond into 140kDa and 176kDa fragments under reducing conditions or into 200kDa and 350kDa fragments under non-reducing conditions (Tsai, et al., 1996; Furlan, Robles & Lämmle, 1996).

ADAMTS13 comprises a short propeptide, a metalloprotease domain (typical reprolysin-like), a disintegrin-like domain, first Thrombospondin type 1 (TSP1) repeat, a Cys-rich domain and a spacer domain. Seven more TSP1 repeats and two CUB domains are present in the carboxyl terminus of ADAMTS13 (Shelat, Ai & Long Zheng, 2005).

The metalloprotease domain of ADAMTS13 has three Histidine residues that co-ordinate essential Zinc or Calcium binding (Zheng, et al., 2001). Also present in the metalloprotease domain is a conserved Met249 which forms a Met-turn and the residues forming a predicted Calcium co-ordination site (Glu 83, Asp173, Cys281, Asp284) (Zheng, et al., 2001).

Refer to Figure 2 for a schematic representation of the ADAMTS13 protein.

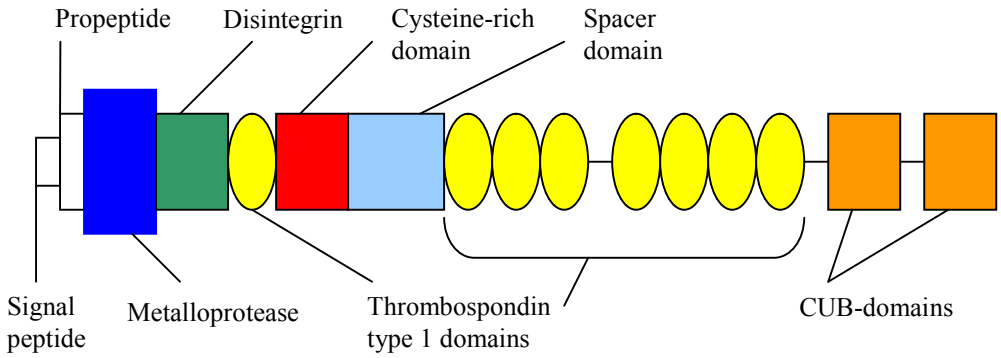

Figure 2. Schematic depiction of the ADAMTS13 protein. Adapted from: Lämmle, Kremer-Hovinga & Alberio, 2005. Thrombotic Thrombocytopenic Purpura. *J Thromb Haemost*, vol. 3, pp. 1665.

All of the proximal carboxyl terminal domains of ADAMTS13 are required for recognition and cleavage of VWF (Ai, et al., 2005). VWF73 (D1659-R1668) has been demonstrated to be the essential and minimal peptide region of VWF which is recognised and cleaved by ADAMTS13 (Kokame, et al., 2003). Proteolysis of VWF and VWF73 by various ADAMTS13 mutants revealed that each of the proximal carboxyl terminal domains of ADAMTS13 not only participate in substrate recognition but also act co-operatively to confer efficient binding (Ai, et al., 2005).

The affinity of ADAMTS13 binding to VWF73 is approximately three times higher than to VWF which suggests that the domains surrounding the central A2 subunit of VWF are involved in negative regulation of the ADAMTS13-VWF interaction (Majerus, et al., 2003; Ai, et al., 2005). Full-length ADAMTS13 can bind to VWF with stoichiometry of 1:2, with equilibrium reached at ~2 hours and a half-life of dissociation of ~4 hours (Ai, et al., 2005; Majerus, et al., 2003). The ADAMTS13 spacer domain has a significant contribution to ADAMTS13-VWF interaction (Majerus, et al., 2003).

Factors modulating VWF cleavage by ADAMTS13 include: shear stress, platelets or platelet glycoprotein-1bα (GP1bα), inflammatory cytokines such as interleukin 6, heparin sulphate or

sodium chloride (Shelat, Ai & Long Zheng, 2005). The Tyr1605-Met1606 bond in the A2 domain of VWF is buried within the core β sheet of the structure (Jenkins, Pasi & Perkins, 1998). Shear force, denaturing agents or binding of VWF to platelets, exposes the ADAMTS13 binding and cleavage site on VWF, thereby enhancing VWF cleavage by ADAMTS13 (Furlan, Robles & Lämmle, 1996; Tsai, 1996; Tsai & Lian, 1998). However, the ADAMTS13 binding site on VWF is not fully exposed in the presence of denaturing agents and the A1 domain of VWF may block access of ADAMTS13 to the cleavage site (Nishio, et al., 2004). The interaction of VWF with platelet glycoprotein 1bα or unfractionated heparin, removes this blockage (Nishio, et al., 2004).

VWF is a large multimeric protein synthesised in endothelial cells and megakaryocytes as a precursor containing a signal peptide and propeptide (Wagner, 1989). Endothelial VWF may be secreted through regulatory and constitutive pathways and VWF transported through the regulated pathway is first stored in the Weibel Palade body (Wagner, 1993). VWF mediates platelet adhesion to the subendothelium and platelet aggregation through binding platelet glycoprotein Ib under conditions of high shear stress (Ruggeri, Dent & Salvidar, 1999). Subsequent to secretion, multimeric VWF is gradually but continuously cleaved to smaller multimers in the circulation (Tsai, 1989; Dent, et al., 1990; Dent, Galbusera & Ruggeri, 1991; Furlan, et al., 1993). VWF is rapidly cleaved by ADAMTS13 under conditions of high shear stress (which probably exposes the cleavage site), however is resistant to cleavage under static conditions (Haberichter & Montgomery, 2006). In the normal degradation process of VWF multimers, the role of ADAMTS13 is not as yet completely distinct (Haberichter & Montgomery, 2006).

Under conditions of high fluid shear stress, VWF unfolds and becomes more adhesive, this is possibly related to tensile force causing unfolding of the A2 domain of VWF and exposure of the otherwise hidden Tyr1605-Met1606 cleavage site which is then accessible to ADAMTS13 for cleavage (Tsai, 1996; Goto, et al., 1998; Siedlecki, et al., 1996).

VWF cleavage by ADAMTS13 is therefore favoured by the relatively high hydrodynamic forces generated when platelets adhere to VWF on endothelial cells (Dong, et al., 2003) or in platelet aggregation (Ajzenberg, et al., 2002; Yagi, et al., 2001). ADAMTS13 binds to the A1 and A3 domains of VWF thereby docking the enzyme in close proximity to the A2 domain to facilitate VWF cleavage (Lopez & Dong, 2004). Larger VWF multimers possess higher affinity receptors for platelet receptors glycoprotein Ib and glycoprotein IIb/IIIa (Federici, et al., 1989), are unfolded more easily under conditions of high shear stress (Tsai 2002) and extend longer so as to bind more platelets (Lian, 2005). High shear stress is observed particularly in pathologically narrowed arteries and arterioles and the unfolded VWF which is unchecked in TTP, easily binds platelets and the platelet aggregates formed, sludge in the arterioles and capillaries as platelet thrombi (Lian, 2005).

Approximately 90% of VWF in a resting solution is present in the form of linear polymers coiled upon themselves in a 'ball of yarn' (globular) form, as shown with electron microscopy (Slayter, et al., 1985). VWF undergoes a conceivably shear-induced conformational transition from a globular state to an extended chain conformation thereby exposing intramolecular globular domains (Siedlecki, et al., 1996). It has been shown that the binding of platelet glycoprotein Ibα the A1 domain of VWF, stimulates the adjacent A2 domain cleavage by ADAMTS13 (Nishio, et al., 2004).

ADAMTS13 functions to regulate the adhesive action of VWF and ADAMTS13 and shear stress regulate VWF activity (Tsai, 2003). VWF mediates initial platelet adhesion to damaged subendothelium in conditions of high shear stress (Furlan & Lämmle, 2001). Brief exposure of VWF to conditions of high shear stress increases the ability of VWF to support platelet aggregation (Tsai, 2002). The conformational response of VWF is size dependent with only the large multimers demonstrating increased activity in response to conditions of high shear stress (Tsai, 2002).

Refer to Figure 3 for a diagrammatic representation of a model for the pathogenesis of TTP with representation of the globular versus the linear from of ULVWF multimers.

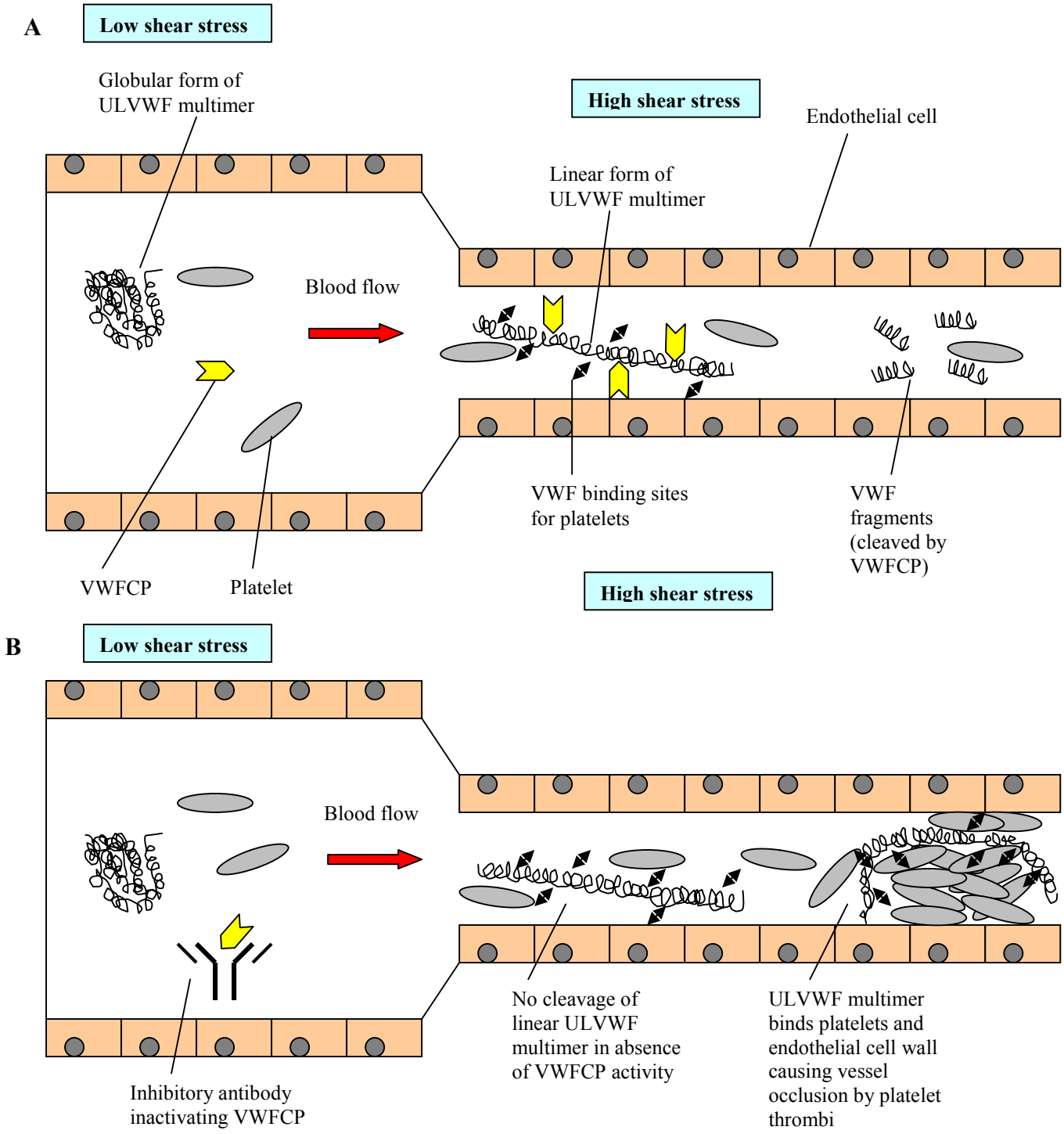

Figure 3. Model for the pathogenesis of TTP. Adapted from: Yarranton & Machin, 2002. Thrombotic Thrombocytopenic Purpura: New Approaches to Diagnosis and Management. *Blood Therapies in Medicine,* Vol 2 (3), pp. 86. **A** reflects normal processing, ULVWF (Ultralarge VWF) multimers are secreted by endothelial cells and circulate in globular formation in large vessels. Under conditions of high shear stress however, the ULVWF multimers unfold to a linear form and expose cleavage sites for VWFCP and platelet binding sites. Less biologically active smaller VWF fragments are generated (ULVWF multimers cleaved at Met-842 and Tyr-843 bond). **B** reflects acquired TTP, where an autoantibody (Immunoglobulin G) inactivates VWFCP. Under conditions of high shear stress there is no processing of ULVWF multimers which then readily bind both platelets and the endothelium. Small vessel occlusion by platelet-rich thrombi then occurs.

1.9.2 Regulation of ADAMTS13 activity

The cleavage of ULVWF multimers is inhibited by Interleukin-6 under flow (Bernardo, et al., 2004) suggesting a possible link between inflammation and coagulation (Shelat, Ai & Long Zheng, 2005). Inflammatory cytokines such as Interleukin-8 and Tumour Necrosis Factor-alpha may stimulate the release of ULVWF multimers (Bernardo, et al., 2004). Chloride ions (De Cristofaro, et al., 2005) and haemoglobin (Studt, et al., 2005) may also regulate the ADAMTS13-VWF interaction. Data by Crawley and co-workers, suggests that ADAMTS13 activity is regulated at the site of thrombus formation by Thrombin, Factor Xa and Plasmin, by proteolytic cleavage (Crawley, et al., 2005). As described by Crawley and co-workers, ADAMTS13 circulates as an active enzyme when secreted into plasma. VWF binds to exposed collagen at sites of endothelial injury and then VWF unfolds in response to shear stress, thereby facilitating platelet tethering which is required for platelet plug formation (Crawley, et al., 2005). If ADAMTS13 were to act unchecked, its VWFCP activity would predictably oppose the crucial tethering of platelets to VWF (Crawley, et al., 2005). The proposed regulatory mechanism of Crawley and co-workers, suggests that ADAMTS13 could be proteolytically inactivated by thrombin at sites of vascular injury, thereby promoting a prothrombotic milieu at a site of vascular perturbation (by inhibiting ADAMTS13, VWF molecules remain intact promoting coagulation) (Crawley, et al., 2005).

In initial platelet plug formation, platelets adhere to both VWF and collagen and the tight association with collagen may assist in the initial resistance of VWF cleavage by ADAMTS13 with subsequent platelet release. However the association of platelets with collagen will be more remote in subsequent layers of the platelet plug (Crawley, et al., 2005).

Imaging of in vivo thrombus formation revealed “blebbing-off” of platelets tethered from the back of the developing thrombus prior to thrombin generation or the deposition of fibrin (Falati, et al., 2002). The latter observation may be explained by the uninhibited action of ADAMTS13 on VWF-bound platelets and highlights the necessity of inhibition of ADAMTS13 at a site of endothelial damage (Crawley, et al., 2005).

Multiple physiological roles and substrates are noted for the Thrombin molecule (Chang, 1985; Di Cera, 2003). After coagulation has been initiated Thrombin has a procoagulant role by cleaving Fibrinogen, by feeding back positively on the coagulation cascade and by activating tethered platelets (Narayanan, 1999).

Thrombin activates clotting factors V, VIII, X and XI (Di Cera, 2003). A novel procoagulant function of Thrombin is described by Crawley and co-workers, which involves ADAMTS13 inactivation which promotes thrombus growth (by preventing cleavage of VWF) (Crawley, et al., 2005).

There has been possible implication of a role of ADAMTS13 deficiency in the pathogenesis of certain thrombotic disorders (Böhm, et al., 2003a; Gao, et al., 2003). However, Crawley and co-workers suggest that during the course of a thrombotic event, increased thrombin-dependent inactivation of ADAMTS13 may occur and result in reduced ADAMTS13 levels. Therefore additional studies would be of value in determining whether a partial ADAMTS13 deficiency is a primary cause or a consequence of thrombosis (Crawley, et al., 2005).

There is physiological modulation of the activity and specificity of thrombin by cofactors which compete for exosite binding, thereby directing either a procoagulant or anticoagulant effect of thrombin (Philippou, et al., 2003). When thrombin binds thrombomodulin on intact endothelium (Thrombin exosite 1 occupied with Thrombomodulin), it has an anticoagulant effect by activating the Protein C pathway, thereby restricting the procoagulant effects (such as fibrinogen cleavage) to the site of vascular injury (Sadler, 1997; Ye, Esmon & Johnson, 1993).

Crawley and co-workers documented that when Thrombin was preincubated with Thrombomodulin, its ability to proteolyse ADAMTS13 was significantly reduced and Thrombin exosite 1 may therefore be implicated in the binding of ADAMTS13 (Crawley, et al., 2005). Crawley and co-workers noted that Plasmin cleaved ADAMTS13 at a faster rate than Thrombin. Plasmin mediates physiological fibrinolysis and is generated subsequent to

thrombin-dependent fibrin deposition (Collen, 1999). As ADAMTS13 is cleaved at low plasmin concentrations, this may suggest a physiologically important role of modulating ADAMTS13 activity at a site of haemostatic plug formation (Crawley, et al., 2005). Plasmin activity is associated with post-injury vessel repair and as there may be a possible role for VWF in tissue repair, the cleavage of ADAMTS13 by Plasmin would prevent cleavage of VWF (Crawley, et al., 2005). Although Plasmin activates several prometalloproteinases, it inactivates ADAMTS13 (Murphy, et al., 1999).

The data from Crawley and co-workers suggests that Thrombin-dependent inactivation of ADAMTS13 may be specifically localised to the site of haemostatic plug formation. The ADAMTS13 activity does occur when Thrombin is bound to Thrombomodulin which constitutes an anticoagulant milieu where ADAMTS13 activity (cleavage of VWF) assists in limiting platelet aggregation from spreading beyond the site of injury (Crawley, et al., 2005). On damaged endothelium, the absence of Thrombomodulin allows Thrombin to bind (proposed via exosite 1), and inactivate ADAMTS13, thereby facilitating the initial platelet adhesion process (Fujimura, 2005).

Platelet adhesion to a site of vascular injury occurs as a two-step process with initial platelet rolling which is mediated by a transient interaction between platelet glycoprotein 1b-IX-V and surface immobilised VWF (which binds exposed collagen at sites of endothelial injury). Subsequently firm platelet adhesion occurs following the binding of platelet integrin αIIbβ3 (Savage, Salvidar & Ruggeri, 1996).

Matsui and co-workers analysed thrombus formation in vitro on a collagen surface under conditions of high sheer stress and the interaction of VWF and fibrinogen (Matsui, et al., 2002). This study revealed that during initial thrombus formation, platelet thrombi are composed of VWF and platelet-derived fibrinogen accumulates mainly inside the growing thrombus thereby serving as a core adhesive ligand, however the thrombus surface exposed to blood flow, is constantly occupied with VWF (Matsui, et al., 2002).

The ability of VWF to bind thrombi that are forming depends on its multimeric size (with multimers ranging into millions of daltons). The multimeric state of VWF is regulated by both ADAMTS13 cleavage in the circulation and the number of N-terminal intersubunit disulfide bonds formed in the Golgi complex (Yarranton & Machin, 2002; Sadler, et al., 2004). ADAMTS13 cleaves ULVWF multimers which are hyperactive, to less active smaller forms (Bernardo, et al., 2004).

VWF is synthesised and stored in the Weibel Palade bodies of endothelial cells and the alpha granules of megakaryocytes and platelets (Wagner & Marder, 1983; Ruggeri & Ware, 1992; Sadler, 1998). The main source of plasma VWF is endothelial cells, which release VWF multimers on stimulation as well as constitutively (Sadler, 1998; Tsai, et al., 1991). VWF forms released upon stimulation are usually rich in the hyperactive ultralarge multimers (Tsai, et al., 1991; Moake, et al., 1982). In the absence of any modulators, the ultralarge multimers form high strength bonds with platelet glycoprotein 1b-IX-V (Arya, et al., 2002; Moake & Chow, 1998; Federici, et al., 1989).

Dong and co-workers showed that the ULVWF multimers secreted from endothelial cells are anchored to the cell surface and form long string-like structures which can induce platelet adhesion and aggregation (Dong, et al., 2002). In vitro, the ULVWF multimer strings are cleaved at more than 1000 fold faster than under static conditions which may imply that in vivo the ULVWF multimer processing occurs on or near the surface of endothelial cells (Bernardo, et al., 2004).

The proteolytic cleavage of ULVWF multimers is in a constant state of homeostatic balance between the proteolytic capacity of ADAMTS13 and the amount of ULVWF multimers released from the endothelium (Bernardo, et al., 2004). Pathological conditions may arise from disruptions of this balance and range from bleeding to thrombosis. Sustained deficiency of ADAMTS13 may be congenital or acquired and results in microangiopathic haemolysis, such as TTP (Moake, 2002), whereas bleeding disorders such as type 2A Von Willebrand disease is associated with increased proteolysis (Batlle, et al., 1994).

The ULVWF / ADAMTS13 control mechanism may also be affected by rapid or excessive release of ULVWF multimers or by a transient inhibition of ADAMTS13 mediated cleavage of ULVWF multimers (Bernardo, et al., 2004). Reported increased levels of VWF occur in a variety of disease entities such as autoimmune diseases (McEntegart, et al., 2001; Porta, Caporali & Montecucco, 1999), infections both bacterial and viral (Kayal, et al., 1998; Tzavara, et al., 1997), trauma (Siemiatkowski, et al., 2000) and peripheral arterial and coronary disease (Jager, et al., 1999, Nielsen, Siegbahn & Swahn, 2000). In these conditions, Inflammation may be the shared cause for release of endothelial cell derived ULVWF multimers which may lead to a consumptive deficiency of ADAMTS13 (Bernardo, et al., 2004).

1.10 Overview of causes of thrombocytopenia in HIV

One of the most common haematological manifestations of HIV infection is thrombocytopenia which may be multifactorial in nature, however it is frequently attributed to the direct effects of HIV (Blazes & Decker, 2004; Sloand, 2005). Thrombocytopenia is also frequently due to immune-related thrombocytopenia (Blazes & Decker, 2004; Miller, et al., 2005). Other causes of thrombocytopenia to consider in HIV include: HIV associated decreased production (direct infection of megakaryocytes), drug-related causes, hypersplenism, marrow infiltration or other infections including opportunistic infections (Geethesh, et al., 2005; Sloand, 2005). TTP, a form of thrombotic microangiopathy (TMA), is another cause for thrombocytopenia. Although TMA is a less common entity than other causes for thrombocytopenia, it is a serious and life-threatening entity and clinicians must be aware of the diagnostic and therapeutic importance (Blazes & Decker, 2004).

TTP is associated with a pentad of features including microangiopathic haemolytic anaemia, thrombocytopenia, fever, neurological dysfunction and renal impairment.

The differential diagnosis of thrombocytopenia, anaemia and neurological dysfunction is broad in patients with Acquired ImmunoDeficiency Syndrome (AIDS) (Cines, Konkle & Furlan, 2000). DIC is a form of microangiopathic haemolysis which must be distinguished from TTP

or HUS and a DIC screen is of value in assessing for the presence of a consumptive coagulopathy.

1.11 TTP and HIV

Literature for HIV-associated TMA is limited. TTP may be the presenting clinical manifestation of HIV infection, although the majority of cases occur in the context of AIDS (Hymes & Karpatkin, 1997). The outcome and survival of TTP in the setting of HIV may be related to the stage of HIV infection and severity of immunosuppression at which TMA occurs (Bell, Chulay & Feinberg, 1997; Blazes & Decker, 2004; Hymes & Karpatkin, 1997; Ripamonti, et al., 1996). Early diagnosis and treatment of TTP is imperative for survival (Cines, Konkle & Furlan, 2000; Rock, et al., 2000).

Blazes and Decker report that the CD4 count at the time of illness appears to affect duration of response to therapy (patients with CD4 counts greater than 100cells/mm^3 had a mean survival of 2 years, whereas those with CD4 counts less than 100cells/ mm^3 had mean survivals of 6 months) (Blazes & Decker, 2004).

A study by Tamkus and co-workers, showed that if plasma exchange is instituted early, clinical remission may be seen in half of HIV positive patients with TMA, although in-patient mortality reached almost 50% in this study (Tamkus, et al., 2006). Early diagnosis and treatment with plasma exchange may be lifesaving in TMA, but the overall prognosis in patients with advanced HIV (with low CD4 counts) who develop TMA is generally considered poor (Bell, Chulay & Feinberg, 1997; Blazes & Decker, 2004; Gadallah, et al., 1996; Gervasoni, et al., 2002). Bell and co-workers identified a group of HIV positive patients with a *TMA-like* syndrome who when compared with previously reported patients with HIV-associated TMA, had lower CD4 counts, more frequent opportunistic infections, less severe thrombocyopenia, less frequent neurological abnormalities and a poor response to therapy appropriate for TMA (Bell, Chulay & Feinberg, 1997) . Potential risk factors for the development of a TMA-like syndrome identified by Bell and co-workers include the use of high dose valacyclovir and the relative risk may be increased in patients receiving fluconazole

(Bell, Chulay & Feinberg, 1997). Bell and co-workers suggest that the short term prognosis of patients with a TMA-like syndrome seems to be better in patients with neurological manifestations, possibly because such patients are more likely to have classical TMA that is responsive to plasma therapy (Bell, Chulay & Feinberg, 1997).

TTP is reported as a relatively rare complication in HIV in the United States in patients taking Highly Active Anti-Retroviral Therapy (HAART), but is seen frequently in the developing world where these drugs are not available (Sloand, 2005). Tamkus and co-workers used a dyad of diagnostic criteria (microangiopathic haemolytic anaemia with fragments on the peripheral smear and thrombocytopenia) to enroll 17 consecutive patients with HIV and TMA (1 HUS, 2 TTP/HUS and 14 TTP). The latter study reported these patients with TMA and HIV, to have advanced HIV infection, as assessed by lower CD4 counts (median=28cells/ mm^3) and higher HIV-1 RNA levels (Tamkus, et al., 2006).

It has long been proposed that HIV infection predisposes to TTP and other TMA (Boccia, et al., 1984; Jokela, Flynn & Henry, 1987). There has been a marked increase in cases reported per year since the early 1990's correlating with the AIDS pandemic (Torok, Holman & Chorba, 1995) and several early case series of reported AIDS cases support this (Hymes & Karpatkin, 1997; Leaf, et al., 1988; Ucar, et al., 1994). Leaf and co-workers documented 4 of 14 patients (between 1985-1987) with TTP to be HIV positive (Leaf, et al., 1988). Ucar and co-workers, noted 7 of 50 patients (from 1979-1990) with TTP to be HIV positive (Ucar, et al., 1994). Subsequently a series by Hymes and Karpatkin documented the percentage of patients with TTP and HIV (between 1990 to 1996) to be over 50% (Hymes & Karpatkin, 1997).
Although some acquired TMA appear to be either idiopathic in nature or secondary to direct effects of HIV infection, there are some reports of co-existing AIDS-defining illnesses in these patients (such as Kaposi sarcoma, cytomegalovirus, cryptococcal meningitis, *Pneumocystis carinii* pneumonia) (Avery, Denuzio & Craig, 1998; Chu, et al., 1995; Leaf, et al., 1988; Thompson, et al., 1992). Medications such as fluconazole, acyclovir and valacyclovir which are used in the management of HIV positive patients have been associated as causal of TMA syndromes (Bell, Chulay & Feinberg, 1997).

The frequency of TMA appears to be even greater in those with more advanced HIV infection and lower CD4 counts (Peraldi, et al., 1995; Ripamonti, et al., 1996; Tamkus, et al., 2006). A prospective study of 350 HIV positive patients, revealed an incidence of TMA of 7%, median CD4 counts were 36cells/mm^3, median HIV-1 RNA levels were 94,000 copies/ml and 72% of patients presented with AIDS-defining illness (Moore, 1999).

Tamkus and co-workers suggest considering TMA as an AIDS-defining illness in order to facilitate early recognition and effective treatment so as to improve the survival of this life-threatening but treatable condition (Tamkus, et al., 2006). In 1984, an association was first documented between HUS and HIV (Boccia, et al., 1984). HUS is a thrombotic microangiopathy affecting glomeruli primarily, however the presenting features of HUS and TTP may be difficult to distinguish (Tamkus, et al., 2006).

Early diagnosis of TTP is imperative for the immediate institution of life-saving plasma exchange/ plasma infusion, however the diagnosis of TTP is complicated in the setting of HIV where numerous other causes of thrombocytopenia may be present. As previously mentioned, other causes for thrombocytopenia to consider in HIV include: defective megakaryopoiesis with direct infection of megakaryocytes, sepsis, infections, antibody-mediated platelet destruction, drugs, lymphoma and haemophagocytosis (Sloand, 2005; Tamkus, et al., 2006).

A high index of suspicion is required for TTP in the setting of HIV in particular, where numerous other causes for the cytopenias may be present. In addition several AIDS-related infections and neoplasms have been shown to be associated with TMA, therefore the cause for the TMA may be difficult to distinguish in HIV positive patients (Tamkus, et al., 2006).

In the presence of anaemia and thrombocytopenia, microangiopathic haemolysis (DIC/TTP/HUS) must be excluded, which is detected by the presence of significant red cell fragmentation on the peripheral smear with other features of haemolysis (such as a high LDH level, raised Reticulocyte Production Index, unconjugated hyperbilirubinaemia, reduced

Haptoglobin level). As the cytopenias in HIV positive patients are often multifactorial in nature, assessment of VWFCP levels would be of value, not only as a baseline in HIV-infected individuals without TTP, but also in HIV positive patients with TTP. VWFCP inhibitor titres may help predict patients with a high possibility of relapse of TTP and these patients may benefit from more aggressive therapy and antiretroviral therapy (Tamkus, et al., 2006).

The effect that HAART will have on the incidence of TMA in the setting of HIV positive patients is not yet evident, but will probably be of significance (Blazes & Decker, 2004). Gervasoni and co-workers prospectively assessed the incidence of TMA syndrome in 347 patients with AIDS during the period 1997 to 2000, during the HAART era, and as no cases of TMA syndrome were observed during this time, they consider that antiretroviral therapy significantly reduced the risk of TMA syndrome development (Gervasoni, et al., 2002). Becker and co-workers revealed that TMA, in a cohort after introduction of HAART, was rare and was associated with HIV infection that was advanced (Becker, et al., 2004).

Miller and co-workers documented relapse of TTP in two patients who discontinued HAART against medical advice, possibly suggesting a causal role for HIV in this setting (Miller, et al., 2005). Any other possible underlying conditions that may have precipitated TMA should also be treated (Blazes & Decker, 2004). Complete deficiency of VWFCP and the presence of a concentration-dependent IgG inhibitor was reported in one patient with HIV-associated TTP (Sahud, et al., 2002).

1.12 Diagnostic utility of ADAMTS13 activity and inhibitor assays

Severe deficiency of ADAMTS13 is considered the primary cause of congenital and acquired TTP (Levy, et al., 2001; Tsai & Lian, 1998; Moake, 2002; Furlan, et al., 1998). ADAMTS13 activity is reported as 50-178% in normal plasma (Veyradier, et al., 2001). Severe deficiency of VWFCP activity (less than 5%) is a key finding confirming the diagnosis of TTP, whereas mild to moderate deficiency is found in various other conditions (Studt, et al., 2003). A universal cut-off value of ADAMTS13 activity for the diagnosis of TTP or ADAMTS13 deficiency is still to be established (Kokame, et al., 2005).

There have been debates in the literature on the specificity of reduced ADAMTS13 levels for the diagnosis of TTP (Mannucci, 2003). Tsai, takes the strongest standpoint that profound ADAMTS13 deficiency (less than or equal to 0.1 UmL $^{-1}$) is specific for TTP (Tsai, 2003). Tsai, explains that other studies may report different findings due to variation in the methods of ADAMTS13 activity measurement and in some studies the definition of TTP and HUS is determined by referring physicians whose criteria of case definition are not specified (Tsai, 2003).

Current assays result in different ranges of values among normal individuals and reported results are not comparable due to different assay methods and case definition (Tsai, 2003). On the other hand, Remuzzi, highlights that a substantial proportion of adult cases with TTP in two different series have normal ADAMTS13 levels (Remuzzi, 2003), ranging from 30% (Veyradier, et al., 2001) to 38% (Raife, et al., 2002). Remuzzi, also discusses that undetectable levels of ADAMTS13 were documented in two adult patients with familial HUS (Remuzzi, et al., 2002), in 4 adults with secondary forms of HUS (Veyradier, et al., 2001), in one adult with post-diarrhoeal HUS (Veyradier, et al., 2001).

However, TTP and HUS are often regarded as variants of one syndrome denoted as TTP/HUS (Furlan & Lämmle, 2001). Furlan and Lämmle, demonstrated that most if not all patients with classic TTP have severe ADAMTS13 deficiency, but not all individuals with congenital ADAMTS13 deficiency develop acute TTP (Furlan & Lämmle, 2001).
An acute event of TTP in a patient with congenital ADAMTS13 deficiency tends to be followed by a chronic and relapsing form of TTP (Furlan & Lämmle, 2001). The prevalence of congenital ADAMTS13 deficiency may be under-diagnosed as some severe ADAMTS13 deficiencies remain undetected in asymptomatic subjects. It is also possible that individuals with acquired ADAMTS13 deficiency may also escape detection as the autoantibodies against ADAMTS13 may disappear before the development of an acute episode of TTP (Furlan & Lämmle, 2001). As reported by Furlan and co-workers, a patient with acquired TTP had his first relapse 3 months subsequent to the antibody-mediated decrease of the protease,

thereby suggesting that a specific pathophysiological condition was required to trigger commencement of the acute episode (Furlan, et al., 1998).

Furlan and co-workers, have also observed that in patients with acquired TTP, remission may occur prior to the complete normalisation of ADAMTS13 and disappearance of the inhibitor (Furlan, et al., 1998). These considerations of acute TTP in severely ADAMTS13 deficient individuals may be paralleled with patients with genetic predispositions to venous thrombophilia (such as Factor V Leiden or deficiencies of Protein C and Protein S) or acquired risk factors (such as oral contraceptives, antiphospholipid antibodies, pregnancy, trauma, malignancy, previous thrombotic episodes), where a single risk factor may not be sufficient to cause thrombosis (Furlan & Lämmle, 2001). However, if several risk factors accumulate then the chance of a thrombotic event increases and the recurrence rate is high thereafter (Furlan & Lämmle, 2001). It is not certain why some individuals with severe congenital ADAMTS13 deficiency experience episodes of acute TTP in childhood, whilst others remain asymptomatic for decades (Furlan & Lämmle, 2001).

ADAMTS13 deficiency is evidently a very strong risk factor for TTP, however the occurrence of an acute microangiopathy requires a trigger, such as alcohol abuse, pregnancy or bacterial or viral infection (Furlan & Lämmle, 2001). In general it is considered that this trigger results in activation or death of the microvascular endothelial cells, thereby resulting in the release of ULVWF multimers from the storage organelles (Furlan & Lämmle, 2001).

1.12.1 VWFCP levels may be altered in settings other than TTP

Mild to moderate reduction in ADAMTS13 levels is reported in other conditions such as inflammation, coronary artery disease (Yoo, et al., 2003), disseminated intravascular coagulopathy (Loof, van Vliet & Kappers-Klunne, 2001), pregnancy, liver disease (Mannucci, et al., 2001), sepsis, heparin-induced thrombocytopenia (Bianchi, et al., 2002) and in patients with metastasizing and malignant tumours (Böhm, et al., 2003b; Oleksowicz, Bhagwati & DeLeon-Fernandez, 1999).

Mannucci et al measured VWFCP levels in 177 control subjects of different ages, in 26 full-term newborns and in 69 pregnant women (Mannucci, et al., 2001). In addition, VWFCP levels were assessed in 42 patients with decompensated liver cirrhosis, 63 patients with chronic uraemia, 15 patients with acute inflammatory states and 24 patients in the post-operative setting (Mannucci, et al., 2001).

VWFCP levels were found to be low in newborns, however levels normalised at 6 months of age and VWFCP levels were lower in the last two trimesters of pregnancy compared to the first trimester (Mannucci, et al., 2001). VWFCP levels were also found to be low in patients with cirrhosis, uraemia, acute inflammation and levels fell in the post-operative period (Mannucci, et al., 2001). Mannucci and co-workers concluded that low VWFCP levels are not specific to TTP as low levels are also found in several physiological and pathological conditions (Mannucci, et al., 2001).

Moore and colleagues also demonstrate reduced ADAMTS13 activity in 6 of 20 patients with Idiopathic Thombocytopenic Purpura (ITP), 5 of 10 patients with Systemic Lupus Erythematosis (SLE), 6 of 10 patients with DIC, 1 of 5 patients with leukaemia, 3 of 25 hospitalised patients and 2 of 20 healthy controls (Moore, et al., 2001).

Lämmle and co-workers discuss that although some studies suggested that VWFCP deficiency was not specific for TTP, VWFCP was either not rigorously quantitated or was only moderately decreased (Lämmle, Kremer Hovinga & Alberio, 2005). In a study by Bianchi and co-workers, VWFCP levels were assessed in 68 patients with thrombocytopenia from various causes except TTP or HUS. Twelve patients were found to have levels lower than 30%. None had levels <10% which is in clear contrast to patients with acute TTP (Bianchi, et al., 2002). A severely deficient VWFCP activity (<5% of normal) is suggested as specific for TTP (Lämmle, Kremer Hovinga & Alberio, 2005).

Subsequent cohort studies on patients diagnosed with acute idiopathic TTP demonstrated marked variability in ADAMTS13 activity (Furlan, et al., 1998; Tsai & Lian, 1998; Vesely, et

al., 2003; Peyvandi, et al., 2004a; Veyradier, et al., 2001; Mori, et al., 2002; Kremer Hovinga, et al., 2004; Matsumoto, et al., 2004; Zheng, et al., 2004). Severe protease deficiency was defined as <5% of normal in all studies, except in the study by Peyvandi and co-workers, where it was defined as <10% (Lämmle, Kremer Hovinga & Alberio, 2005).

Lämmle and co-workers discuss that not all patients clinically diagnosed with idiopathic TTP may have severe ADAMTS13 deficiency as measured with static protease activity assays (Lämmle, Kremer Hovinga & Alberio, 2005). Lian attributes the variation in reports of ADAMTS13 deficiency in idiopathic TTP to the fact that ADAMTS13 deficiency may not be the cause of platelet thrombi in approximately 40% of patients and that other contributing pathogenic factors may be involved in these cases of idiopathic TTP (Lian, 2005). Kokame and co-workers, demonstrated an association of ADAMTS13 activity with gender and age suggesting that ADAMTS13 activity of women should be significantly higher than that of men and that plasma ADAMTS13 activity should decrease at least after the early 40s (Kokame, et al., 2005).

Galbusera and co-workers, reported that nearly all patients during the acute phase of thrombotic microangiopathy had increased proteolytic fragments of VWF (176kDa and 140kDa), independently of normal or deficient ADAMTS13 activity (Galbusera, et al., 1999a). ADAMTS13 cleavage of VWF is known to generate 176kDa and 140 kDa fragments, and Remuzzi, emphasizes that other proteins may be involved in the cleavage of VWF in thrombotic microangiopathy patients. Although ADAMTS13 inhibits VWF function *in vitro*, it may also function to prevent microangiopathy by cleaving other unknown proteins involved in the coagulation system or endothelial integrity regulation (Remuzzi, 2003).

ADAMTS13 activity as low as 5-10% may be sufficient for the prevention of microvascular platelet thrombi and parents of TTP patients with ADAMTS13 activity as low as 6-20% are generally asymptomatic (Sasahara, et al., 2001).The ability of assays to screen for ADAMTS13 deficiency and to distinguish between hereditary and acquired deficiency is relevant due to differences in patients' therapeutic management. However further work is

required to improve functional methods as well as the standardisation of ADAMTS13 activity (Wyrick-Glatzel, 2004).

The ULVWF multimers appear to undergo proteolytic processing to smaller multimers directly on the surface of the endothelial cell (Wyrick-Glatzel, 2004). Abnormal processing of the ULVWF multimers and microthrombi formation may occur due to either autoantibody formation or a structural defect of the ADAMTS13 molecule that prevents the binding of ADAMTS13 to the endothelial cell receptor (Wyrick-Glatzel, 2004).

Current assays available do not detect defective binding of ADAMTS13. Further development of endothelial based assays of ADAMTS13 activity may better define cases of TTP without severe deficiency of ADAMTS13 that results from impaired binding (Wyrick-Glatzel, 2004). The clinical utility of an ADAMTS13 assay still remains a matter of considerable debate (Remuzzi, 2003; Tsai, 2003; Mannucci, 2003) which may in part be related to interassay variability (Shelat, Ai & Long Zheng, 2005). Laboratory assays for ADAMTS13 activity must reliably assess severe deficiency and inhibitory antibodies (Studt, et al., 2003).

Although the specificity of low ADAMTS13 levels for the diagnosis of TTP, HUS or other thrombotic microangiopathies has been debated in the literature (Tsai, 2003; Remuzzi, 2003), measurement of ADAMTS13 may be of value in patient management (Tripodi, et al., 2004). Severe deficiency of ADAMTS13 activity of less than 5% in a patient with clinical symptoms of an acute thrombocytopenia and evidence of a microangiopathic haemolytic anaemia (in particular red cell fragments on peripheral smear), appropriately defines the diagnosis of TTP. However severely deficient ADAMTS13 activity may not always produce the clinical entity of TTP (Wyrick-Glatzel, 2004). Therefore correlation of ADAMTS13 activity with other laboratory and clinical findings is essential.

1.13 Overview of VWFCP assays

ADAMTS13 assays can be divided into static versus flow based and direct versus indirect *(*Shelat, Ai & Long Zheng, 2005). Refer to Table 1 for a summary of VWFCP assays.

Table 1. Summary of Direct versus Indirect assays available for the measurement of VWFCP levels.

Direct Assays	**Indirect Assays**
SDS-PAGE & Western Blotting	Collagen-Binding Assay
Fluorescent Resonance Energy Transfer (FRETS) assay	Ristocetin-Induced Aggregation
	Flow-based Assays
	New ELISA using recombinant VWF-A2 domain as substrate

Central to each assay is two distinct steps. The first step involves proteolysis of a substrate by plasma containing ADAMTS13. Substrates include exogenous, endogenous, purified fragments or recombinant VWF. The substrate usually requires unfolding with the use of either urea or guanidine and ADAMTS13 is usually activated with the use of divalent cations. Various test plasma concentrations are utilised (Wyrick-Glatzel, 2004).

The second step involves the quantitation of the remaining substrate or the residual VWF after proteolytic cleavage. Quantitation of substrate may be performed by, electrophoresis (detection of fragments or the generation of smaller multimers), immunological methods (ELISA, Immunoradiometric assays/ IRMA which use monoclonal antibodies to VWF), or the measurement of functional activity (Ristocetin cofactor activity of VWF or the decrease of collagen binding) (Wyrick-Glatzel, 2004).

Assays for detecting ADAMTS13 activity in TTP have been in use from 1998. The method by Tsai and co-workers, is the only method that measures the generation of VWF degradation products by ADAMTS13 proteolysis (Tsai & Lian, 1998; Wyrick-Glatzel, 2004). Direct assays detect the cleavage products of a particular substrate such as macromolecular VWF, the A2 domain of VWF or the small synthetic 73-amino-acid peptide FRETS-VWF73 (fluorescence resonance energy transfer) (Shelat, Ai & Long Zheng, 2005; Kokame, et al., 2005). The cleavage products of the substrate have been detected by Agarose or polyacrylamide gel

electrophoresis (PAGE), Western blotting and the FRETS techniques (Shelat, Ai & Long Zheng, 2005).

Indirect assays depend on the measurement of residual substrate or on disappearance of the substrates. Examples of indirect assays include the Collagen–binding assay (Gerritsen, et al., 1999) and modifications of this assay (Rick, et al., 2002; Zheng, et al., 2004; Mannucci, et al., 2001), Ristocetin-induced aggregation, flow-based assays and Enzyme-linked immunosorbent assay (Shelat, Ai & Long Zheng, 2005).

Tsai and co-workers, used a direct method which measures the product of ADAMTS13 enzymatic activity by means of SDS-PAGE which detects the cleavage products of purified VWF as dimers of 176-kd and 140-kd fragments with a normal range of VWFCP activity of 68%-126% in the control group (Tsai & Lian, 1998).

The collagen binding assay by Gerritsen and co-workers (normal range of 28%-119%), the two-site immunoradiometric assay by Obert and co-workers (normal range of 50%-150%), the method of quantitative immunoblotting of cleaved or degraded VWF multimers by Furlan and co-workers (normal range of >50%) and the method using ristocetin cofactor activity by Böhm and co-workers (normal range of 52%-134%), are all indirect methods for the measurement of ADAMTS13 activity (Böhm, Vigh & Scharrer, 2002; Gerritsen, et al., 1999; Obert, et al., 1999; Furlan, et al., 1997).

However all the above-mentioned indirect methods and the direct method by Tsai and co-workers, are based upon static and non-physiological settings (with the use of denaturing conditions with either urea or guanidine, barium ions and long incubation periods), instead of more physiological conditions on the endothelial cell surface under fluid shear forces (Amar, et al., 2002; Dong, et al., 2002; Shenkman, et al., 2002; Shenkman, et al., 2003). Inhibitory antibodies to ADAMTS13 may be detected by all of the functional assays for ADAMTS13 with a wide range of sensitivities (Shelat, Ai & Long Zheng, 2005). ADAMTS13 autoantibodies may be classified into either inhibitory or noninhibitory antibodies, which is based on whether

the antibodies block proteolytic cleavage of VWF in an in vitro assay (Shelat, Ai & Long Zheng, 2005).

Proteolytic activity of ADAMTS13 may be blocked in vitro if the autoantibodies target ADAMTS13 proximally, whereas in vitro activity of ADAMTS13 may not be blocked if the autoantibodies target the middle and distal carboxyl terminus of ADAMTS13 (Soejima, et al., 2003). Inhibitory antibodies thus far react with the Cys-rich and spacer domains and have some specificity to other parts of the ADAMTS13 molecule including the catalytic domain (Klaus, et al., 2004). This supports the essential role of the Cys-rich and spacer domains in substrate recognition (Zheng, et al., 2003a; Soejima, et al., 2003; Ai, et al., 2005).

1.13.1 Inhibitor Assays

As per an unpublished observation by Shelat and co-workers, the FRETS-VWF73 assay appears the most sensitive out of three evaluated assays (collagen-binding assay, GST-VWF73 and FRETS-VWF73) of detecting inhibitory autoantibodies (Shelat, Ai & Long Zheng, 2005).

Both inhibitory and non-inhibitory autoantibodies may be detected by an ELISA assay along with Western blotting using recombinant ADAMTS13 as an antigen (Rieger, et al., 2005). Rieger and co-workers report IgG antibodies in 97% of untreated patients with acute acquired TTP whose ADAMTS13 plasma levels are below 10% and a corresponding prevalence of IgM antibodies of 11% (Rieger, et al., 2005). The presence of ADAMTS13 autoantibodies is considered fairly specific for making a diagnosis of acquired TTP (Tsai & Lian, 1998; Tsai, 2003). A high inhibitory autoantibody titre correlates with a greater number of relapses (Shelat, Ai & Long Zheng, 2005).

Adjunctive immune therapies including Rituximab ^{TM} (anti-CD20 chimeric monoclonal antibody) or cyclophosphamide are considerations in patients who chronically relapse or who do not respond adequately to plasmapheresis (Zheng, et al., 2003b; Zheng, et al., 2004; Fakhouri, et al., 2004; Fakhouri, et al., 2005). A robust ADAMTS13 inhibitor assay is essential

for both understanding the mechanism of TTP and for adjusting therapeutic modalities (Shelat, Ai & Long Zheng, 2005).

1.13.2 Direct Assays

- **SDS-PAGE and Western blotting**

This assay was initially developed by Tsai and co-workers and is based on detection of the 170kDa and 140kDa VWF fragments on denatured, but unreduced SDS-PAGE and Western blotting (Tsai, 1996; Tsai & Lian, 1998; Tsai, et al., 1997).

This assay differs from the SDS-Agarose method developed by Furlan and co-workers by: no requirement for barium chloride activation; easier to work with gels from SDS-PAGE than agarose gel electrophoresis; relatively short incubation period of 1-3 hours; the cleavage product is visualised directly thereby eliminating the possibility of non-specific degradation of the multimers (Shelat, Ai & Long Zheng, 2005; Tsai & Lian, 1998; Furlan, Robles & Lämmle, 1996; Furlan, et al., 1997; Furlan, et al., 1998).

After the reaction products have been separated by SDS-Agarose or SDS-PAGE gel electrophoresis, this is followed by Western blotting analysis with anti-VWF antibodies (Furlan, Robles & Lämmle, 1996; Tsai, 1996).

This SDS-PAGE assay detected less than 5% of normal ADAMTS13 activity (severe deficiency) in all 37 patients with idiopathic TTP and inhibitory activity against ADAMTS13 was detected in 67% (Tsai & Lian, 1998). This assay is sensitive enough to distinguish between patients with congenital TTP, asymptomatic carriers and healthy individuals and precise mapping of the defective ADAMTS13 gene can be performed (Levy, et al., 2001). Although these assays have significantly increased the understanding of ADAMTS13 and the pathogenesis of TTP, due to technical complications these assays are not widely used at the clinical level (Kokame, et al., 2005).

- **Fluorescent Resonance Energy Transfer (FRETS) assay**

Kokame and co-workers, developed a synthetic 73-amino-acid fluorogenic peptide, FRETS-VWF73, containing the 73 amino acids from D1596 to R1668 of VWF. The Q1599 residue at the P7 position was converted to a 2,3-diaminopropionic residue (A2pr) which was modified with a 2-(N-methylaminobenzoyl group (Nma) and the N1610 residue of the P5' position was converted to A2pr which was modified with a 2,4-dinitrophenyl group (Dnp). At 340nm, the Nma group is excited and fluorescence resonance energy is transferred to Dnp, the neighbouring quencher. However if the bond between Y1605 and M1606 is cleaved, the fluorescence energy transfer quenching does not occur and this allows the emission of fluorescence from Nma at 440nm (Kokame, et al., 2005). Refer to Figure 4 for a graphical depiction of the structure of the FRETS substrate (as per Kokame, et al., 2005).

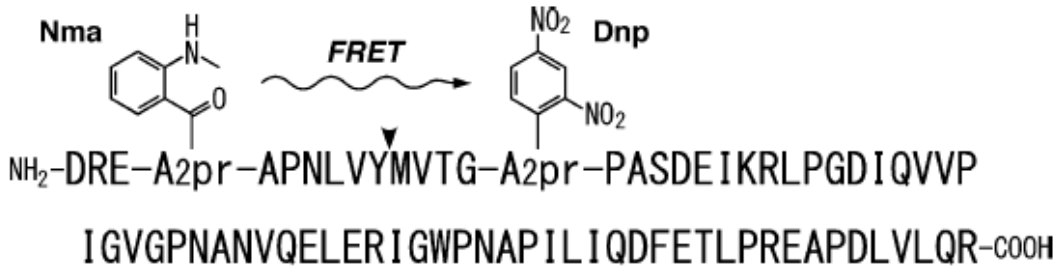

Figure 4. Schematic representation of structure of fluorescence resonance energy transfer (FRETS)-Von Willebrand factor (VWF)-73 substrate. *Amino acid residues Q1599 and N1610 (which are within the 73 amino acid peptide corresponding to the region D1595-R1668of VWF) are replaced by A2pr (Nma) and A2pr (Dnp) respectively. The arrow indicates the cleavage site of ADAMTS13. Diagram from:* Kokame, K., Nobe, Y., Kokubo, Y., et al. 2005. FRETS-VWF73, a first fluorogenic substrate for ADAMTS13 assay. *Br J Haematol,* vol.129, pp. 94.

FRETS-VWF73 is a monomeric molecule which has a single cleavage site as opposed to the previously established substrate, purified plasma VWF which comprised of non-uniform multimers with multiple cleavage sites (Kokame, et al., 2005). Denaturants are not required for the reaction, thereby making this assay closer to physiological conditions. This assay follows a simple procedure and special reagents or equipment is not required other than a fluorescence spectrophotometer. Proposed uses for this assay include potential clinical diagnostics and selection of plasma with high titre ADAMTS13 activity for treatment purposes (Kokame, et al., 2005).

A universally applicable cut-off value for ADAMTS13 activity for the diagnosis of TTP or ADAMTS13 deficiency is still to be established and the definition of standard plasma is of primary importance (Kokame, et al., 2005). Purified or recombinant ADAMTS13 availability may assist in the standardisation of ADAMTS13 assays and the age and gender distribution of ADAMTS13 will need to be established in the general population (Kokame, et al., 2005). The FRETS-VWF73 assay is a high-throughput assay that would facilitate performing large population-based studies (Kokame, et al., 2005).

1.13.3 Indirect Assays

- **Collagen-Binding Assay**

Collagen-binding assays are based on the preferential binding of large VWF multimers to type III human collagen (Gerritsen, et al., 1999; Mannucci, et al., 2001; Rick, et al., 2002; Zheng, et al., 2003a). These assays have some variations in the initial method, with some employing the use of incubation with purified VWF (Gerritsen, et al., 1999) but all culminate in quantitation of the residual amount of VWF binding to collagen by means of peroxidase conjugated anti-VWF antibody on an ELISA plate (Gerritsen, et al., 1999; Rick, et al., 2002; Zheng, et al., 2003a; Zheng, et al., 2004).

This assay can accommodate many samples simultaneously and is considered quite robust in the detection of severe deficiency of ADAMTS13 (16 of 20 patients) and high titre inhibitors (7 of 16 patients) in idiopathic TTP (Zheng, et al., 2004). This assay correlates well with other indirect assays (Tripodi, et al., 2004). We have set up the rapid collagen binding assay as per Rick and co-workers.

A modification of this assay is used to assess for the presence of an inhibitor to ADAMTS13, by incubation of patient sample with pooled normal plasma (Rick, et al., 2002; Zheng, et al., 2004). The presence of high-titre inhibitors in TTP patients is associated with a delayed response to plasmapheresis and refractory disease (Zheng, et al., 2004; Vesely, et al., 2003; Veyradier, et al., 2001). Assessment of ADAMTS13 inhibitor titres may be useful for the

identification of patients with a high possibility of relapse as this group of TTP patients may benefit from intensive immunotherapy in addition to plasmapheresis (Zheng, et al., 2004). This assay may be used for diagnostic purposes, therapeutic monitoring and for predicting outcome (Shelat, Ai & Long Zheng, 2005).

The collagen binding assay (without the dialysis step) is also of value in the diagnosis of Type 2A and Type 2B Von Willebrand's Disease, where high molecular weight multimers of VWF are either absent or deficient (Favaloro, 2000). An ELISA test for the binding of VWF to collagen was first described in 1986 by Brown & Bosak and has subsequently been modified (Brown & Bosak, 1986; Favaloro, 2000).With respect to Von Willebrand disease assessment, the collagen source is of importance (Favaloro, 2000).

A collagen binding assay can be used in the diagnosis and subtype discrimination of Von Willebrand disease depending on two variables: the generation of an adequate VWF-dose-dependent calibration curve and the ability to detect only the most functional VWF forms (the high and intermediate weight molecular weight multimers of VWF are preferentially detected) (Favaloro, 2000).

Not all collagen preparations provide an effective VWF-dose-dependent calibration curve and the ability of the collagen-based assay to preferentially detect high and intermediate molecular weight VWF multimers also depends on the type of collagen used and there may be batch/ lot dependency (Favaloro, 2000). Therefore optimal assay conditions need to be established with respect to collagen concentration, buffer choice and collagen coating incubation time (Favaloro, 2000).

Favaloro makes recommendations for ELISA collagen-binding assays with respect to Von Willebrand disease assessment including the use of a Type I/III collagen mixture product (used at 10-50µg/ml) or a pure Type III collagen (used at 1-5µg/ml) (Favaloro, 2000). Bovine and equine collagen sources have shown comparable results (Favaloro, 2000; Favaloro, 2002).

For the purposes of Von Willebrand disease detection, the use of purified Type I collagen is not recommended, due to low efficacy, reproducibility or both (Favaloro, 2000; Favaloro, 2002). However, for the purposes of this study, Vitrogen ^{TM} Collagen was used which is a purified bovine Type I Collagen, as used by Rick et al, for the purposes of VWFCP activity (as measured by % residual collagen binding activity). This differs from VWF antigen detection where generation of an adequate VWF-dose-dependent calibration curve is required. It should be noted that data on different collagen sources for the purpose of the residual collagen binding assay for VWFCP activity is not available.

- **Ristocetin-Induced Agglutination**

This assay utilises purified VWF which is digested in buffer and the residual VWF is determined with the use of ristocetin cofactor activity in a platelet aggregometer (Shelat, Ai & Long Zheng, 2005). The method has been shown to be reproducible and severe ADAMTS13 deficiency in patients with acute TTP is detected (Böhm, Vigh & Scharrer, 2002). However this assay has not been widely used as only a limited number of samples can be analysed on the aggregometer at a time (Shelat, Ai & Long Zheng, 2005).

- **Flow-based assays**

Shenkman and colleagues, used a cone and platelet analyser to determine the ability of TTP plasma versus normal plasma, to increase platelet deposition on a polystyrene surface under flow conditions (Shenkman, et al., 2003). The assay is sensitive and specific for the assessment of ADAMTS13 deficiency and inhibitors. The deposition of normal platelets was increased following addition of plasma in all 15 patients with acute TTP but only in three of 14 patients in remission (Shenkman, et al., 2003).

Dong and colleagues, described that when released from cultured endothelial cells, ULVWF multimers form extremely long, platelet-decorated string-like structures on the surface of endothelial cells under conditions of fluid shear stress (Dong, et al., 2002). When perfused with plasma from patients with TTP, the string-like structures are not cleaved, whereas in the

presence of normal plasma, the string-like structures disappear rapidly due to the cleavage of ULVWF multimers by ADAMTS13 in the perfused plasma (Dong, et al., 2002).

This suggests that ADAMTS13 binds to the endothelial cell and locally cleaves the exceptionally adhesive ULVWF multimers (Dong, et al., 2002). An endothelial cell dependant assay may shorten the method and reflect the function of ADAMTS13 in a more physiological manner (Amar, et al., 2002).

Moake hypothesised that there may be defective anchoring of ADAMTS13 to the endothelial cell surface (Moake, 2002), this may be related to possible structural defects of the ADAMTS13 molecule (Studt, et al., 2003) or to anti-CD36 antibodies (Tandon, Rock & Jamieson, 1994). In such cases severe ADAMTS13 dysfunction may not be detected by the other methods discussed but may only be detected by an endothelial-based assay. This could also explain the presence of clinically distinct TTP patients with normal ADAMTS13 activity (Mori, et al., 2002; Vesely, et al., 2003; Studt, et al., 2003; Wyrick-Glatzel, 2004).

- **New Enzyme-Linked Immunosorbent Assay using recombinant VWF-A2 domain as a substrate**

Whitelock et al, succeeded in culturing bacteria which expressed a recombinant VWF-A2 peptide (amino acid 718—905). This peptide contains both a 6xHis tag at the N-terminal end and a Tag-100 epitope at the C-terminal end and is used as a substrate for this assay (Whitelock, et al., 2004; Zhou & Tsai, 2004). The VWF substrate is immobilised on a microtitre plate. A Ni^{2+}-coated microtitre plate is utilised as the Ni^{2+} captures the VWF-A2 peptide by its 6xHis tag and cleavage of the A2 peptide is ascertained by the removal of the C-terminus of the peptide that contains the Tag-100 (Whitelock, et al., 2004).

Samples are incubated with the substrate, the cleavage activity is defined by low detection of the A2 peptide containing the Tag-100 epitope by means of an antiTag-100 monoclonal antibody thereby detecting the residual substrate present (Whitelock, et al., 2004).

The proteolytic activity of ADAMTS13 is inversely proportional to the amount of residual VWF substrate present. The VWF A2 domain ELISA detected residual ADAMTS13 activity in TTP patients of 2.4 + /- 0.7% compared with 40 + /- 4.2% in normal plasma and these results were validated with the use of a traditional long incubation immunoblotting method (Whitelock, et al., 2004). TTP patients can be distinguished from carriers of ADAMTS13 gene mutation and normal healthy individuals (Zhou & Tsai, 2004).

This assay is reproducible, cost-effective, can be performed in less than 5 hours and measures ADAMTS13 activity under non-denaturing and physiological conditions (Whitelock, et al., 2004). As this is an ELISA-based method, lengthy electrophoresis is avoided (Whitelock, et al., 2004). Another advantage of this assay is the use of purified recombinant VWF-A2 peptide as a substrate which allows standardisation of the substrate, thereby avoiding variability among various batches of VWF preparation including antigen levels and multimer distribution. In addition, the cleavage of recombinant VWF-A2 peptide occurs without the addition of metal ions and under physiological buffer saline with a pH of 7.4.

Other assays require addition of specialised buffers containing Ba^{2+} or Ca^{2+} ions and a higher pH to promote the enzyme to cleave full-length VWF. Urea is not required to unfold the substrate due to the constitutively exposed cleavage site of the A2 peptide (Whitelock, et al., 2004).

Significantly shorter incubation time and less plasma (activity at 1:50 dilution detected) is required for cleavage of the VWF-A2 domain. This is related to the readily accessible open conformation of VWF-A2. The A2 domain appears to be masked in native VWF multimers by other adjacent structures such as the A1 and A3 domains (Whitelock, et al., 2004).

This may explain why ADAMTS13 cleaves the ULVWF formed under fluid shear stress, one thousand fold faster than under static conditions (Whitelock, et al., 2004; Dong, et al., 2002). As the ULVWF strings tethered with platelets are subjected to noteworthy amounts of wall shear stress which may stretch VWF to the extent of exposing the A2 domain (Whitelock, et

al., 2004). This concurs with previous studies by Tsai et al., which demonstrated that fluid shear stress accelerates VWF cleavage by ADAMTS13 (Tsai, Sussman & Nagel, 1994). Rapid diagnosis of TTP and other conditions that may result in ADAMTS13 deficiency may be enhanced by the fast turnaround time of the ELISA method (Whitelock, et al., 2004).

1.14 Studies comparing different assays

Studt et al, published a study based upon the comparison of the results obtained from five different laboratories on the same samples: one used the immunoblotting assay, two utilised the residual collagen binding activity, one used the residual ristocetin cofactor activity and one used the immunoradiometric assay (Studt, et al., 2003).

The purpose of this study was to assess whether or not there was concordance of the 4 different techniques used in 5 different laboratories to assess 30 plasma samples from patients with hereditary and acquired TTP and other clinical conditions associated with altered ADAMTS13 activity with levels ranging from <3% to >100% (Studt, et al., 2003). This study also evaluated whether or not plasma containing ADAMTS13 inhibitory antibodies could be distinguished from plasma without antibodies (Studt, et al., 2003).

With few exceptions, the methods used by all laboratories identified plasma with severe ADAMTS-13 deficiency (<5%) and plasma with strong inhibitor activity (Studt, et al., 2003). There was less consistency of interassay and inter-laboratory results for plasma samples with normal or moderately reduced ADAMTS13 activity (Studt, et al., 2003). However this study demonstrated that these methods developed for ADAMTS13 detection showed good general agreement between the assays concerning the identification of severe ADAMTS13 deficiency and did not indicate a systematic deviation for any method. These assays are therefore considered clinically useful and relevant as a screening test in suspected TTP patients (Studt, et al., 2003).

The collagen binding assay is widely used due to its relative simplicity, however in this study three samples were misclassified by two laboratories (one false negative and two falsely

positive) (Studt, et al., 2003). The false negative result was in a confirmed patient with hereditary TTP, where one laboratory obtained a result of ADAMTS13 activity of 44% (all other laboratories diagnosed a severe deficiency of ADAMTS13 with less than 5% ADAMTS13 activity). One laboratory obtained two false positive results where a severe ADAMTS13 deficiency was diagnosed in two patients, whereas all other laboratories found only a mild to moderate decrease in ADAMTS13 activity (10-25% in the one patient and 15-35% in the other patient) (Studt, et al., 2003).

The collagen binding assay appears to be subject to disturbance and is delicate and some improvement in terms of accuracy is required, although no systematic error was identified (Studt, et al., 2003). Substantial discrepancies may also occur with other methods (Mannucci, 2003).

The immunoblotting assay is reproducible in the lowest activity range, is very sensitive and allows distinction between 3% and 0% activity and depending on the VWF substrate quality, this assay may distinguish between 1% and 0% activity (Studt, et al., 2003). However the disadvantage of the immunoblotting assay is the labour-intensive and time-consuming method which limits the application of this method to use in a specialised laboratory setting (Studt, et al., 2003).

The limitation of this study, is that precision and reproducibility were not specifically evaluated which are important criteria for laboratory method validation (Mannucci, 2003). Thereafter another multicentre study was co-ordinated to include a wider array of tests (Mannucci, 2003; Tripodi, et al., 2004). Tripodi and co-workers, performed an international collaborative study of eleven methods for ADAMTS13 measurement in different laboratories testing the same set of coded plasmas (Tripodi, et al., 2004). Two plasmas, one from a patient with familial TTP and one normal were mixed at the co-ordinating centre to obtain 6 plasmas with varying concentration of ADAMTS13 activity ranging from 0%, 10%, 20%, 40%, 80% and 100% (Tripodi, et al., 2004). Nine of the ten methods performed under static conditions were quantitative and one was semi-quantitative (Tripodi, et al., 2004).

Four laboratories used the collagen binding assay (as per Gerritsen, et al., 1999), one used the semi-quantitative rapid collagen binding assay (as per Rick, et al., 2002), one used residual ristocetin cofactor activity (as per Böhm Vigh & Scharrer, 2002), one used quantitative immunoblotting of degraded substrate of VWF multimers (as per Furlan, et al., 1998), one used immunoblotting of unreduced VWF multimers (as per Amar, et al., 2002), one used an immunoradiometric assay (IRMA) with monoclonal antibodies to the N and C-terminal subunits (as per Obert, et al., 1999), and one used SDS-PAGE and quantitative immunoblotting of a recombinant VWF polypeptide which includes domains A1-A2-A3 of VWF (as per Remuzzi, et al., 2002).

One method was not static and was carried out under flow conditions to assess the extent of cleavage of endothelial cell derived ULVWF string-like structures with attached platelets under fluid shear stress (as per Amar, et al., 2002). The latter method expressed results as deficient, normal or borderline (Tripodi, et al., 2004).

Linearity of the expected versus the observed levels with the use of the squared correlation coefficient (r^2), ranged from 0.98 to 0.39. Reproducibility for repeated measurements assessed with the coefficient of variation ranged from <10% to 83% (Tripodi, et al., 2004). The majority of the methods were successful at discriminating between the different levels of ADAMTS13 and this is an important characteristic as both reproducibility and accuracy are taken into account (Tripodi, et al., 2004).

The discrimination between different ADAMTS13 levels was performed best by two of the collagen binding assays, the ristocetin cofactor assay and the SDS-agarose electrophoresis and immunoblotting of degraded VWF multimers. However these methods have little in common in terms of assay design (Tripodi, et al., 2004).

Another important characteristic of methods measuring ADAMTS13 is the ability to detect severe ADAMTS13 deficiency and the majority of the assays could detect ADAMTS13 levels of 0% (Tripodi, et al., 2004).

Some assays could distinguish between levels of 0% and 10% of ADAMTS13 namely one of the collagen binding assays, the ristocetin cofactor activity assay and the assay using SDS agarose electrophoresis and immunoblotting of degraded VWF multimers (Tripodi, et al., 2004).

The overall best performance was noted for three methods: the ristocetin cofactor activity, the collagen binding assay and the immunoblotting of degraded multimers of VWF substrate (Tripodi, et al., 2004). The method performed under flow conditions identified the plasma samples of 80% and 100% as normal in all 10 repeated measurements and the 0%, 10%, 20% and 40% activity was detected respectively as deficient in 7, 5, 1 and 3 of the 10 repeated measurements (Tripodi, at al., 2004). This method may be reliable in distinguishing ADAMTS13 levels higher or lower than 20%. As the latter assay reported results as normal, borderline or deficient based on the percentage of strings cleave after 2 minutes of perfusion, reproducibility and linearity of this method could not be assessed (Tripodi, et al., 2004).

The best concordance between expected and achieved ADAMTS13 levels was found in two methods. These include one of the laboratories using the collagen binding assay (by Gerritsen, et al., 1999, using purified VWF, not semi-quantitative method) with r^2=0.97, and one of the laboratories using the ristocetin cofactor activity assay (by Böhm, Vigh & Scharrer, 2002, using purified VWF) with r^2=0.98 (Tripodi, et al., 2004).

Reproducibility studies revealed that the most precise methods were the semi-quantitative collagen binding assay (as per Rick, et al., 2002) and the ristocetin cofactor activity where the average coefficient of variation was less than 10% (Tripodi, et al., 2004). Intermediate precision was achieved by 2 collagen binding assay and the SDS agarose electrophoresis and immunoblotting of VWF multimers (average coefficient of variation between 12-15%). Two other collagen binding assays, the IRMA, the SDS-PAGE and quantitative immunoblotting of recombinant VWF polypeptide and the SDS-agarose and immunoblotting of ULVWF multimers yielded average coefficient of variation results from 30-83% (Tripodi, et al., 2004).

In terms of the overall ability of each method to discriminate between different ADAMTS13 levels, the ristocetin cofactor activity, two collagen binding assays and the SDS agarose electrophoresis and immunoblotting of degraded VWF multimers revealed good discrimination (Tripodi, et al., 2004). Intermediate discrimination between different ADAMTS13 levels was noted with the semi-quantitative rapid collagen binding assay, two collagen binding assays and the SDS-PAGE and quantitative immunoblotting of a recombinant VWF polypeptide. Low discrimination was noted with the IRMA method and the SDS-agarose and immunoblotting of unreduced VWF multimers (Tripodi, et al., 2004).

The ability of each method to distinguish between the 0% and 10% ADAMTS13 plasmas could not be statistically determined as the results obtained by the majority of methods for the 0% plasma were below detection limits (Tripodi, et al., 2004).However, the subjectively rated ability of one collagen binding assay, the ristocetin cofactor assay and the SDS agarose electrophoresis and immunoblotting of degraded multimers of VWF substrate was excellent and the ability of a second collagen binding assay was subjectively rated as good (Tripodi, et al., 2004). The only method that directly evaluated ADAMTS13 protease activity was the SDS-PAGE and quantitative immunoblotting of a recombinant VWF polypeptide including the A1-A2-A3 domains of VWF. For the latter assay, the linearity (r^2=0.77) between expected and observed results and ability to detect severe ADAMTS13 deficiency were acceptable, however these results were no better than that of the other methods which indirectly assess VWF cleavage (Tripodi, et al., 2004).

The only method using ULVWF multimers (the likely physiological substrate for ADAMTS13) as a substrate (SDS-agarose and immunoblotting), had a high detection rate for 0% ADAMTS13 levels. Further methodological improvement is required in terms of the reproducibility and the linearity (r^2=0.39) of the expected and the observed results (Tripodi, et al., 2004).

There is poor agreement between the results obtained on plasma samples with intermediate and normal ADAMTS13 levels with different methods (Tripodi, et al., 2004). This may be improved to some extent with the use of a common standard to construct calibration curves. The use of different standards did not appear, however, to be a main determinant of the variability between laboratories. High variability was noted even for different laboratories employing the same techniques for ascertaining ADAMTS13 levels (Tripodi, et al., 2004). Thus, the inter-laboratory variability may be due to the way in which the different laboratories have set up the same technique rather than due to differences between techniques (Tripodi, et al., 2004).

Tripodi and co-workers conclude that a varied performance of the methods is noted, the results by Studt and co-workers are extended and confirmed, and an overall optimistic view of the reliability of currently available methods for ADAMTS13 detection is supported (Tripodi, et al., 2004). Tripodi and co-workers recommend further multicentre clinical studies to further assess and clarify the role of assaying ADAMTS13 levels with respect to management of patients with thrombotic microangiopathies (Tripodi, et al.,2004). The subsequent more recent assays for assessing ADAMTS13 activity including the Enzyme-Linked Immunosorbent Assay using recombinant VWF-A2 domain as a substrate used by Whitelock et al, and Zhou et al, and the FRETS-VWF73 assay used by Kokame et al, have not yet been compared in a multicentre trial (Whitelock, et al., 2004; Zhou & Tsai, 2004; Kokame, et al., 2005).

2. Study Objectives

- To establish a practical assay for VWFCP activity for routine use in our laboratory. The rapid collagen binding ELISA assay based on Rick and co-workers, and Aronson and co-workers, was selected (Aronson, Krizek & Rick, 2001; Rick, et al., 2002).

- To measure VWFCP activity using a group of HIV negative controls and two HIV positive groups, those with normal platelet counts and those with thrombocytopenia (of any cause except TTP), in order to establish cut-off values for normal VWFCP activity in these groups and to assess any significant differences between groups in patients from our local setting.

- The diagnostic utility of assessment of VWFCP activity in patients with HIV related TTP, would depend on the outcome of the above investigation.

Should a wide range of VWFCP activity be found in HIV positive patients (particularly comparing those with low platelet counts versus normal platelet counts), the assay is unlikely to be of diagnostic utility in the individual HIV positive patient, where levels of VWFCP may be altered in the absence of TTP (Reiter, et al., 2003; Mannucci, et al., 2001).
However, should VWFCP activity in unselected patients with HIV and thrombocytopenia be found to fall generally within the normal range, or if a specific cut-off value can be established for HIV positive patients in general (regardless of platelet count), then further investigation into levels in patients with HIV related TTP would be warranted and patients with HIV and TTP would be assessed using this assay.

Endpoints of study

The study will have been completed once the assay has been established and sufficient samples have been analysed to determine the range of VWFCP activity in HIV positive patients with and without thrombocytopenia.
However, depending on the outcome of the initial assessments, further assessment of VWFCP activity in patients with HIV-related TTP would be performed.

3.. Materials and Methods

3.1 Specimen collection

The study was a prospective observational study.

Study samples were obtained from Johannesburg General Hospital and Helen Joseph Hospital.

- Samples from HIV negative control patients with normal platelet counts (Group 1), were collected from consecutive patients attending the Coagulation/INR clinic at Johannesburg General Hospital.
- Samples from HIV positive patients with both normal platelet counts (Group 2) and low platelet counts but without TTP (group 3), were collected from consecutive patients attending the HIV / ARV (Anti-RetroViral) clinic at Helen Joseph Hospital.
- Samples from HIV positive patients with TTP (prior to the start of plasma therapy) were collected from consecutive patients presenting at the Johannesburg General Hospital or Helen Joseph Hospital between November 2003 and December 2005 (n=20).

Summary of Inclusion criteria:

Group 1: HIV negative, normal platelet count (n=77)

Group 2: HIV positive, normal platelet count (n=74)

Group 3: HIV positive, low platelet count (platelet count less than 100 x10^9/l) (n=58)

Patient groups seen at the above-mentioned clinics were identified by review of the laboratory data available on DISA (the hospital computer system). The number of patients chosen was based on logistical constraints.

Venous blood samples taken for routine purposes in citrated tubes were utilised, with informed consent. Samples were processed anonymously with a laboratory number. Once the samples were collected, they were centrifuged and the plasma taken off. The Plasma was stored at -70 ºC.

Ethics approval and informed patient consent were obtained. HIV positive patients were known patients who were aware of their HIV status and who were attending the ARV-clinic.

All patients were confirmed to be HIV positive or negative by enzyme-linked immunosorbent assay.

Minimum diagnostic criteria for TTP were thrombocytopenia (platelet count < $100X10^9$/l), microangiopathic haemolytic anaemia and high serum lactate dehydrogenase levels with no other identifiable cause (Moake, 2002). In each case the presence of red cell fragments and thrombocytopenia was confirmed by a haematopathologist. Coagulation studies to exclude a DIC were performed on all patients at presentation as well as serum chemistry to assess renal function. The presence of opportunistic infections or HIV-associated malignancies were excluded as far as possible by the treating clinician, using clinical examination with serology, radiological imaging and microbiological or histological assessment where appropriate. None of the 20 patients were on antiretroviral therapy at the time of presentation. Patients were treated at the discretion of the managing physician. Therapy was commenced immediately after diagnosis and the majority of patients were treated with daily therapeutic plasma exchange.

3.2 Overview of the principles of the collagen binding assay

The collagen binding assay used by Rick and co-workers, and Aronson and co-workers, was set up in this study (Aronson, Krizek & Rick, 2001; Rick, et al., 2002). This is an ELISA-based assay where the microtitre plate well is coated with collagen. Any remaining sites on the microtitre plate are blocked with blocking agent. Both undialysed and dialysed (this serves to "activate" VWFCP which cleaves VWF) patient sample is added to the microtitre plate. The patient's VWF binds the collagen covering the microtitre well surface. The wells are then washed, and horseradish peroxidase labelled anti-VWF antibody is added. Another wash step is performed, and substrate (ABTS ^{TM}) is added which is enzymatically converted to product. The increase in absorbance is then measured spectrophotometrically at 405nm.

The dialysed absorbance reading is divided by the undialysed absorbance reading and the value is multiplied by 100 to give a percentage of Residual Collagen Binding Activity (RCBA %). See section 3.3 on interpretation of results for more information in this regard.

In 1983, Santoro, described the preferential binding of high molecular weight multimers of VWF to fibrillar collagen (Santoro, 1983). Santoro, documented that high molecular weight forms of VWF bind even at low collagen concentrations, high and intermediate molecular weight forms of VWF bind at high collagen concentrations and very low molecular weight forms of VWF do not bind even at high collagen concentrations (Santoro, 1983). Therefore higher molecular weight forms of VWF have a greater affinity for fibrillar collagen and under conditions where the concentration of collagen is limiting, there is preferential binding of the higher molecular weight forms of VWF (Santoro, 1983).

The latter finding explains the underlying principle of the ELISA-based collagen binding assay, whereby the undigested high molecular weight multimers of VWF that have not been cleaved by activated VWFCP (during the dialysis step), then bind to the collagen on the microtitre plate (low collagen concentrations used 3µg/ml). The ratio of the dialysed to the undialysed sample is then used to assess for the % residual collagen binding activity which is indirectly proportional to the VWFCP activity.

This assay therefore functions due to the preferential binding of high molecular weight VWF multimers (Favaloro, Facey & Grispo, 1995; Ramasamy, et al., 1998; Fischer, et al., 1996; Fischer, Thomas & Dorner, 1998; Casonato, et al., 1997).

Optimal conditions were established for this assay in our laboratory which concurred with those used by Rick and co-workers. The use of low concentration collagen in our assay (3µg/ml) results in preferential binding of the high molecular weight multimers as documented in the literature (Santoro, 1983).

Vitrogen ™ Collagen was used, which is an ultrapure purified bovine Type I Collagen, as used by Rick and co-workers, for the purposes of VWFCP activity (as measured by % residual collagen binding activity). The use of other different types of collagen was not explored in this study. This would be a costly exercise and there is documented successful use of Vitrogen ™ Collagen by Rick and co-workers, upon which this assay is based. Of note is that subsequent to this study, the manufacturer of Vitrogen ™ Collagen is apparently no longer in business and the replacement is PureCol™ which is apparently identical to Vitrogen ™ Collagen (http://www.vitrogen.nl/). This collagen replacement has not as yet been used in our laboratory setting.

3.3 Interpretation of the rapid collagen binding assay results

The cut-off values for % RCBA indicated here are as per Rick and co-workers, this study established our own cut-off values which are discussed in section 4. See Figure 5, 6 and 7 for a schematic depiction and explanation of the reaction in the microtitre well. The absorbance reading of the microtitre plate reflects the collagen binding activity of the sample. Each sample was run in duplicate. The average dialysed absorbance reading was divided by the average undialysed absorbance reading and multiplied by 100 to give a percentage. This value is termed % RCBA.

The % RCBA is inversely related to the VWFCP activity. % RCBA above the cut-off value translates to reduced VWFCP activity (VWFCP deficiency) and % RCBA below the cut-off value translates to a normal VWFCP activity.

Normal VWFCP activity leads to a decrease in collagen binding by VWF, due to a decrease in the high molecular weight multimers of VWF (VWFCP cleaves these high molecular weight multimers). Dialysis of the sample serves to “activate” the VWFCP, therefore the dialysed reading reflects RCBA post VWFCP activation.

The dialysed sample reading should be lower than the undialysed sample reading (undialysed sample has not been exposed to VWFCP activity) which will have more high molecular weight multimers of VWF which bind to collagen resulting in a higher reading. Therefore the normal ratio according to Rick and co-workers, should be less than 33% (Rick, et al., 2002).

As per Rick et al., a ratio of >33%, reflects reduced VWFCP activity which comes about as a result of increased binding of high molecular weight VWF multimers which have not been cleaved by VWFCP (either due to a deficiency of VWFCP or an autoantibody to VWFCP). In this setting, the dialysed reading is not lower than the undialysed or baseline reading and a greater ratio is achieved (>33%).

- Normal VWFCP activity detected by ratio:
  $\frac{\text{(Residual CBA) Dialysed absorbance reading}}{\text{(CBA at t=0) Undialysed absorbance reading}} < 33\%$ *

- Decreased VWFCP activity detected by ratio
  $\frac{\text{(Residual CBA) Dialysed absorbance reading}}{\text{(CBA at t=0) Undialysed absorbance reading}} > 33\%$ *

* Calculation and cut-off values as per Rick, et al., 2002. This MMED study established our own cut-off values in our laboratory.

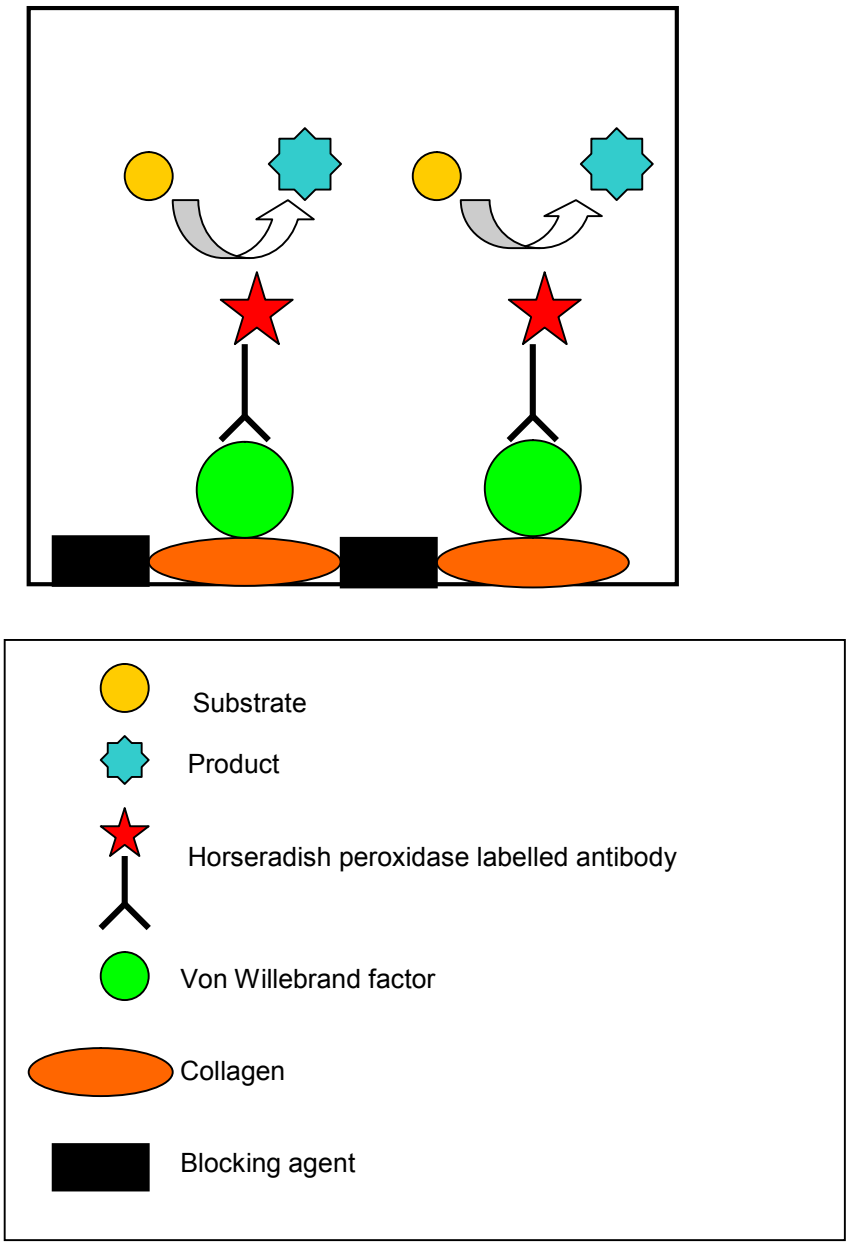

Figure 5. Schematic representation of the VWF binding ELISA assay. The microtitre plate well is coated with collagen. Any remaining sites on the microtitre plate are blocked with blocking agent. When the patient sample is added to the microtitre plate well, the patient's VWF binds the collagen covering the well surface. The wells are then washed, and horseradish peroxidase labelled anti-VWF antibody is added. Another wash step is performed, and substrate (ABTS ^{TM}) is added which is enzymatically converted to product. The increase in absorbance is then measured spectrophotometrically at 405nm.

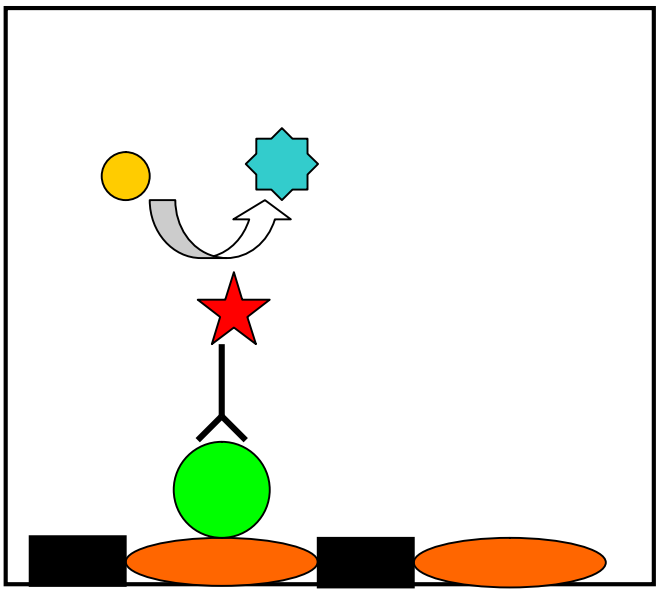

Figure 6. Schematic representation of microtitre plate well with normal VWFCP activity. Under normal circumstances, when there is no deficiency of VWFCP, the VWF is cleaved by the VWFCP which leaves less VWF remaining capable of binding to the collagen on the microtitre plate well. This results in a dialysed: undialysed ratio of RCBA below the cut-off value.

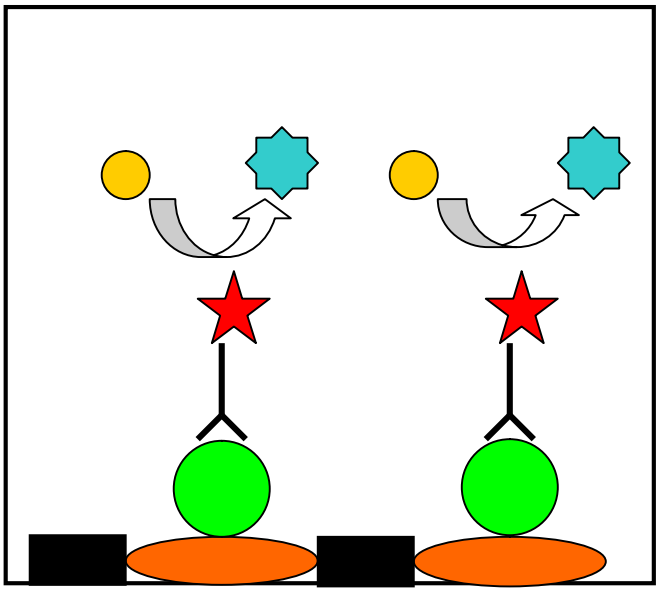

Figure 7. Schematic representation of microtitre plate well with deficient VWFCP activity. When a deficiency of VWFCP exists, such as in TTP, less VWF is cleaved by the VWFCP, leaving more VWF to bind to the collagen coating the microtitre plate well. This results in a dialysed: undialysed ratio of RCBA above the cut-off value.

Please refer to Figure 8 for a flow diagram of the laboratory methods of the rapid collagen binding assay (as per Rick, et al., 2002).

3.4 Flow Diagram of assay procedure

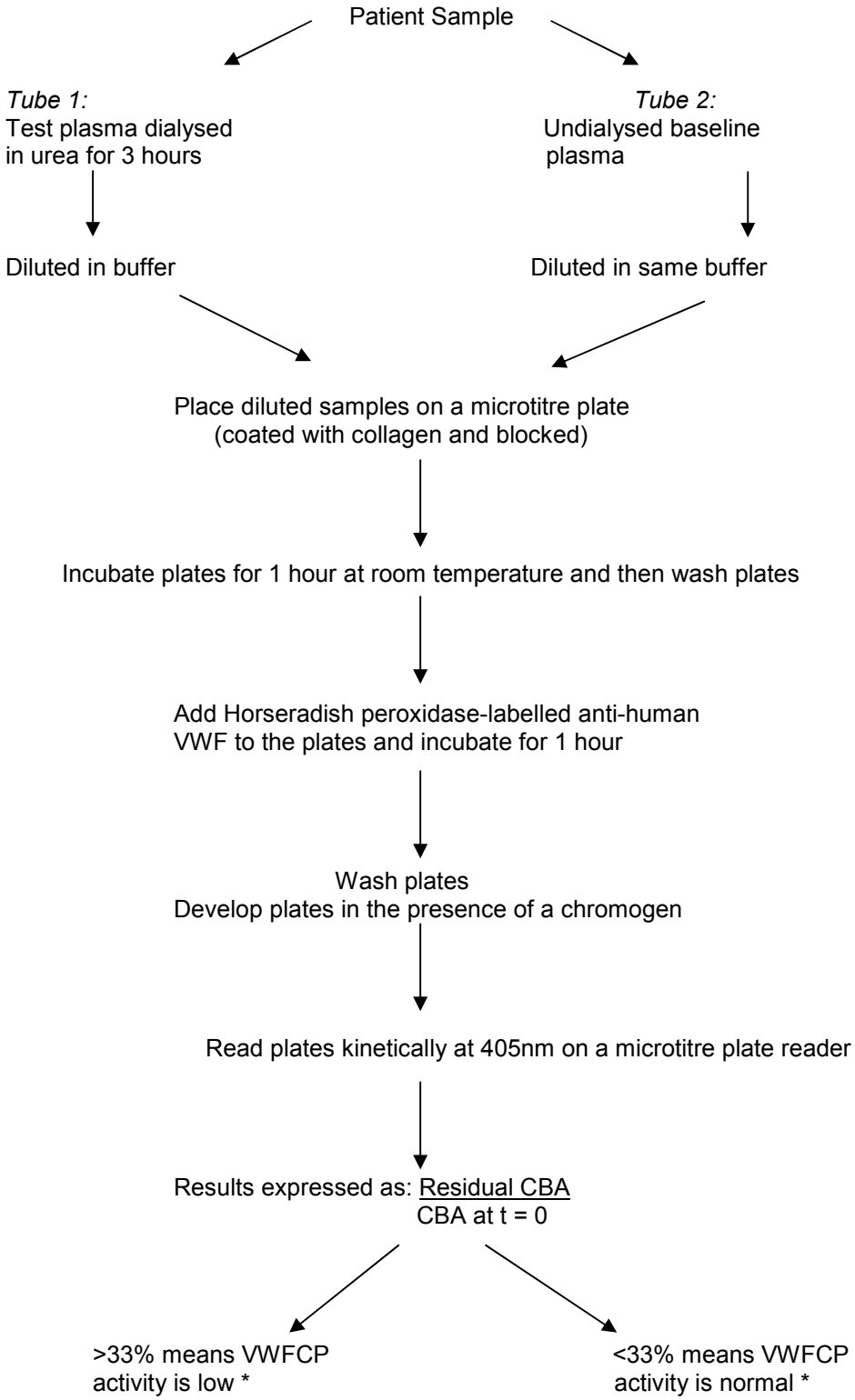

Figure 8. Flow diagram of the residual collagen binding assay laboratory methods (as per the method described by Rick, et al., 2002). * % RCBA cut-off as established by Rick, et al., 2002.

Explanation of the flow diagram of the assay procedure

The assay used, is as per Rick and co-workers, who developed a simple rapid assay for the detection of VWFCP activity based on the collagen binding properties of VWF. The assay does not require specialized reagents and can be completed within 8 hours (Rick, et al., 2002). The plasma of the patient is used as both the source of the enzyme and the substrate, negating the need for purified VWF or other preparation of normal plasma VWF (Rick, et al., 2002; Aronson, Krizek & Rick, 2001). Test plasma (approximately 0.5ml) is dialysed for 3 hours in urea (which facilitates the VWFCP with proteolytic cleavage of VWF multimers) and is then diluted in buffer.

Undialysed plasma samples are diluted in the same buffer. The diluted samples are then placed on a microtitre plate, previously coated with 3µg/ml of collagen and blocked. After a 1hour incubation period at room temperature, the plates are washed and horseradish peroxidase-labelled anti-human VWF is added to the plate and incubated for 1 hour. The plates are then washed, developed in the presence of a chromogen and read kinetically at 405nm on a microtitre plate reader. Results are then expressed as the (dialysed) residual collagen binding activity (CBA) divided by the (undialysed) baseline CBA (at t = 0).

Clinical utility would probably involve providing a result indicating either a normal or decreased protease activity, rather than a quantitative result.

A normal level of protease activity leads to a decrease in collagen binding by VWF due to a decrease in the high molecular weight multimers of VWF (VWFCP cleaves these high molecular weight multimers). Only VWF which has not been sufficiently cleaved by VWFCP, will bind to the collagen coated microtitre plates. Reduced VWFCP activity results in increased collagen binding of VWF multimers, as they are not proteolytically cleaved by VWFCP (either due to a deficiency of VWFCP or autoantibody).

3.5 Assay development

Refer to Figures 9, 10, 11, 12 and 13 for photographs of some of the laboratory equipment used.

3.5.1 Buffer Preparation

Buffers were prepared as follows:

Collagen Buffer: To Prepare 500mls:

- 3,0285g Tris; 2,922g NaCl
- Distilled Water was added up to 300mls at first as HCL needs to be added to achieve a pH of 7,4, thereafter more distilled water could be added up to a total volume of 500mls.
- 50µl was used per well therefore 5mls of this buffer was required per microtitre plate.

Sample/Dilution Buffer: To prepare One Litre:

- 3,0285g Tris; 2,922g NaCl; 1000µl Tween
- Distilled Water was added up to one litre, but pH of 7,4 was required before the full volume was added.
- The end of the pipette tip was cut off when working with Tween due to its viscosity.

-this buffer was used for dilution of patient samples and antibody solution.

Reaction/Dialysis Buffer: To Prepare One Litre:

- 0,6057g Tris; 1000µl Tween
- 90,75g Urea-Distilled Water was added up to one Litre, but a pH of 8,3 was required before full volume was added.

Wash Buffer: To Prepare One Litre:

- 6,057g Tris; 5,844g NaCl; 1000µl Tween
- Distilled Water was added up to One Litre but a pH of 7,4 was required before full the volume is added.

Phosphate Buffered Saline:

- A prepared tablet form was used, dissolved in 100mls distilled water and mixed with a magnetic stirrer (PBS Dulbecco A 100 tablets from C A Milch™).

3.5.2 Preparation of Collagen-coated and blocked microtitre plates

- Collagen Solution was made up per number of plates required at 50 µl per well, using Collagen buffer with Vitrogen ™ Collagen: from Ilex Medical Systems ™ (stored in fridge) at a concentration of 3µg/ml. A concentration of 3µg/ml Collagen is required, per plate (50µlx96wells =5mls), therefore the total amount of collagen required was 15µg (5mls x 3µg). One µl of Vitrogen ™ Collagen is equivalent to 3µg of Vitrogen ™ Collagen. Therefore per microtitre plate: 5µl Vitrogen ™ Collagen was required per 5mls collagen buffer to have a collagen concentration of 3µg/ml.
- Once the above collagen solution was prepared, 50µl of this solution was pipetted into each well and incubated for one hour at room temperature on a rocker. ELISA plates used: from NUNC ™: Immunomodule F16 Maxisorp. Therefore for running of patient samples, a full 96 well plate does not have to be used (F16 individual strips placed in NUNC ™ frames).
- The plates were washed 3 times with Wash buffer (using multichannel pipette at 200µl per well).
- Blocking agent was prepared to block the microtitre plates. Superblock ™ Dry Blend Blocking Buffer in TBS (from Pierce ™, 37545) was used. Each pouch of dry Superblock ™ was reconstituted (make sure completely dissolved) with 200 mls distilled water just before use.
- 200µl of the above blocking solution was pipetted into each well. The plate was immediately emptied by inversion. This step was repeated twice.
- The plates were washed 3 times in Wash buffer.
- Storage:

  -Phosphate Buffered Saline (PBS) was added at 200µl per well to fill up the wells so as to prevent them from drying out.

  -Plates were covered with Parafilm/ NUNC ™ sealing tape.

-Plates were placed in plastic packet labelled with date and content (stored upright).

-Stored at 4 ºC in the fridge overnight (can be stored for 4 weeks).

3.5.3 Sample Preparation and Dialysis

- The baseline patient plasma samples were Barium Chloride exposed which "activated" the VWFCP enzyme in the sample prior to dialysis in urea (in reaction/dialysis buffer). Mild denaturing conditions with urea and exposure to Barium Chloride promoted VWFCP interaction with VWF (Tripodi, et al., 2004). ADAMTS13 requires divalent metal ions for proteolytic activity (Kokame, et al., 2005) such as Ba^{2+} or Ca^{2+} ions.

  To make up 50mls Barium Chloride solution the following was used:

  One part $BaCl_2$ to 9 parts collagen coating buffer:

  1.1g $BaCl_2$ up to 50mls with collagen coating buffer.

  120uls baseline patient plasma taken and 12ul of the $BaCl_2$ solution added. Incubated for 5 minutes and then proceeded with the assay where 50ul of the sample was dialysed and the remainder was left at room temperature (undialysed sample).
- 60µl of each sample was placed in a minidialysis unit: Slide-A-Lyzer MINI-Dialysis ^{TM} units 10,000 MWCO from Separations ^{TM} used (label each sample), inserted into the floater and immersed in approximately 100mls (although Rick and co-workers suggest one litre of reaction/dialysis buffer, however, we used a smaller container.
- The volume of bath to sample should be >30:1 of reaction/dialysis buffer which was prewarmed to 37 ºC in a sealed glass beaker/container in a water bath. These samples were dialysed for three hours in the above mentioned sealed beaker/container which was placed in the waterbath (which should remain at 37 ºC).
- Dialysis for 3 hours in a buffer containing urea facilitates partial VWF unfolding to expose the cleavage site at Tyr 842 – Met 843 in VWF multimers (Furlan & Lämmle, 2001). Hypotonic salt solution, urea, guanidium chloride or shear stress cause conformational change in the VWF molecule, thereby exposing the cleavage site (Furlan & Lämmle, 2001).

- The undialysed samples remained at room temperature during this time.
- 1:20 dilutions of both the dialysed and undialysed samples were prepared, using sample plasma and sample/dilution buffer.

  1:20 dilution: 50µl patient sample and 950µl sample/dilution buffer.

  buffer (each undialysed and each dialysed sample were run in duplicate on the microtitre plate).
- 50µl of the 1:20 of both the dialysed and undialysed sample were added to the each microtitre well and this was performed in duplicate.

3.5.4 Addition of samples to Microtitre Plate

- Refer to Table 2 and Table 3 which show the numbering of 96 well ELISA plate.
- Undialysed samples (50µl/well) were run in rows A, C, E and G with 1:20 dilutions (each in duplicate). 20 patient samples could be run per 96 well microtitre plate (as 8 wells were used for both dialysed and undialysed normal pool control sample, 4 wells were used for background antibody and substrate and 4 wells were used for background substrate, see below).
- Dialysed samples (50µl/well) were run in rows B, D, F and H with 1:20 dilutions (each sample run in duplicate).
- A1, A2, G11, G12, were used for only Antibody and substrate (which are added at a later stage) and no patient sample was added to these wells.
- At this point in time only PBS is added to these wells (50ul/well).
- B1, B2, H11, H12 were used for only Substrate (which is added at a later stage) and no patient sample was added to these wells.

  At this point in time only PBS was added to these wells (50ul/well).
- Undialysed normal pool control sample was added to wells C1, C2, E11, E12.

  Dialysed normal pool control sample was added to wells D1, D2, F11, F12.
- Samples were incubated in the microtitre plate for one hour at room temperature, on a rocker.
- The microtitre plate was washed 3 times with Wash buffer.

3.5.5 Addition of Peroxidase-labelled Anti-human Von Willebrand Factor Antibody to microtitre plate

- Peroxidase-labelled Anti-human Von Willebrand Factor (VWF) antibody: Rb a Hu Von Willebrand Factor / HRP 2ml from Diagnostech ™ (stored in fridge) was added to the microtitre plate at a 1:400 dilution.

  92 wells require antibody (the remaining 4 wells have substrate only) at 50µl per well (made up a little extra though, therefore for 96 wells per plate) 96 x 50 = 4800µl antibody solution was required.

  For a 1:400 dilution of the antibody for one microtitre plate:

  Required 12µl of antibody and 4788µl buffer (Use sample buffer).
- 50µl of the above antibody solution was added to each well, except B1, B2, H11, H12 which would only have substrate added at a later stage (these wells were filled with only Phosphate Buffered Saline at 50µl/ well at this point in time).
- Incubated for one hour at room temperature on a rocker.
- Washed plate 3 times with Wash Buffer.

3.5.6 Addition of Substrate to Microtitre Plate

- One substrate tablet was added (ABTS™ tablets from Roche™ catalogue number: 1204521. 5mg x20 tablets, light sensitive and stored in fridge), to 5mls of commercial substrate solution (Buffer for ABTS ™ from Roche ™ catalogue number: 1204530 125ml, also stored in fridge) in a test tube and immediately covered with foil. This was stored in a dark cupboard while the tablet was dissolving.
- 50µl of the above substrate solution was added to each well, using the multichannel pipette (this is quicker as the substrate is light sensitive). Allowed for slightly more than 5mls of the above solution per plate.
- Incubated the microtitre plate in a dark cupboard for 30 minutes.
- Read the plate on a Microtitre Plate Reader at precisely 45 minutes (at 405nm wavelength). 30 minute absorbance readings were also taken for comparison, however the 45 minute absorbance readings were used.

- Divided dialysed absorbance reading by undialysed absorbance reading and multiplied by 100 to obtain percentage RCBA . As each sample is run in duplicate, the average of the two readings were used.

Table 2. Depiction of numbering of 96 well ELISA plate for addition of patient sample.

A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

Table 3. Depiction of sample placement per 96 well ELISA plate. Ab+S indicates Antibody and Substrate only; S indicates Substrate only; NPu indicates Normal Pool Undialysed; NPd indicates Normal Pool Dialysed; P1u-P20u indicates Patient sample 1-20 Undialysed; P1d-P20d indicates Patient sample 1-20 Dialysed.

Ab+S	Ab+S	P3u	P3u	P7u	P7u	P11u	P11u	P15u	P15u	P19u	P19u
S	S	P3d	P3d	P7d	P7d	P11d	P11d	P15d	P15d	P19d	P19d
NPu	NPu	P4u	P4u	P8u	P8u	P12u	P12u	P16u	P16u	P20u	P20u
NPd	NPd	P4d	P4d	P8d	P8d	P12d	P12d	P16d	P16d	P20d	P20d
P1u	P1u	P5u	P5u	P9u	P9u	P13u	P13u	P17u	P17u	NPu	NPu
P1d	P1d	P5d	P5d	P9d	P9d	P13d	P13d	P17d	P17d	NPd	NPd
P2u	P2u	P6u	P6u	P10u	P10u	P14u	P14u	P18u	P18u	Ab+S	Ab+S
P2d	P2d	P6d	P6d	P10d	P10d	P14d	P14d	P18d	P18d	S	S

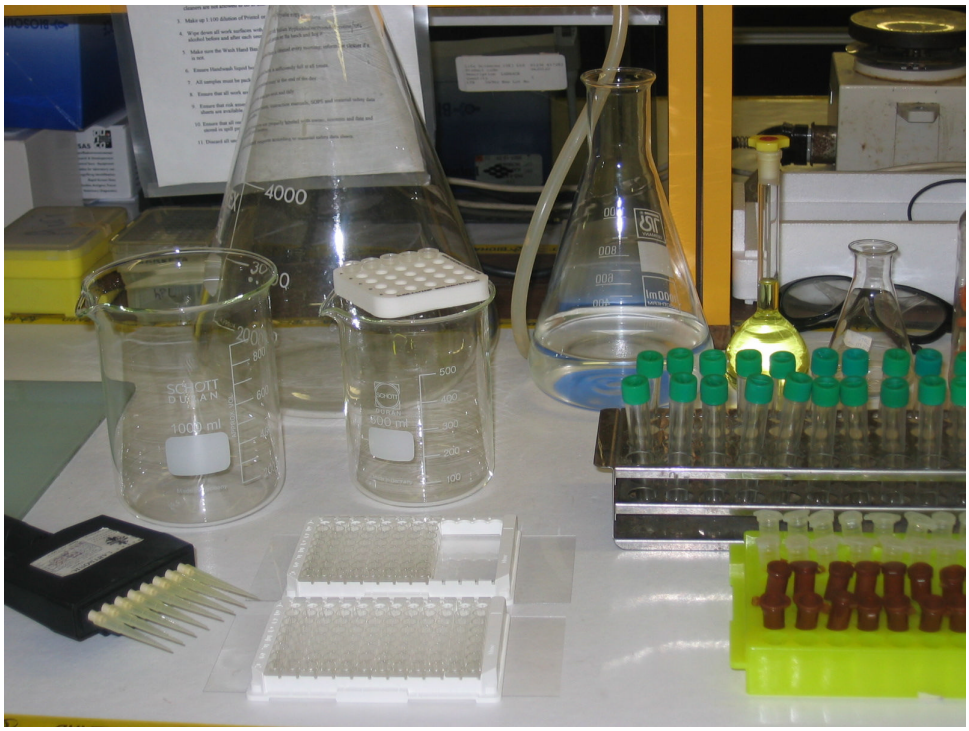

Figure 9. Photograph of some of the laboratory equipment used.

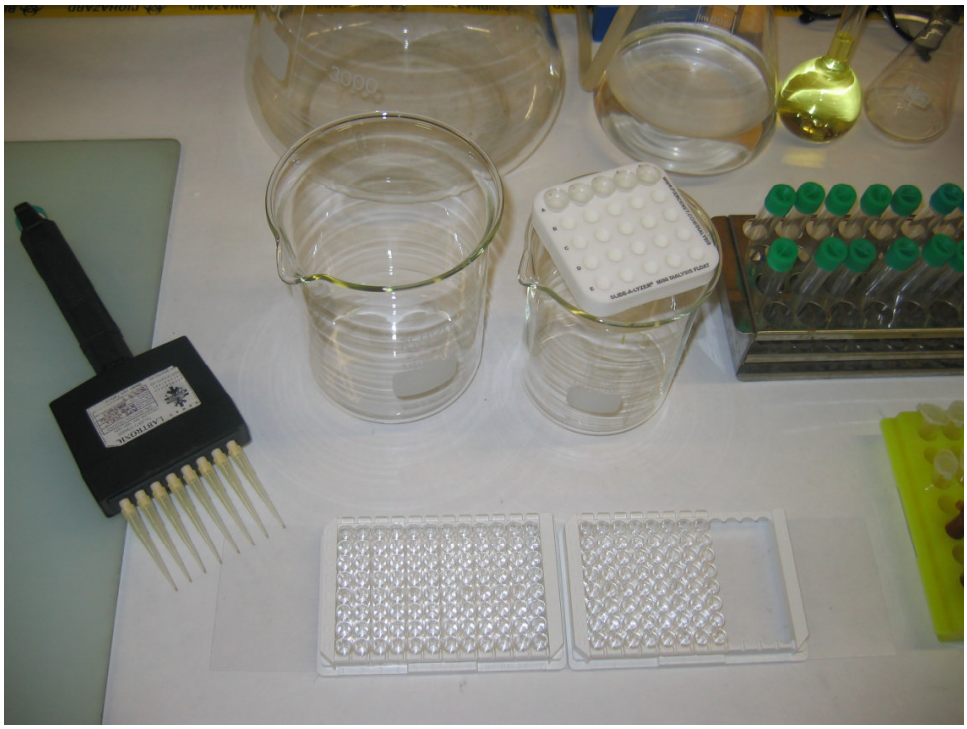

Figure 10. Photograph of NUNC ™ ELISA plates and minidialysis float ™.

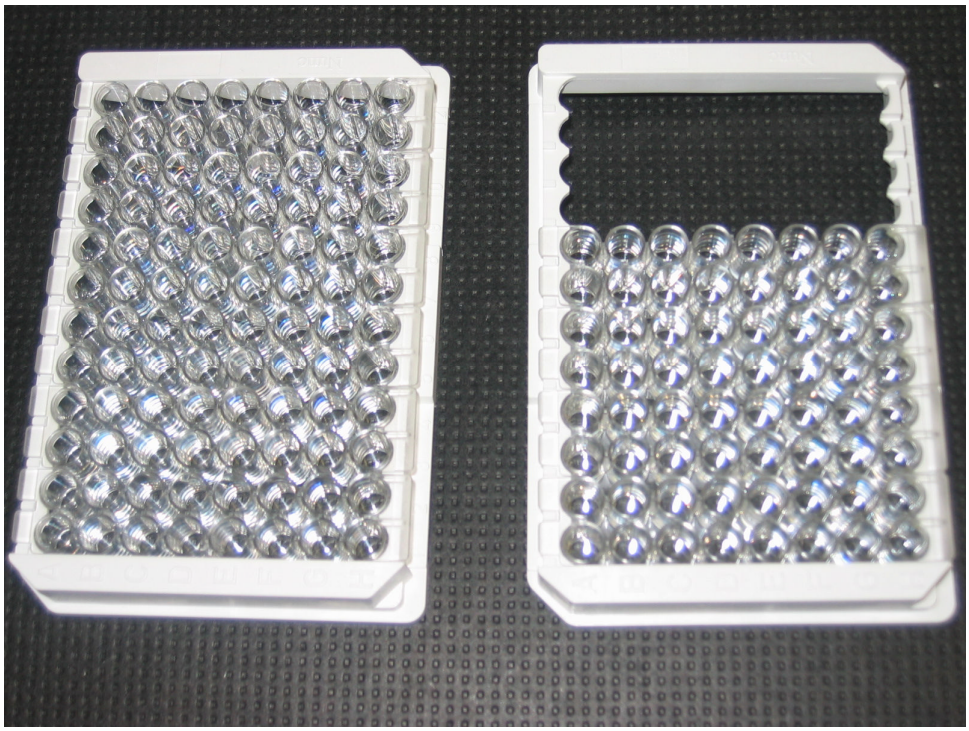

Figure 11. Photograph of NUNC [TM] ELISA immunomodules and frames used.

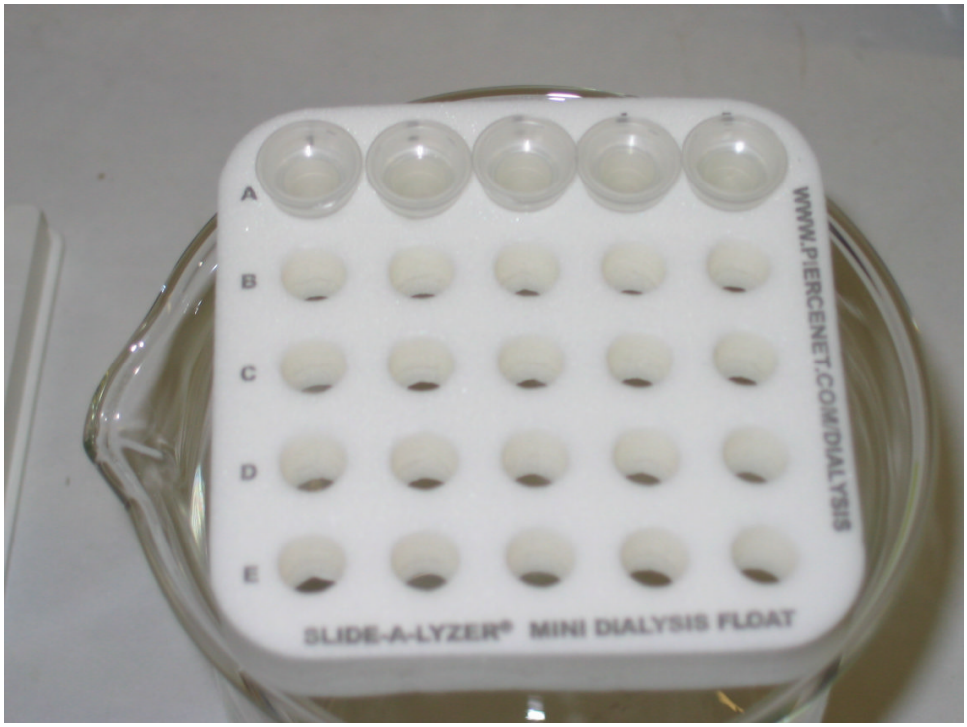

Figure12. Photograph of Slide-A-Lyzer [TM] Minidialysis float and minidialysis units.

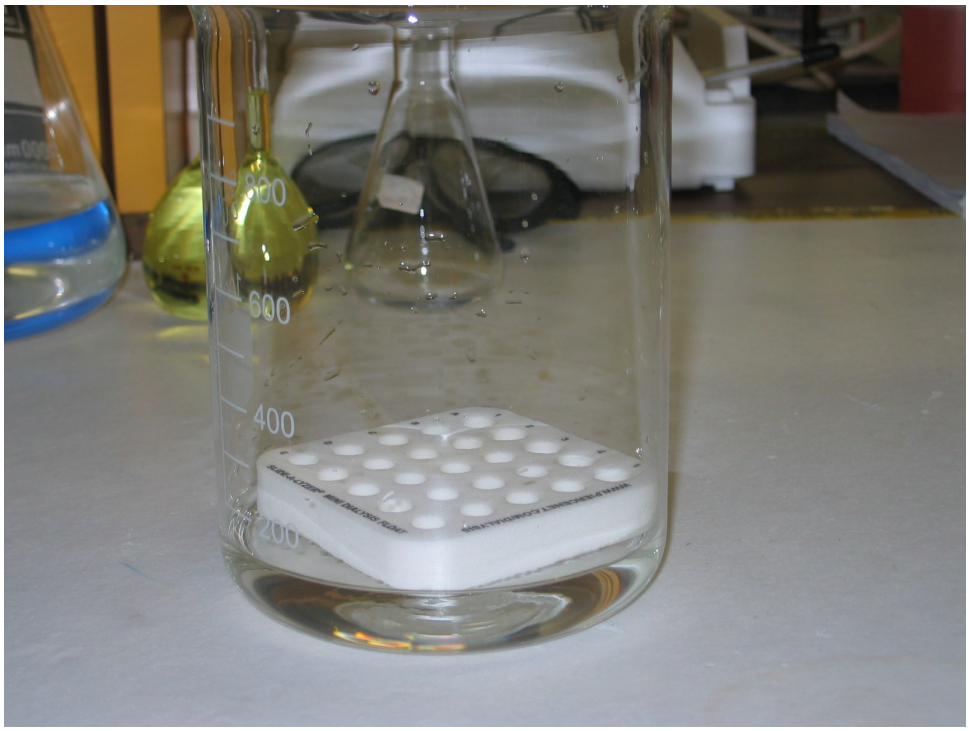

Figure 13. Photograph of minidialysis float [TM] immersed in dialysis buffer prior to placement in a waterbath at 37 ºC for 3 hours.

3.5.7 Mixing Studies

This is a modification of the assay which was used to evaluate the presence of an inhibitor to VWFCP activity. Samples of plasma from patients with high % RCBA (i.e. low VWFCP activity) were mixed with equal volumes of pooled normal plasma (confirmed to have normal enzyme activity) and incubated at room temperature for 30 minutes prior to the dialysis step. Correction of the % RCBA back into the normal range due to activity of the added protease from a documented normal pool control sample was taken to indicate absence of an inhibitor. Conversely, persistent elevation of the % RCBA suggested the presence of an antibody inhibitor limiting the activity of the added extrinsic protease from the control sample.

3.6 Quantitation of Von Willebrand Factor antigen levels in patients with TTP and HIV (Group 4)

Quantitative determination of VWF antigen levels was done on the same citrated plasma samples collected before therapy from each patient. A commercial automated latex enhanced immunoassay was used for the quantitative determination of VWF antigen in citrated plasma on IL Coagulation Systems. VWF antigen levels are reported in % of normality.

3.7 Statistical analysis

Statistical programmes used include SAS Version 9.1 and STATA Version 9.
Statistical significance was assessed at the 5% level.

Non-parametric procedures namely the Kruskal-Wallis test and the Wilcoxon rank-sum test were used to assess the three patient groups:
The Kruskal-Wallis test was used to compare the three groups (Group 1, Group 2, Group 3).
The Wilcoxon rank-sum test was then used to do pair-wise comparisons of these groups (Group 1 versus Group 2; Group 1 versus Group 3; Group 2 versus Group 3).
Analysis of Variance was used to assess and compare the background non-specific binding of antibody and Substrate reactions between ELISA plates.

To find correlations, Pearsons and (Spearmans) Rank correlations were used which are parametric and non-parametric procedures respectively.

The null hypothesis Ho states: There are no statistically significant differences between the 3 patient groups (Group 1= Group 2= Group 3). *The alternate hypothesis H1 states:* There is at least one statistically significant difference between the various pairs of the 3 groups (p=0.0001).

Our Data will be visualized according to a plot of "% Residual Collagen Binding Activity" on the vertical axis and various patient groups on the horizontal axis.

4. Results

4.1 Summary of established optimal conditions for the ELISA

Refer to Table 4 which lists the parameters that were optimised for the ELISA. Further detail regarding optimisation is discussed further in the following section.

Table 4. Summary of the parameters that were optimised for the ELISA

Parameter	Optimisation step	Not Optimal
Patient samples	Citrated patient samples	Red top tubes (no additive)
Collagen concentration	3µg/ml for coating ELISA plate	4µg/l, 5µg/l, 6µg/l
Blocking agent	Superblock ™ Dry Blend	Casein
ELISA plate	NUNC ™ immunomodule ELISA plates & frames (batching samples)	Full ELISA plates
Patient sample dilution	1:20 (run in duplicate)	1:40
Primary antibody dilution	1:400	1:100, 1:200, 1:1000
Incubation times	One hour with patient samples and anti-VWF antibody	45 minutes
Reading ELISA plate	45 minutes after loading substrate	30 minutes, 60 minutes
Control	Normal pooled plasma as a standard for the ELISA plate	

4.1.1 Blocking of ELISA plates

The ELISA plates used by Rick and co-workers were pre-blocked. As pre-blocked plates could not be sourced and for cost-effectiveness, our own microtitre plates were blocked. Superblock ™ Dry Blend was compared to Casein (0.5g) as blocking agents.

Casein (0.5g) was made up 100 mls in both PBS and TBS/wash buffer and 200ul was pipetted into each microtitre well, incubated for various times ranging from 15 minutes to one hour.

As indicated in the section 3, each pouch of dry Superblock ™ was reconstituted (making sure it was completely dissolved) with 200 mls distilled water just before use. 200µl of the above blocking solution was pipetted into each well. The plate was immediately emptied by inversion. This step was repeated twice.

Overall the use of Casein was not found to be successful compared to Superblock ™. Refer to Appendix D for data obtained for ELISA plates blocked with Superblock ™ Dry Blend versus Casein (0.5g). The % RCBA values were overall greater for ELISA plates blocked with Casein with lower absorbance readings for both Undialysed and Dialysed samples. The background antibody and substrate and background substrate readings were however comparable for ELISA plates blocked with both Superblock ™ versus Casein. With the use of Casein, the higher RCBA percentages were due to the smaller difference between the Undialysed absorbance reading and the dialysed absorbance reading. Superblock ™ Dry Blend was therefore elected for use in this study.

In a split sample comparison of thirty eight samples, plates blocked with Superblock ™ compared to plates blocked with Casein as a blocking agent showed a mean % RCBA of 33.12% versus 43.9% respectively. This indicated that Superblock ™ was a more effective blocking agent.

4.1.2 Background non-specific binding

Background Antibody and Substrate reaction

The background Antibody and Substrate reaction was assessed in four microtitre wells per ELISA plate where only peroxidase-labelled Anti-human Von Willebrand Factor (VWF) antibody (Rb a Hu Von Willebrand Factor / HRP 2ml from Diagnostech ™) and subsequently Substrate was added with no addition of patient sample. Please refer to section 3 for further detail.

Established the background non-specific binding (Antibody + Substrate) absorbance readings with a mean value of =0.12, a minimum value of 0.11 and a maximum value of 0.13 (standard deviation=0.01). The 95% Confidence Interval for background non-specific binding is noted to be 0.118 to 0.124.

Using analysis of variance (ANOVA) no statistically significant difference was noted for the background antibody and substrate reactions between 13 ELISA plates (p=0.0825).

Background Substrate reaction

The background Substrate reaction was assessed in four microtitre wells per ELISA plate where only Substrate was added with no addition of patient sample or peroxidase-labelled Anti-human VWF antibody. We established the background substrate reaction with a mean value of 0.074, a minimum value of 0.069 and a maximum value of 0.08 (standard deviation=0.003).

4.2 Troubleshooting the Assay

The data from 2 ELISA plates were excluded, as the background non-specific binding of antibody and substrate was higher on these plates (0.1855 and 0.173 respectively). Thus instead of n=77 for Group 1, n=70, as 7 samples were excluded and instead of n=74 for Group 2, n=55, as 19 samples were excluded. However statistical analysis performed comparing the 3 patient groups before exclusion of these samples, revealed the same statistical end-points, however the % RCBA cut-off values for *Group 1:* was 1.41% higher at 38.53%, *Group 2:* was 1.86% higher at 52.04%, *Group 3*: was the same at 50.88%

The combined % RCBA cut-off value for Group 2 and Group 3 was 0.95% higher at 51.46% with inclusion of the data from the 2 excluded plates.

4.3 Reproducibility of assay

Inter-assay Reproducibility

Normal pool sample was run in quadruplicate on each ELISA plate and this data were used to assess the reproducibility of the assay. The inter-assay coefficient of variation is 8.80%.
For the normal pool sample, the interassay % RCBA ranged from a minimum of 24% to a maximum of 31.57% with a mean of 27.44%.

Intra-assay reproducibility

The data of normal pool sample run in quadruplicate on each ELISA plate were also used to assess intra-assay reproducibility. This revealed a coefficient of variation of 4.83%. As this is a manual technique, the variability may be related to pipetting error.

4.4 Data for HIV negative controls, HIV positive patients with normal platelet counts and low platelet counts

To establish whether the collagen binding assay would be of clinical utility in the setting of HIV, results of percentage RCBA in HIV positive (both low platelet counts and normal platelet counts) and HIV negative patients (with normal platelet counts) were compared. Results are given in percentage (absorbance readings of dialysed/ undialysed x 100) of RCBA. Group 1 constitutes HIV negative patients with normal platelet counts, Group 2 constitutes HIV positive patients with normal platelet counts and group 3 constitutes HIV positive patients with low platelet counts.

We established our own cut-off value for % RCBA in both HIV positive and HIV negative patients. The upper limit or cut-off of % RCBA for each group was established by the mean plus 2 standard deviations as per Rick and colleagues (Rick, et al., 2002).

In Group 1 (HIV negative patients with normal platelets), the % RCBA cut off value we obtained is 37.12%, which is comparable to the cut-off value used by Rick and co-workers (33.02%).

In group 2 (HIV positive patients with normal platelets), the cut-off value we obtained is 50.18% and in group 3 (HIV positive patients with low platelets), a similar cut-off value of 50.85% is noted.

The combined cut-off for the HIV positive patients with both normal and low platelets (Group 2 and Group 3) was 50.51% which is higher than that for HIV negative controls (Group 1).

Please refer to Table 5 for the standard deviation values used to calculate the cut-off values. Figure 14 depicts a box and whisker plot of the 3 groups which illustrates the higher % RCBA in the setting of HIV (Group 2 and Group 3).

Statistical analysis using the Kruskal-Wallis test and Wilcoxon rank-sum test showed a significant difference between Group 1 and Group 2 (p=0.0001) and between Group 1 and Group 3 (p=0.0006). However, there was no significant difference between Group 2 and Group 3 (p=0.7783). Refer to Table 7 for a summary of this information.

Table 5. Depiction of % Residual Collagen Binding Activity data from 3 groups of patients

Group	Number	Mean	Median	Minimum	Maximum	SD	cut-off
1	70	27.06%	26.97%	17.79%	38.62%	5.03%	37.12%
2	55	33.02%	30.45%	17.19%	55.12%	8.59%	50.18%
3	58	32.41%	30.55%	14%	55.24%	9.22%	50.85%

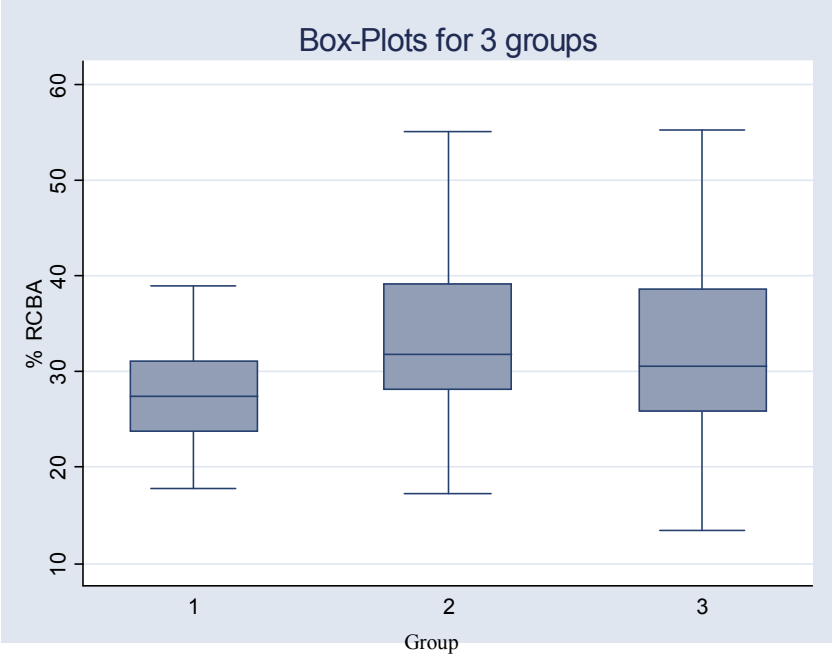

Figure 14. Box-plots of % Residual Collagen Binding Activity (RCBA) for 3 patient groups:
Group 1: HIV negative control patients;
Group 2: HIV positive patients with normal platelet counts;
Group 3: HIV positive patients with low platelet counts.

The % RCBA cut-off values are inversely related to the VWFCP activity. In HIV negative patients (Group 1), a result of less than 37.12% indicates normal VWFCP activity and a result greater than 37.12% reflects reduced VWFCP activity. In HIV positive patient groups regardless of the platelet count (Group 2 and Group 3), a result of less than 50.51% indicates normal VWFCP activity and a result above 50.51% indicates reduced VWFCP activity. The latter is depicted in Table 6.

Table 6. Depiction of % Residual Collagen Binding Activity cut-off value in HIV negative (Group 1) versus average for HIV positive (Group 2 and Group 3) patients and interpretation of Von Willebrand Factor Cleaving Protease (VWFCP) activity.

	Group 1: HIV negative	**Group 2 & 3: HIV positive**
% RCBA cut-off	37.12%	50.51%
Normal VWFCP activity	Less than 37.12%	Less than 50.51%
Reduced VWFCP activity	Greater than 37.12%	Greater than 50.51%

The Shapiro-Wilk test of normality revealed the data for Group 1 to be normally distributed ($p > 0.05$), the data for Group 2 is not normally distributed ($p < 0.05$) and the data for Group 3 is noted to be normally distributed ($p > 0.05$).

The data for Group 2 could be normalised on log-transformation and parametric analysis could therefore be applied to all groups. Student's T-test revealed that the level of significance remained unchanged, compared to Wilcoxon-rank sum, for the group to group comparisons as assessed by p-value. This therefore confirmed the trueness in the analysis of this data. Refer to Table 7 for a summary of this data.

Table 7. Summary of p-values for Group comparisons

Pair	Student's T-test	Wilcoxon rank-sum	Significance
Group 1 vs Group 2		p=0.0001	Different
Group 1 vs Group 3		p=0.0006	Different
Group 2 vs Group 3		p=0.7783	Same
Log-Group 1 vs Log- Group 2	p=0.000004		Different
Log-Group 1 vs Log- Group 3	p=0.00029		Different
Log-Group 2 vs Log- Group 3	p=0.7136		Same

4.5 Data for twenty HIV positive patients with TTP (Group 4)

Using this assay, a specific cut-off value could be established for HIV positive patients in general (regardless of platelet count), and further investigation into VWFCP levels in patients with HIV related TTP was warranted. Samples from twenty HIV positive patients with TTP were assessed using this assay and these patients constitute Group 4.

Table 8 shows the laboratory findings at presentation in the latter patients as well as the results and interpretation of the collagen bonding assay and mixing studies. The mixing studies are a modification of the collagen binding assay which was used to evaluate the presence of an inhibitor to VWFCP activity in patients with a high % RCBA.

Of the 20 patients, 6 (30%) had normal VWFCP activity (<50.5% RCBA). One patient had a borderline normal result of 51.2% which was included in the normal VWFCP activity group as the other 14 patients (70%) had severely reduced activity (as indicated by >90% RCBA). Loss of the plasma sample precluded assessment of inhibitors in one patient. Of the remaining 13 patients with severely reduced VWFCP activity, 8 (62%) had no inhibitors (%

RCBA within normal range post mixing) while 5 (38%) had a definite inhibitor. Refer to Table 9 for a summary of this data.

VWF antigen levels were high in all patients but the majority of the patients with severely reduced VWFCP activity had higher levels than patients with normal/ borderline VWFCP activity and this achieved statistical significance.

Table 8. Laboratory Data of 20 HIV positive patients with Thrombotic Thrombocytopenic Purpura (TTP). CD4 indicates CD4 count; Hb, haemoglobin; LDH, lactate dehydrogenase; INR, international normalized ratio; PTT, partial thromboplastin time; VWF Ag, Von Willebrand Factor antigen; %RCBA, percentage residual collagen binding activity; %RCBAc, corrected percentage residual collagen binding activity post mixing with 1:1 pooled normal serum; VWFCP, Von Willebrand Factor Cleaving Protease activity, Inhibitor, VWFCP inhibitor; ND, not done; SR, severely reduced.

	CD4	Hb	Platelets	LDH	INR	PTT	D-Dimers	Creatinine	VWF Ag	%RCBA	VWFCP	%RCBAc	Inhibitor
	$x10^6$/l	g/dl	$x10^9$/l	U/l			mg/l	µmol/l	%				
1	242	4.6	14	15077	1.28	28.6	0.98	60	252	110.9	SR	72.7	yes
2	389	6.7	9	2335	1.2	37	4.98	136	179	98.8	SR	38.5	no
3	193	4.3	15	4772	1.45	30.9	1.22	65	263	92.8	SR	38.1	no
4	69	3.6	15	4962	1.08	27	0.9	68	114	34	Normal		
5	283	6.3	12	4737	1.01	28.2	1.5	75	248	118.4	SR	24.3	no
6	ND	9.1	11	5085	1.03	27.6	1.38	97	265	109.9	SR	87.3	yes
7	144	5.4	36	6468	1.35	32.6	5.45	279	422	93.1	SR	41.6	no
8	87	4.2	5	3833	1.22	46.8	1.68	116	405	99.7	SR	23.5	no
9	138	4.1	17	1335	1.12	46.2	2.94	107	167	51.2	Borderline		
10	17	5.9	22	1015	1.28	22.5	1.67	87	335	21.8	Normal		
11	163	5.9	5	3166	1	29.7	4.14	95	181	113.3	SR	47.7	no
12	408	8.3	53	737	1.13	26.2	0.85	47	161	24.5	Normal		
13	102	4.8	11	4052	1.27	32.6	4.93	1221	251	91.9	SR	44.8	no
14	89	5.2	12	2317	1.34	29.3	0.3	122	289	96.1	SR	71.2	yes
15	112	5.7	11	1948	1.22	26.5	ND	97	278	90.9	SR	75.3	yes
16	6	8.3	37	949	1.21	40	4.06	183	207	22.6	Normal		
17	210	5.2	12	3400	1.14	39.2	3.27	78	294	103.3	SR	43.2	no
18	65	4.7	8	3086	1.1	25.5	2.02	81	202	40.1	Normal		
19	818	4.9	10	4112	1.19	32.6	4.3	36	289	107.6	SR	ND	ND
20	ND	5.2	10	9750	0.84	22.8	0.6	90	279	100.5	SR	87.8	yes

Table 9. Depiction of normal and reduced Von Willebrand Factor Cleaving Protease (VWFCP) activity in 20 HIV positive patients with Thrombotic Thrombocytopenic Purpura.

	Normal VWFCP activity <50.5%RCBA	**Reduced VWFCP activity >50.5% RCBA**
Patients	6	14
Inhibitors	Not applicable	5

Figure 15 depicts that within the HIV positive group with TTP (Group 4) there is distinct separation between a group with normal VWFCP activity (% RCBA less than 50.51%) and a group with reduced VWFCP activity (% RCBA greater than 50.51%).

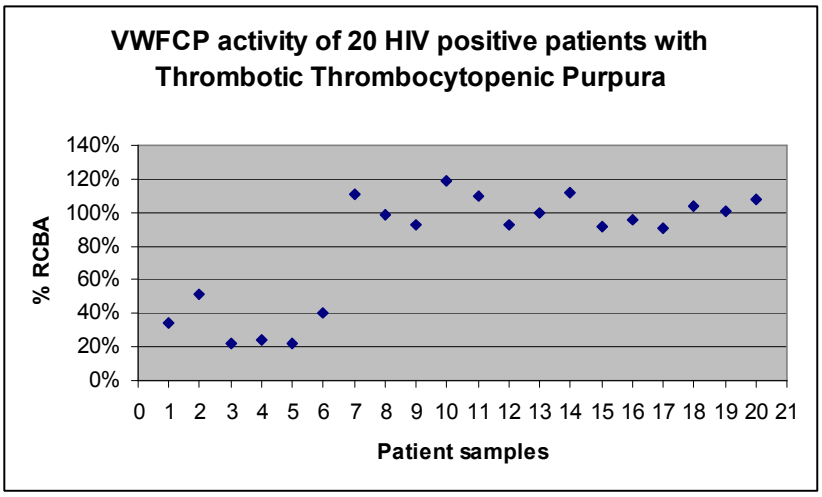

Figure 15. Scatter plot of Von Willebrand Factor Cleaving Protease Activity (VWFCP) in 20 HIV positive patients with Thrombotic Thrombocytopenic Purpura (TTP). Normal VWFCP activity is <50.5% RCBA) which is noted in 6 patients and Reduced VWFCP activity (>50.5% RCBA) is noted in 14 patients.

As seen in Figure 16, within group 4, 13 patients showed reduced VWFCP activity (% RCBA greater than 50.51%) and of these patients, in 5 patients there was no correction of % RCBA to below the cut-off value upon mixing studies (indicating the presence of an inhibitor) whereas in 8 patients there was correction of VWFCP activity (indicating the absence of an inhibitor).

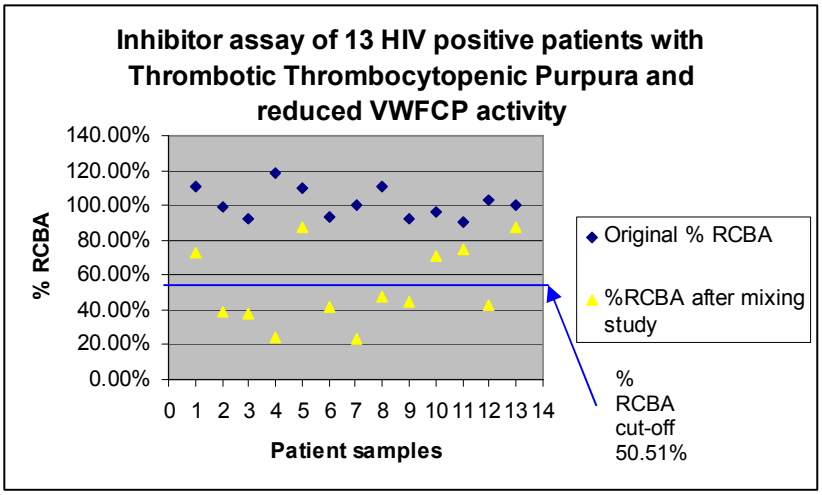

Figure 16. Scatter plot of Inhibitor assay (50:50 mixing study of Residual Collagen Binding Activity assay with normal pool sample) of 13 HIV positive patients with reduced Von Willebrand Factor Cleaving Protease (VWFCP) activity. In 5 patients the % RCBA did not correct to below 50.5% (inhibitor therefore present) and in 8 patients the % RCBA did correct to below 50.5% (inhibitor therefore not present).

The data for Group 4 did not have a normal distribution (Shapiro-Wilk test) and could not be normalised on log–transformation or square-root transformation. Non-parametric statistical analysis was therefore applied to compare groups. Using the Wilcoxon rank-sum test, a statistically significant difference was noted for the % RCBA between Group 4 (HIV positive patients with TTP) and Group 1 (HIV negative controls), $p<0.0001$, between Group 4 and Group 2 (HIV normal platelets) $p=0.0001$ and between Group 4 and Group 3 (HIV low platelets) $p=0.0003$. Refer to Table 10 for a summary of this data.

Table 10: Summary of statistical analysis for Group 4 versus other Groups using the Wilcoxon rank-sum test.

Pair	Wilcoxon rank-sum	Significance
Group 1 versus 4	p<0.0001	Different
Group 2 versus 4	p=0.0001	Different
Group 3 versus 4	p=0.0003	Different

Statistical analysis of the data for Group 4 (HIV positive patients with TTP) using the Kruskal-Wallis test and Wilcoxon rank-sum test revealed significantly lower CD4 counts (p=0.0492) and LDH levels (p=0.0168) and higher platelet counts (p=0.0282) at presentation in patients with normal VWFCP activity. Reduced VWFCP activity in the absence of a detectable inhibitor was associated with significantly higher D-Dimer levels (p=0.0108).

VWF antigen levels were high in all patients but those patients with severely reduced VWFCP activity had significantly higher levels (p=0.0320) than patients with normal VWFCP activity. No significant difference was found between patients with reduced VWFCP levels with and without inhibitors (p=0.5582).

4.6 Graphical depiction of the summary of the four groups

Figure 17 depicts the four patient groups and highlight the similar distribution for groups 2 and 3 (HIV positive patients with both normal and low platelets) which is different from Group 1 (the HIV negative control group). The latter graph also highlights that Group 4 (HIV positive patients with TTP) is distinctly different from the other groups.

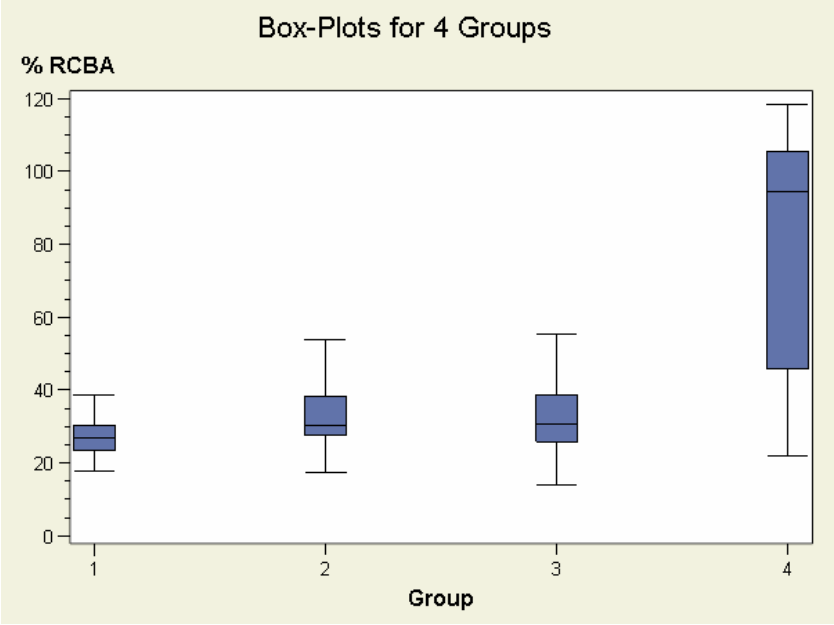

Figure 17. Box and whisker plot comparing the % RCBA between the four different groups.
Group 1: HIV negative control patients;
Group 2: HIV positive patients with normal platelet counts;
Group 3: HIV positive patients with low platelet counts;
Group 4: HIV positive patients with TTP.

4.7 Robustness of the assay

Various samples were re-run to assess the robustness of the assay.
When samples of 17 HIV positive patients with TTP were re-run, this showed results which remained consistently below the defined % RCBA cut-off value for HIV positive patients (50.51%).

Despite small differences in % RCBA in some cases, the results consistently fell below the % RCBA cut-off value and thus clinical decisions would not be affected by these small differences.

When samples of 20 HIV positive patients with TTP (from Group 4) were re-run, the % RCBA remained consistently either above or below the % RCBA cut-off value in each patient.
Re-run of mixing studies in 5 HIV positive patients with TTP showed consistent results.

Of note is that undialysed and dialysed samples should be run on the ELISA plates immediately, as freezing these samples and subsequently thawing and re-running at a later point reveals significant discrepancies in % RCBA, such that the values for the same patient in some cases are either below or above the designated % RCBA cut-off value. Thus freezing and thawing of undialysed and dialysed samples affects analysis and is thus not recommended as misclassification of the result (for example below the cut-off as opposed to above the cut-off for % RCBA) would result in misdiagnosis.

5. Discussion

5.1 TTP and VWFCP

Thrombotic Thrombocytopenic Purpura (TTP) is a disease characterized by microvascular platelet aggregation and thrombus formation in selected tissue beds resulting in a pentad of: thrombocytopenia, microangiopathic haemolytic anaemia, renal failure, neurological symptoms and fever (Amorosi & Ultman, 1966; Cines, Konkle & Furlan, 2000; Moake, 2002; Rock, et al., 2000). One cannot wait for the full pentad of symptoms of TTP to develop, as early diagnosis full pentad and treatment are imperative for survival.

A possible key role for Von Willebrand Factor in the pathogenesis of TTP was first suggested by Moake et al, in 1982 who postulated a deficiency of a VWFCP as the cause for the presence of ULVWF multimers documented in the plasma of four patients with chronic relapsing TTP (Moake, et al., 1982). In 2001, several groups identified ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1 motif 13) as the VWFCP (Furlan, Robles & Lämmle, 1996; Tsai, et al., 1996) and deficiency of ADAMTS13 was implicated in the pathogenesis of TTP (Furlan, et al., 1998).

The measurement of VWFCP activity in the setting of TTP as well as other pathological states is a focus of much research (Levy, Motto & Ginsburg, 2005). One of the most common haematological manifestations of HIV infection is thrombocytopenia which may be multifactorial in nature. There is a broad differential diagnosis of thrombocytopenia, anaemia and neurological dysfunction in patients with AIDS (Cines, Konkle & Furlan, 2000). It has long been proposed that HIV infection predisposes to TTP and other TMA (Boccia, et al., 1984; Jokela, Flynn & Henry, 1987). TTP may be the presenting clinical manifestation of HIV infection, although the majority of cases occur in the context of AIDS (Hymes & Karpatkin, 1997).

Tamkus and colleagues, suggest consideration of TMA as an AIDS-defining illness so as to facilitate the early recognition, effective treatment and improve survival of this life-threatening but treatable condition (Tamkus, et al., 2006). The pathophysiology of TTP, specifically in the

setting of HIV is not known and only limited data is available regarding VWFCP activity or the presence of autoantibodies in these patients. Early diagnosis and treatment of TTP is imperative for survival (Cines, Konkle & Furlan, 2000; Rock, et al., 2000; Levy, Motto & Ginsburg, 2005) and assay of VWFCP levels can be useful in this regard. However, as the therapy for TTP is very costly, improved diagnostic tools would be of value in confirming the diagnosis of TTP.

5.2 The rapid collagen binding assay

There is currently no assay available in our laboratory setting for the measurement of VWFCP. The semi-quantitative rapid collagen binding assay described by Rick and co-workers, is an ELISA-based assay which relies on assessment of the residual collagen binding activity (RCBA) of the patient's own plasma-derived VWF.

Although this is only a semi-quantitative assay, it was chosen for use in our laboratory because of its practical advantages in terms of cost-effectiveness (refer to Appendix A for a breakdown of cost) and time effectiveness. It would therefore be suitable for routine use. This assay is effective and reliable in the distinction between very low VWFCP activity and normal or borderline VWFCP activity.

Samples can be stored frozen at -70 ºC and if required the samples can be batched, thereby optimising resources. With the use of NUNC ^{TM} immunomodules, only the required number of wells can be selected as opposed to using a full ELISA plate for a few patient samples, thereby optimising cost.

With the use of very small concentrations of collagen (3µg/ml) batch/ lot variability is eliminated as one purchase of Vitrogen ^{TM} Collagen (100mls) can be utilised for a very large number of tests. A derivation of this assay is used to assess for the presence of an associated inhibitor and is based on a mixing study. Inhibitor detection may have therapeutic implications such as consideration of the benefit of associated steroid therapy (which may be controversial in the setting of already immunocompromised individuals).

In the multicentre study by Tripodi et al., comparing the results of measuring ADAMTS13 in the same coded plasmas by 11 methods, the semi-quantitative collagen binding assay by Rick et al., showed excellent reproducibility (average coefficient of variation of less than 10%), relatively good linearity of expected versus observed results (r^2 value 0.81) and intermediate ability to discriminate between different VWFCP levels (Tripodi, et al., 2004).

This MMED study obtained an inter-assay coefficient of variation of 8.80% and an intra-assay coefficient of variation of 4.83%.

The assay used was chosen for its practical advantages. Some limitations were identified. It is a semi-quantitative assay based on residual VWF-collagen binding activity rather than direct VWFCP measurements. This assay requires a 3 hour incubation period of plasma samples in a denaturing environment (urea) in order to detect metalloprotease activity and may not accurately reflect enzymatic activity under physiological flow conditions in vivo. The method used for inhibitor analysis may not be sufficiently sensitive to detect weak inhibitors especially as correction of RCBA back into the normal range for HIV positive patients (who have mildly reduced protease levels compared to non HIV infected individuals) was taken as the defining criteria to exclude an inhibitor. Moreover, non-neutralizing antibodies may be present which accelerate ADAMTS13 clearance or inhibit binding (Scheiflinger, et al., 2003) but do not interfere with its activity in vitro.

This assay tended to give disproportionately higher VWFCP measurements at the lowest levels (Tripodi, et al., 2004) and other quantitative assays may have a more accurate minimum detection limit. This may result in some false negative results, which may be problematic and consideration of other laboratory and clinical correlation is imperative. Tripodi and co-workers, do not report false positive results with the use of this assay, which could result in patients with borderline clinical findings receiving inappropriate therapy. As a semi-quantitative screening assay, this assay has advantages in terms of cost-effectiveness and accommodation of many samples simultaneously.

Initial studies by Studt and co-workers, revealed that the collagen binding assay appears to be subject to disturbance and is delicate and some improvement in terms of accuracy is required, although no systematic error was identified (Studt, et al., 2003). However, as per an international collaborative study of eleven methods for ADAMTS13 by Tripodi and co-workers, the best overall performance was noted for three methods: the ristocetin cofactor activity, the collagen binding assay and the immunoblotting of degraded multimers of VWF substrate (Tripodi, et al., 2004). Reproducibility studies demonstrated that the most precise methods were the semi-quantitative collagen binding assay (as per Rick, et al., 2002) and the ristocetin cofactor activity where the average coefficient of variation was less than 10% (Tripodi, et al., 2004).

Tripodi and co-workers, conclude that a varied performance of the methods is noted and an overall optimistic view of the reliability of currently available methods for ADAMTS13 detection is supported (Tripodi, et al., 2004).The subsequent more recent assays for assessing ADAMTS13 activity including the Enzyme-Linked Immunosorbent Assay using recombinant VWF-A2 domain as a substrate used by Whitelock et al., and Zhou et al., and the FRETS-VWF73 assay, used by Kokame et al., have not yet been compared in a multicentre trial (Whitelock, et al., 2004; Zhou & Tsai, 2004; Kokame, et al., 2005). The latter two assays appear to have practical advantages in terms of efficiency, although may not be as cost-effective as the rapid collagen binding assay.

Currently there is no gold standard assay available in our laboratory setting for comparison. Although other assays have subsequently become available since the commencement of this MMED project, the rapid collagen binding assay remains a very cost-effective assay and is a practical tool for screening VWFCP activity.

Freezing and thawing of undialysed and dialysed samples affected analysis in this study. Of note, however is that as per Tsai & Lian, analysis of control plasma that was stored frozen for at least 20 years did not have an adverse effect on VWFCP activity (Tsai & Lian, 1998).

In the literature there are some references to the use of a serine protease inhibitor such as Pefabloc SC ^{TM}, which is incubated with the plasma sample so as to prevent non-specific proteolysis of VWF by other enzymes in plasma. Pefabloc SC ^{TM} (or other serine protease inhibitors) serves to inhibit serine proteases in plasma samples (Shelat, Ai & Long Zheng, 2005; Furlan, et al., 1998; Furlan, et al., 1997; Zheng, et al., 2004). As per Rick and co-workers, we did not use a serine protease inhibitor.

5.3 Findings in HIV positive patients

This MMED study established the optimal conditions for this assay in our laboratory and ascertained the cut-off values for % RCBA (inversely proportional to VWFCP activity) in our laboratory in the setting of HIV negative and HIV positive patients. VWFCP activity was also measured in patients with HIV related thrombocytopenia (of any cause except TTP) in order to assess whether this is the same as in HIV positive patients with normal platelet counts and not markedly decreased which would preclude any diagnostic utility in HIV related TTP.

This study then compared VWFCP activity in HIV positive patients with low platelet counts with HIV positive patients with normal platelet counts and HIV negative control patients.

The % RCBA for the HIV negative group (Group 1) was statistically significantly different from the HIV positive group with normal platelets (Group 2), p=0.0001 and from the HIV positive group with low platelets (Group 3), p=0.0006. However, the two HIV positive groups with normal platelets (Group 2) and low platelets (Group 3) were not statistically significantly different (p=0.7783).

The cut-off value of % RCBA for normal VWFCP activity in HIV positive patients was 50.51%, as determined from the mean plus two standard deviations (as per Rick, et al., 2002) in 113 control patients with HIV infection but no TTP (Group 2 and Group 3 combined). This cut-off value is higher than for HIV negative controls (37.12% determined from the mean plus two standard deviations in Group 1) and this indicates mildly reduced enzyme activity in HIV positive patients regardless of the platelet count. This concurs with the finding that VWF

levels are increased in HIV positive patients (Aukrust, et al., 2000) and that there is a negative association between VWF antigen levels and VWFCP activity (Mannucci, Capoferri & Canciani, 2004).

Mildly decreased VWFCP activity has been described in numerous other disease states, many of which may occur in association with HIV infection. Decreased VWFCP activity in many of these pathological states, may also be on the basis of continued elevation of plasma VWF due to chronic inflammation. However, additional factors such as proteolytic inactivation of VWFCP by thrombin (Crawley, et al., 2005) in conditions which are associated with coagulation activation (such as DIC), may contribute to down-regulation of VWFCP activity and have relevance in the context of HIV infection.

Our finding of mildly reduced VWFCP activity in the setting of HIV infection (regardless of the platelet count) does not preclude the diagnosis of TTP as only severely reduced VWFCP activity is relevant. The mildly reduced VWFCP activity noted in HIV positive patients is unlikely to affect the clinical utility in the diagnosis of TTP.

The specificity of low ADAMTS13 levels for the diagnosis of TTP has been debated in the literature (Tsai 2003; Remuzzi, 2003). Severe deficiency of ADAMTS13 activity of less than 5% in a patient with clinical symptoms of an acute thrombocytopenia and evidence of a microangiopathic haemolytic anaemia (in particular red cell fragments on peripheral smear) appropriately defines the diagnosis of TTP. As severely deficient ADAMTS13 activity may not always produce the clinical entity of TTP (Wyrick-Glatzel, 2004) the specificity of severely reduced ADAMTS13 levels for TTP has been questioned. It is therefore essential to correlate ADAMTS13 activity with other laboratory and clinical findings.

5.4 Findings in HIV positive patients with TTP

Importantly, our study revealed that thrombocytopenia in the setting of HIV due to causes other than TTP is not associated with severely reduced VWFCP levels thereby giving this assay potential clinical utility in the diagnosis of HIV-related TTP. Therefore HIV positive patients with TTP (Group 4) could be assessed as an additional component of this MMED project

There was marked heterogeneity with respect to VWFCP activity and inhibitors in the setting of TTP in HIV positive patients. Whilst the majority of such patients (70% in this study) have severely reduced VWFCP activity (>90% RCBA), a significant minority (30% in this study) had normal/borderline VWFCP activity (<50.5% RCBA). A statistically significant difference was noted for the % RCBA between Group 4 (HIV positive patients with TTP) and Group 1 (HIV negative controls), $p<0.0001$, between Group 4 and Group 2 (HIV normal platelets) $p=0.0001$ and between Group 4 and Group 3 (HIV low platelets) $p=0.0003$.

In acquired TTP such as that associated with HIV, it has been suggested that decreased VWFCP activity is mediated by an inhibitor. In this study inhibitors were demonstrated in only 5 of 13 patients (~38%).

In a previous series of patients with HIV-related TTP, where VWFCP levels and autoantibody levels were assessed, all 6 of the patients tested had reduced VWFCP levels, however none had detectable inhibitors (Miller, et al., 2005). The absence of inhibitors in a subset of our patients concurs with the findings of Miller and co-workers.

A case report by Sahud and co-workers, describes an IgG inhibitor associated with complete absence of VWFCP activity in the plasma of one patient with HIV-related TTP (Sahud, et al., 2002). An additional two patients with HIV-related TTP with complete deficiency of VWFCP and a strong inhibitor to VWFCP were apparently later identified by the same group (Sahud, et al., 2002).

Although the rapid collagen binding assay has some limitations, in view of the apparent heterogeneity of HIV-related TTP, it seems probable that factors other than immune inhibition of VWFCP activity may result in the development of TTP, at least in some HIV positive patients. One consideration is that there is endothelial cell injury due to either direct infection by the HIV virus (Del Arco, et al., 1993; Miller, et al., 2005) or mediated by inflammatory cytokines (Lafrenie, et al., 1996) which may result in release of large quantities of stored VWF. This overwhelms VWFCP capacity and a 'consumptive deficiency' of the enzyme ensues. A more focal or milder process may result in a relative VWFCP deficiency which is restricted to the milieu of the affected tissue bed, despite overall normal circulating levels of VWFCP.

A more diffuse or more severe process may result in absolute VWFCP deficiency thereby resulting in significantly reduced measured VWFCP activity in the absence of an inhibitor. In either scenario, high molecular weight VWF multimers would accumulate with activation of platelets in affected tissue beds. In addition, endothelial cell damage with associated loss of the inherent endothelial thromboresistant properties would enhance the deposition of platelet rich thrombi which result in the development of thrombotic microangiopathy. There is supporting evidence of this idea. Markedly elevated VWF plasma levels have been documented in HIV-infected patients (Aukrust, et al., 2000).

Reiter and co-workers, demonstrate transient appearance of ULVWF multimers and loss of VWFCP activity following the infusion of DDAVP which stimulates the release of stored VWF from endothelial cells (Reiter, et al., 2003). The ability of overwhelming VWF release to exhaust VWFCP activity is supported by the latter study by Reiter and co-workers.

In this MMED study, increased VWF antigen levels were documented in all patients and as would be predicted, patients with severely reduced VWFCP activity had significantly higher VWF antigen levels than patients with normal/borderline VWFCP activity ($p=0.0320$).
In the absence of any other coagulation parameter abnormalities, elevated D-Dimer levels is reported in HIV-related TTP (Gunther & Dhlamini, 2007). This may reflect localized

coagulation activation secondary to endothelial injury with resultant overwhelming VWF release and loss of endothelial thromboresistance. This finding would therefore support the above considered mechanism.

Notably in this MMED study, D-Dimer levels were significantly higher in patients with reduced VWFCP activity without inhibitors (p=0.0108). In the latter patients, VWF release may constitute the primary pathogenic event. In contrast, in patients with inhibitors, the release of VWF may only provide the necessary 'second hit' precipitating an acute episode of TTP.

Crawley and co-workers, described the down-regulation of VWFCP activity by thrombin and plasmin (Crawley, et al., 2005). Thrombin and Plasmin (generated during coagulation activation) may assist in explaining the association between raised D-Dimer levels and reduced VWFCP activity in the absence of inhibitors and may also contribute to thrombotic microangiopathy development in these patients.

In this MMED study, normal VWFCP activity was significantly associated with lower CD4 counts (p=0.0492), higher platelet counts (p=0.0282), lower LDH levels (p=0.0168).
In TTP patients with normal VWFCP levels, an alternative possibility is that mechanisms unrelated to VWF cleavage may be responsible for the formation of platelet thrombi.
The degree of endothelial injury may be great enough to eliminate the normal endothelial thromboprotective anticoagulant properties and induce platelet adhesion, aggregation and thrombus formation in very severely affected patients. Therefore, even in the absence of accumulation of ULVWF multimers, this could give rise to the clinical features of TTP.
In this MMED study, this is supported by the significant correlation of normal VWFCP levels with lower CD4 counts and more advanced retroviral disease and the pathogenesis of TTP in these patients may be related to significant endothelial damage.

The existence of alternate pathogenic mechanisms for TTP, have also been suggested outside of HIV-related TTP. In approximately 40% of patients with acquired idiopathic TTP, VWFCP deficiency may not be the cause of microvascular platelet thrombi (Lian, 2005). The

formation of microvascular platelet thrombi in patients with acquired TTP without VWFCP deficiency may be caused by endothelial injury and/or platelet aggregation triggered by various stimuli that may be related to inflammation, infectious agents, immune response or chemicals (Lian, 2005). Some triggering agents may cause vascular injury/platelet aggregation and result in TTP without VWFCP autoantibody formation (Lian, 2005).
A larger sample size of TTP patients would have been of value and further studies of TTP both in the setting of HIV positive and negative individuals is required.

This MMED study demonstrates a fairly limited number of patients with inhibitors to VWFCP in the setting of HIV and TTP. This may explain the clinical observation that TTP in patients with HIV is highly responsive to simple plasma infusion therapy and may not require plasma exchange (Novitsky, et al., 2005). However, further studies would be necessary to attempt to correlate VWFCP activity and inhibitors (possibly using assays that directly measure VWFCP) with specific clinical data and clinical response in HIV-related TTP. This may be of value in further elucidating and confirming the pathogenesis of HIV-related TTP and in establishing the utility of such investigations as a routine tool to guide and monitor response to therapy.

Lämmle and co-workers, suggest that due to problems with static protease activity assays, not all patients diagnosed with idiopathic TTP may have severe VWFCP deficiency (Lämmle, Kremer Hovinga & Alberio, 2005). In contrast however, Lian, suggests that as not all patients diagnosed with idiopathic TTP have severe VWFCP deficiency, there may be other different aetiological causes for idiopathic TTP aside from severe VWFCP deficiency (Lian, 2005).

Although standardisation of VWFCP assays is required with the establishment of a universal cut-off value for the diagnosis of TTP, data from this MMED study supports the heterogeneity of the pathogenesis of TTP. This assay has potential as a routine tool to guide therapy, monitor response to therapy and assess prognostic significance of both HIV positive and HIV negative TTP patients.

6. Conclusion

The rapid collagen binding assay is a practical cost-effective assay for the assessment of VWFCP activity which could be instituted as a routine laboratory assay to enhance diagnostic, therapeutic follow-up and research aspects pertaining to TTP. A derivation of this assay based on mixing studies for the assessment of VWFCP inhibitors, may have therapeutic implications such as consideration of concomitant steroid therapy as well as provide further information regarding the pathogenesis of TTP.

This study demonstrates that although semi-quantitative, the rapid collagen binding assay described by Rick and co-workers is practical and effective in the distinction between very low VWFCP activity and normal/borderline VWFCP activity, for which various explanations have been proposed. We established that in the setting of HIV, regardless of the platelet count, there is a baseline reduction in VWFCP activity. This study established our own cut-off values for VWFCP activity in the setting of HIV negative (% RCBA > 37.12%) and HIV positive patients (% RCBA > 50.51%) and showed that there is no difference in VWFCP levels in HIV positive patients with low platelet counts versus normal platelet counts.

This study showed that thrombocytopenia in the setting of HIV due to causes other than TTP is associated with only mildly reduced VWFCP levels and it is not associated with severely reduced VWFCP levels. The baseline mild deficiency of VWFCP in the setting of HIV would not impair the use of this assay for the diagnosis of TTP, as it is severe deficiency of VWFCP (associated with microangiopathic haemolytic anaemia, thrombocytopenia and appropriate clinical findings) that is associated with TTP. Thus this assay has potential clinical utility in the diagnosis of HIV-related TTP.

However, use of this assay in the setting of HIV-related TTP demonstrates clear heterogeneity with regard to VWFCP activity and inhibitors in the context of HIV positive patients with TTP, suggesting that other factors are likely to interact in the setting of HIV infection to produce the same clinical end-point.

Thus VWFCP levels cannot be used in isolation to exclude a diagnosis of TTP in HIV. Despite the heterogeneity of the pathogenesis of TTP in the setting of HIV, assessment of VWFCP activity is still relevant to the diagnosis of TTP, especially to assess for severely reduced VWFCP activity. Moreover, determining VWFCP activity and inhibitors with this assay is of relevance in elucidating the pathogenesis of TTP in HIV, which appears to be a heterogeneous disease.

Further studies are needed to confirm this as the number of HIV positive TTP samples obtained were small and it would be useful to run similar studies using alternate assays to verify these findings. No specific assay is as yet accepted in the literature as a gold standard and ongoing research with standardisation of VWFCP assays is required in order to attempt to establish a universal cut-off value for VWFCP activity for the diagnosis of TTP.

7. Appendices

Appendix A: Estimated Cost per VWFCP test excluding labour

Table 11: Estimated cost per VWFCP test excluding labour: January 2006-01-22

Slide-A-Lyser ™ per plate (20 patients & 1 control=21)	R357-00
Superblock ™ per plate	R93-00
Vitrogen ™ Collagen per plate	R0-28
NUNC ™ ELISA per plate	R11-00
NUNC ™ Sealing tape per plate(R2.15x4=R8.60)	R8.60
Rb a Hu Von Willebrand Factor /HRP per plate	R18-98
ABTS ™ tablets per plate	R18-65
ABTS ™ buffer per plate	R10-28
Estimated cost of Urea, Barium Chloride, NaCl, Tris, PBS tablets per plate	R5-00
Estimated Cost of Consumables per plate (pipette tips, gloves)	R5-00
Total	R527.79
20 tests per plate therefore:	
Estimated cost per VWFCP test excluding labour	R26.38

Appendix B: Summary of statistical analysis for Group 4

Table 12: Depiction of summary of statistical analysis of significant correlations for Group 4. The Kruskal-Wallis test and Wilcoxon rank-sum test were used.

	Number	**Mean score**	**p-value**
Variable: CD4 count			
Reduced VWFCP	12	11.25	
Normal VWFCP	6	6	p=0.0492
Variable: Platelet count			
Reduced VWFCP	14	8.607143	
Normal VWFCP	6	14.916667	p=0.0282
Variable: LDH level			
Reduced VWFCP	14	12.571429	
Normal VWFCP	6	5.666667	p=0.0168
Variable: D-Dimer level			
Inhibitor present	4	2.75	
No inhibitor	8	8.375	p=0.0108
Variable: PTT			
Inhibitor present	5	3.4	
No inhibitor	8	9.25	p=0.0083

Table 13: Depiction of summary of statistical analysis of correlations of Von Willebrand Factor antigen levels for Group 4. This was assessed in HIV positive patients with Thrombotic Thrombocytopenic Purpura (TTP), in the setting of reduced versus normal Von Willebrand Factor Protease activity and then with and without an inhibitor. Analysis performed using the Wilcoxon rank-sum test.

	Number	Rank Sum	p-value
Variable: VWF antigen			
Reduced VWFCP	14	173	p=0.0320
Normal / Borderline VWFCP	6	37	
Variable: VWF antigen			
Inhibitor present	5	39	p=0.5582
No inhibitor	8	52	

Appendix C: Summary of data of % RCBA for Group 1, Group 2 and Group 3

Table 14: Summary for average, median, standard deviation and cut-off values of % RCBA for Group 1, Group 2 and Group 3.

	Group 1	Group 2	Group 3
average	27.06%	33.02%	32.41%
n	70	55	58
median	26.97%	30.45%	30.55%
stdev	0.050308	0.085894	0.092216
cut-off	37.12%	50.18%	50.85%
average cut-off group 2 & 3			50.51%

Appendix D: Data of 4 ELISA plates blocked with Superblock ™ versus Casein.

Table 15: Comparison of data from 4 ELISA plates blocked with Superblock ™ versus Casein.

	Superblock ™ Dry Blend (Samples 1-38)	**Casein (0.5g) (Samples 1-38)**
Average % RCBA	33.12%	43.9%
Minimum % RCBA	19.4%	30.8%
Maximum % RCBA	53.3%	67.9%
Average Undialysed Absorbance	0.59	0.32
Average Dialysed Absorbance	0.19	0.14
Antibody + Substrate	0.12	0.11
Substrate	0.07	0.08

Appendix E: Ethical Clearance Certificates

M0
UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Garizio

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M070740**
PROJECT	Von Willebrand Factor Cleaving Protease Levels in Patients with HIV Related Thrombo-cytopenia (Incorporating M030311)
INVESTIGATORS	Dr DG Garizio
DEPARTMENT	School of Pathology
DATE CONSIDERED	
DECISION OF THE COMMITTEE*	APPROVED UNCONDITIONALLY

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 07.08.24 **CHAIRPERSON** .....
(Professors PE Cleaton-Jones, A Dhai, M Vorster, C Feldman, A Woodiwiss)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr K Gunther

.....

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Garizio

CLEARANCE CERTIFICATE **PROTOCOL NUMBER** M03-03-11

PROJECT An Investigation nto Von Willenbrand Factor Cleaving Protease Levels in Retroviral Related Thrombotic Thrombocytoppenic Purpura (TTP)

INVESTIGATORS Dr DG Garizio

DEPARTMENT School of Pathology, Johannesburg Hospital

DATE CONSIDERED 03-03-28

DECISION OF THE COMMITTEE Approved unconditionally

Unles otherwise specified the ethical clearance is valid for 5 years but may be renewed upon application This ethical clearance will expire on 1 January 2008.

DATE 03-05-30 CHAIRMAN.....(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor: Dr K Gunther
Dept of School of Pathology, Johannesburg Hospital
Works2\lain0015\HumEth97.wdb\M 03-03-11

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DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. I agree to a completion of a yearly progress form. I/we agree to inform the Committee once the study is completed.

DATE 2/7/03 SIGNATURE .....Garizio

PLEASE QUOTE THE PROTOCOL NO IN ALL QUERIES .: M 03-03-11

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

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