The pathophysiology of thrombotic thrombocytopenic purpura

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A thesis submitted to the University of London for the degree of Doctor of Philosophy

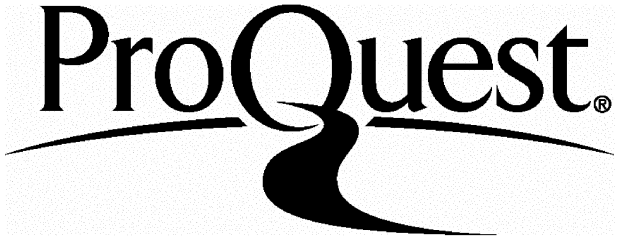
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Abstract

It has been hypothesised that thrombotic thrombocytopenic purpura (TTP) may be a disease of primary platelet aggregation mediated by the presence of ultra large von Willebrand factor (ULVWF) multimers. The principle aims of this thesis were to investigate platelet activation and VWF-cleaving protease activity in adult-onset and congenital TTP.

There was evidence of significantly increased *in vivo* platelet activation in idiopathic TTP as detected by platelet degranulation using flow cytometric techniques. However other novel markers of platelet activation: platelet microparticles and platelet-leucocyte aggregates, were not elevated. This likely reflects the complex relationship between platelet activation and recruitment to areas of thrombus formation. Protease activity was significantly reduced at presentation of idiopathic TTP as compared to haemolytic uraemic syndrome (HUS) and healthy controls but was highly variable in secondary TTP. A significant positive correlation between clinical remission and recovery of protease activity was demonstrated in idiopathic TTP suggesting a pivotal pathogenic role for the latter. However persistently depressed activity did not preclude durable remission nor did it appear to confer an increased risk of relapse. Protease activity was uniformly significantly decreased in all congenital TTP samples despite variations in clinical severity. Correction studies were consistent with a constitutional deficiency. Unidentified factors may modulate phenotype since intrinsic protease activity of plasma products used in the management of congenital TTP correlated with clinical efficacy. In contrast, IgG with inhibitory protease activity was detected in some cases of idiopathic TTP both at presentation and in durable remission. The pathogenesis of TTP thus appears heterogeneous. Preliminary data is presented suggesting that this may explain in part the wide response to treatment seen in clinical practice. Since severe protease deficiency may not necessarily lead to clinical disease,

it appears hitherto unidentified factors are also involved in the pathogenesis of TTP. Antiphospholipid antibodies were not implicated.

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Abbreviations

5-HT	5-hydroxytryptamine
ACL	Anticardiolipin antibody
ADP	Adenine diphosphate
AECA	Anti-endothelial cell antibody
aPA	Activated platelet aggregate
APA	Antiphospholipid antibody
APS	Antiphospholipid syndrome
AT	Antithrombin
β_2GPI	β_2-glycoprotein
β-TG	β-thromoglobulin
BMT	Bone marrow transplant
Ca^{2+}	Calcium ions
CNS	Central nervous system
DIC	Disseminated intravascular coagulation
EDRF	Endothelium-derived relaxing factor
ELISA	Enzyme linked immunoabsorbent assay
F 1.2	Prothrombin fragment 1.2
FCγRII	Human immunoglobulin G receptor class II
FDP's	Fibrin degradation products
FFP	Fresh frozen plasma
GMP-140	Granule membrane protein-140
GP	Glycoprotein
GvHD	Graft versus host disease
HELLP	Haemolysis, Elevated Liver function tests, Low Platelets
HIV	Human immunodeficiency virus
HUS	Haemolytic uraemic syndrome
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule-1

Ig	Immunoglobulin
ITP	Idiopathic thrombocytopenic purpura
IUGR	Intra-uterine growth retardation
LA	Lupus anticoagulant
LAMP	Lysosome associated membrane protein
LDH	Lactate dehydrogenase
MAHA	Microangiopathic haemolytic anaemia
MB	Methylene blue
OCS	Open canalicular system
PADGEM	Platelet activation dependent granule-external membrane protein
PAI-1	Plasminogen activator inhibitor –1
PAPS	Primary antiphospholipid syndrome
PDGF	Platelet-derived growth factor
PECAM-1	Platelet-endothelial cell adhesion molecule-1
PF4	Platelet factor 4
PGI_2	Prostacyclin
PIP_2	Phosphatidylinositol 4,5-biphosphate
PK	Prekallikrein
PKC	Protein kinase C
PL	Phospholipid
PLA	Platelet-leucocyte aggregate
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PMP	Platelet microparticles
PNP	Pooled normal plasma
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
PRP	Platelet rich plasma
RGD	Arg-Gly-Asp
S/D	Solvent-detergent treated
SLE	Systemic lupus erythematosus

TAT	Thrombin-antithrombin complex
TBI	Total body irradiation
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGN	Trans Golgi network
TM	Thrombomodulin
tPA	Tissue plasminogen activator
TRAP	Thrombin receptor agonist peptide
TSP	Thrombospondin
TTP	Thrombotic thrombocytopenic purpura
TXA_2	Thromboxane A_2
TXB_2	Thromboxane B_2
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VT	Verotoxin
VTEC	Verotoxin enterococcal
VWF	Von Willebrand factor
VWF:Ag	Von Willebrand factor antigen
VWF-CP	Von Willebrand factor cleaving protease

Publications arising from thesis

S.L.Allford, P.Harrison, H.Cohen, I.J.Mackie and S.J.Machin. Platelet activation in thrombotic thrombocytopenic purpura. (Poster) XVII Congress of the International Society on Thrombosis and Haemostasis 1999 *Thrombosis and Haemostasis* Supp Abstract 1144

S.L.Allford, P.Harrison, A.Lawrie, R.Liesner, I.J.Mackie and S.J.Machin. VWF-cleaving protease activity in congenital thrombotic thrombocytopenic purpura (TTP). (Oral) British Society for Haematology 40th Annual Meeting 2000 *British Journal of Haematology* 108 Supp 1 Abstract 94

S.L.Allford, P.Harrison, A.Lawrie, H.Cohen, I.J.Mackie and S.J.Machin. VWF-cleaving protease activity in acute thrombotic thrombocytopenic purpura. (Poster) First North Sea Conference on Thrombosis and Haemostasis 2000 *Haemostasis* 30(1 – 2): 85 Abstract 124

S.L.Allford, P.Harrison, A.Lawrie, I.J.Mackie and S.J.Machin. The role of solvent-detergent treated plasma (Octaplas) in acute thrombotic thrombocytopenic purpura. (Oral) 26th Congress of the International Society of Blood Transfusion 2000 *Vox Sanguinis* 78 Supp 1 Abstract OO43

S.L.Allford, P.Harrison, A.S.Lawrie, H.Cohen, I.J.Mackie and S.J.Machin. Recurrent thrombotic thrombocytopenic purpura: association of durable remission with cyclosporin and recovery in vWF-cleaving protease activity. (Poster). American Society of Hematology 42nd Annual Meeting 2000 *Blood* 96(11): 629a

S.L.Allford, P.Harrison, A.S.Lawrie, I.J.Mackie, H.Cohen and S.J.Machin. The role of vWF-cleaving protease activity in acute thrombotic thrombocytopenic purpura. (Oral) British Society for Haematology 41st Annual Meeting 2001 *British Journal of Haematology* 113 Supp 1 Abstract 5

S.L.Allford, P.Harrison, A.S.Lawrie, I.J.Mackie and S.J.Machin. Inhibitory vWF-cleaving protease activity may predict clinical response in acute thrombotic thrombocytopenic purpura. (Oral) British Society for Haemostasis and Thrombosis 2001 *Blood Coagulation and Fibrinolysis* 12(7): A3 Abstract O-6

S.L.Allford, P.Harrison, A.S.Lawrie, R.Liesner, I.J.Mackie and S.J.Machin. Von Willebrand factor-cleaving protease activity in congenital thrombotic thrombocytopenic purpura. *British Journal of Haematology* 2000 111: 1215-1222.

R.Cardigan, **S.Allford** and L.Williamson. Levels of von Willebrand factor cleaving protease are normal in methylene blue treated fresh frozen plasma (in press).

G.L.Stark, J.P.Wallis, **S.L.Allford**, J.Hanley. Chronic relapsing thrombotic thrombocytopenic purpura due to a deficiency of von Willebrand factor-cleaving protease. *British Journal of Haematology* (in press)

W.A.Lester, M.D.Williams, **S.L.Allford**, M.S.Enayat and S.J.Machin. Successful treatment of congenital thrombotic thrombocytopenic purpura using the intermediate purity factor VIII concentrate *BPL 8Y.* (submitted)

Acknowledgements

The work in this thesis was performed during a three year period (1998 – 2001) whilst I was employed as a Research Registrar in the Haemostasis Research Unit, University College London. I would like to thank my supervisor Professor Samuel J. Machin for the opportunity to perform this study as well as his support and encouragement during this time. Many thanks is also due to Dr. Ian Mackie (Senior Lecturer) and Dr. Paul Harrison (Lecturer) whose scientific knowledge and guidance has been invaluable. I am also indebted to my clinical colleagues, especially the staff of the Haematology Day Care Unit, University College Hospital, for their help in collecting samples. Finally I am extremely grateful to my fellow colleagues in the Haemostasis Research Unit for their practical assistance and camaraderie and my husband for his steadfast support.

1. General Introduction

1.1 Historical perspective and definition of thrombotic thrombocytopenic purpura

Thrombotic thrombocytopenic purpura (TTP) was first described by Moschowitz in 1924 (Moschowitz, 1924). In this paper a 16 year old girl was described presenting with thrombocytopenia, fever and stupor. At post mortem widespread hyaline thrombi formation was noted. However the condition thrombotic thrombocytopenic purpura was not coined until 1947 (Singer *et al*, 1947). A pentad of features: microangiopathic haemolysis, thrombocytopenia, fluctuating neurological dysfunction, renal impairment and fever typify TTP. However, several of the other microangiopathic syndromes, notably haemolytic uraemic syndrome (HUS) and the haemolytic, elevated liver enzymes and low platelets (HELLP) syndrome, are also characterised by similar features. This leads to diagnostic confusion. The concept has arisen that they might represent an overlapping spectrum of disease, although with varying pathophysiological features. Literature therefore may refer to the syndrome TTP-HUS.

In contrast, since the histological appearances of TTP and HUS are not identical, it has also been proposed that the two conditions are distinct pathophysiological entities and should be differentiated. The characteristic pathological lesion in TTP is widespread occlusion of terminal arterioles or capillaries by intravascular thrombi and subendothelial hyaline deposits (Neame *et al*, 1973;Feldman *et al*, 1966). This occurs predominantly in the renal and cerebral circulation thus accounting for the clinical features of the disease. However, other anatomical sites may be affected: for instance, abdominal pain and serous retinal detachment are recognised complications of TTP and are thought to be secondary to mesenteric and retinal ischaemia respectively. The microvascular thrombi formed have been shown to be composed almost entirely from platelets which show varying degrees of cytoplasmic alteration and degranulation

(Asada *et al*, 1985;Neame *et al*, 1973) (Feldman *et al*, 1966). Endothelial proliferation may be observed in many of these lesions. Since neither significant perivascular inflammation, obvious endothelial cell desquamation nor subendothelial exposure have been demonstrated in TTP, it has been hypothesised that TTP may be a disease of primary platelet aggregation within the microvasculature (Harkness *et al*, 1981). In support of this immunohistochemistry shows that thrombi formed in TTP contain abundant von Willebrand factor (VWF) and little fibrinogen or fibrin in contrast to those found in disseminated intravascular coagulation (DIC) when the reverse is seen (Asada *et al*, 1985). This has lead to the hypothesis that VWF multimers, perhaps in conjunction with high shear stress, might promote platelet aggregation during episodes of TTP.

1.2 Platelets

1.2.1 Platelet production, structure and function

Platelets are fragments of megakaryocyte cytoplasm. Megakaryocytes in turn are derived from pluripotential stem cells. The earliest committed cell lineage progenitor cell is the burst-forming unit, BFU-Meg. Under the influence of thrombopoietin (Tpo) and cytokines Il-3, Il-6 and Il-11, it develops into megakaryocyte colony forming units (CFU-Meg). Maturation of CFU-Meg is accompanied by nuclear and cytoplasmic proliferation and platelet granule and glycoprotein proliferation. Pseudopodia form when the megakaryocyte is fully mature and these penetrate the marrow sinusoids. It is unclear whether platelets are the immediate progeny of megakaryocytes by detachment secondary to the mechanical forces of blood flow. It has been suggested that platelets may be synthesised via the intermediate proplatelet. These are ultra-large megakaryocyte cytoplasmic fragments that are thought to fragment further in the peripheral or pulmonary circulation. Megakaryocytes have been identified in the pulmonary vasculature and it has therefore been proposed that the lungs rather than the marrow represent the primary site of platelet production.

Whatever the site of platelet production, newly synthesised platelets or "reticulated platelets" contain rough endoplasmic reticulum and are therefore able to synthesise small amounts of proteins. Consequently they contain some residual mRNA which has largely degraded by 24 hours (Dale *et al*, 1995). Messenger RNA in individual platelets may be identified by staining with a variety of fluorochromes or supravital dyes. It has been suggested that such reticulated platelets are haemostatically more effective than platelets of a normal age distribution (Shulman *et al*, 1968;Karpatkin, 1969). Although platelets usually circulate for approximately ten days, the mechanism of platelet clearance from the circulation is unclear. There appears to be an age dependent process selectively removing senescent platelets in addition to preferential elimination of functionally "exhausted" platelets *in vivo* (Rinder *et al*, 1991a).

Mature platelets are smooth biconcave anuclear discs of diameter 2-4μm and volume 5-8fl. The platelet membrane has a typical bilammellar structure and is composed of phospholipid. It is contiguous with the surface-connected open cannalicular system (OCS) which effectively increases the platelet surface area and promotes membrane transport. Specific platelet glycoproteins (GP) are expressed on both the platelet plasma membrane and the OCS. These include the integrins GPIa and GPIIbIIIa in addition to the leucine-rich GP family. Those recognised as central to haemostasis are detailed in *Table 1.1.* Many are heterodimers which span the membrane and are therefore able to effect signal transduction. Signal transduction is dependent on intracellular calcium concentration (see below). The dense tubular system is the equivalent of the smooth endoplasmic reticulum in other cells and is the site of calcium sequestration. It lies in close contact with channels of the OCS.

Gene family	Glycoprotein	Major ligand	Major function
Leucine-rich glycoprotein	Ib-IX	VWF Thrombin	Adhesion
Integrin	IaIIa	Collagen	Adhesion
	IIbIIIa	Fibrinogen, VWF Fibronectin Vitronectin	Aggregation
Selectin	GMP-140	Leucocyte oligosaccharide chain	Cell-cell interactions
Immunoglobulin-domain	PECAM-1		
	FcγRII	IgG (Fc fragment)	Cell-cell interactions

Table 1.1 *Platelet membrane glycoproteins, their ligands and major functions*

A variety of secretory granules are found within the cytoplasm. The most abundant are α-granules which contain a number of platelet specific peptides and coagulation factors. They thus promote platelet function, chemotaxis, mitogenesis and vessel repair. However their physiological importance is questioned since there is no well defined bleeding diathesis in those patients whose platelets lack α-granules (grey platelet syndrome). In contrast a moderate haemorrhagic tendency is apparent if there is a quantitative or functional deficiency in dense bodies such as is found in the Hermansky-Pudlak or Chediak-Higashi syndromes. Dense bodies contain 5-hydroxytryptamine (5-HT), adenine nucletides (primarily adenine diphosphate, ADP) and calcium ions and play a pivotal role in the formation and maintenance of the platelet plug.

Platelets possess a complex cytoskeleton. A peripheral band of microtubules is a prominent structure in the quiescent platelet and maintains its discoid shape. However,

when the platelet is activated, it disappears only to reappear centrally. The exact function of this central apposition is unclear but is thought to expedite the release reaction. Cytoplasmic microfilaments are another component of the platelet contractile system. They are found throughout the platelet but may be intimately related to the peripheral microtubules. They are composed of actin and myosin filaments and are involved in maintenance of platelet shape, pseudopod formation and platelet secretion.

1.2.2 Platelet glycoproteins

The GPIb-IX-V complex expressed on the platelet membrane is composed of four distinct gene products: GP1bα (135kDa) disulphide bonded to GPIbβ (25 kDa) and the non-covalently associated subunits GPIX (22kDa) and GPV (82 kDa). GPIbα, GPIbβ, GPIX and GPV are all members of the leucine rich protein family. They are expressed on the platelet surface in an apparent ratio 2:2:2:1 and there are approximately 25,000 copies per platelet. Although all the units of the GPIb-IX complex are required for stable surface expression of functional receptor (López *et al*, 1993), the precise role of GPV is less well defined (López, 1994). A major physiological role of GPIb-IX-V is to mediate initial adhesion of circulating platelets to VWF in the endothelial matrix at high shear (López, 1994;Kroll *et al*, 1996). At low shear, adhesion is essentially independent of GPIb-IX-V and involves other adhesion molecules such as collagen receptors, the $\alpha_2\beta_1$ integrin (GPI-IIa), GPIV or GPVI. The VWF binding site is localised to the N-terminal domain of GPIbα. It has become evident that VWF binds to more than one site within this domain and that binding is dependent on the way in which the interaction is induced (Ward *et al*, 1996). However when constitutively expressed on the platelet surface membrane, GPIb-IX-V does not appear to recognise VWF spontaneously but requires either association of VWF with the subendothelium or exposure of VWF and platelets to high shear (Kroll *et al*, 1996;López, 1994). Ultrastructural studies have demonstrated that GPIb complexes are distributed on external and internal platelet membranes (Hourdillé *et al*, 1990;Escolar *et al*, 1994). Redistribution of GPIb-IX-V receptors occurs following platelet activation (Hourdillé *et al*, 1990).

GPIIbIIIa is a heterodimeric transmembrane molecule and a member of the integrin family. Approximately 45,000 copies are expressed on the external platelet membrane with additional complexes located in the OCS and in the α granules. Upon platelet activation and following α granule secretion, these intraplatelet GPIIbIIIa complexes are translocated to the cell surface exterior. GPIIbIIIa has a low affinity for binding soluble fibrinogen on quiescent platelets but following activation platelets are able to bind fibrinogen relatively strongly. Activation induces a conformational change in the GPIIbIIIa receptor that is responsible for this change in fibrinogen binding affinity (Shattil *et al*, 1985). The amino acid sequence recognised is Arg-Gly-Asp (RGD). Consequently GPIIbIIIa interacts with several other adhesive proteins that also contain an RGD sequence including VWF, thrombospondin, laminin, vitronectin and collagen.

1.2.3 Platelet adhesion

Although platelets circulate in close proximity with the endothelial cell wall, endothelial adhesion and subsequent platelet aggregation normally only occurs following endothelial damage. High local concentrations of prostacyclin (PGI_2) and endothelium derived relaxing factor (EDRF) are thought to reduce intracellular calcium concentration via a cAMP dependent mechanism and thereby inhibit calcium dependent functions such as release and aggregation. However subendothelial exposure swiftly leads to the formation of a primary platelet plug. Two complimentary mechanisms are responsible for platelet adhesion. One is VWF-dependent and is primarily responsible for adhesion under high shear pressures (Peterson *et al*, 1987). The other mediates adhesion under low shear conditions and is VWF-independent (Ikeda *et al*, 1991). Aggregation under low shear conditions may be simulated using an aggregometer in which platelet rich plasma (PRP) is stirred with a bar rotating at the bottom of the cuvette containing the suspension. Normal platelets do not aggregate unless an agonist is added. Using a cone and plate viscometer, platelets may be exposed to high shear stress. Stable platelet aggregation can be achieved under such conditions (shear stress greater than 60 – 80 dynes/cm^2) without the need for exogenous agonists. This demonstrates that distinct mechanisms of activation may

operate under varying shear (Brown III *et al*, 1975;Belval *et al*, 1984;Anderson *et al*, 1978).

In all blood vessels, blood flow is faster towards the centre than close to the vessel wall. As a consequence, there is a shear effect between adjacent layers of fluid moving at different speeds that is maximal nearest the wall. Shear forces of up to 500-5,000s^{-1} can be attained within arterioles. Under these conditions, platelet adhesion must occur rapidly and the adhered cell must be able to resist shear stress. VWF is integral to this process. Although collagens types I, III and IV are powerful inducers of platelet adhesion and aggregate formation under flow conditions at high shear rates (Saelman *et al*, 1993;Saelman *et al*, 1994) this process is still VWF-dependent (Houdijk *et al*, 1985). Primary platelet adhesion is mediated via the GPIb receptor which binds the A1 domain of VWF deposited on the subendothelial extracellular matrix. As discussed above, a striking feature of GPIb is its apparent inability to bind human VWF in plasma whereas it avidly binds immobilised VWF. It has been suggested that plasma VWF has a non-functional ("non-adhesive") conformation that must switch to "functional" to initiate platelet adhesion, thus supporting the concept of conformational change. However, the interaction has an extremely fast association rate. This may not only facilitate binding at the high shear pressures which exist within the microvasculature but also permit binding of soluble VWF in the plasma. In the absence of other thrombogenic stimuli, the latter may be nonreactive since any interaction is short lived. It therefore follows that irreversible platelet adhesion to subendothelial VWF cannot be achieved due to the fast dissociation rate of the VWF-GPIbα interaction. Thus platelets continue to move constantly in the direction of flow, albeit at a lower velocity.

Despite this, as demonstrated by the cone and plate viscometer *in vitro*, if platelets are exposed to high shear pressures, direct platelet aggregation may occur in the fluid phase. This phenomenon has an absolute requirement for VWF, GpIb, GpIIbIIIa, calcium and ADP (Moake *et al*, 1986;Moake *et al*, 1988;Peterson *et al*, 1987). The reasons why VWF is required for aggregation under high shear are not fully

understood but are likely to be explained by specific mechanical and structural properties of the molecule. High shear forces have a profound effect on the three dimensional structure of VWF promoting the formation of extended filaments (Siedlecki *et al*, 1996). The repeating subunit structure of the large multimers may offer an array of interaction sites capable of binding in a multivalent manner to receptors on the platelet membrane, increasing the strength of interaction and promoting linkage of platelets to one another.

VWF binding to GPIb-IX-V activates platelets triggering the cytoskeletal changes that control shape change, spreading, secretion, aggregation and contraction (Ruggeri, 1997). Although the GPIb-IX complex is not an integrin, it serves as a conduit between VWF in the extracellular matrix and the platelet membrane cytoskeleton. It has been demonstrated that the cytoplasmic tail of GPIb bears contact sites for actin binding protein (Andrews & Fox, 1991). Binding of VWF to GPIb in the presence of ristocetin increases intracellular calcium concentration (Kroll *et al*, 1991). This is associated with secretion and potentiation of platelet aggregation. Whether a similar response is evoked on first contact of platelets with VWF in the extracellular matrix is yet to be determined. There is circumstantial evidence that signaling may involve cross-linking of one or more GPIbα molecules. Firstly there are at least two copies of the VWF binding GP Ibα subunit in each GP Ib-IX-V complex (López, 1994). Secondly, although a monomeric proteolytic fragment of VWF may bind to GPIbα it does not induce platelet activation in contrast to native multimeric VWF (Andrews & Berndt, 1998). In any event, binding of multimeric VWF to GPIb with shear forces above 60 – 80 dynes/cm^2 causes a transmembrane flux of calcium ions. Platelet activation and secretion of ADP and other agonists follows. Additional platelets are thus stimulated promoting the calcium dependent aggregation mediated by the platelet membrane integrin αIIbβ3 (GpIIbIIIa) which binds fibrinogen or VWF (Du & Ginsberg, 1997). Crosslinkage of platelets follows secondary to the dimeric nature of fibrinogen and aggregation results.

Collagen, fibrinogen and fibronectin may mediate platelet adhesion under conditions of low shear stress. Adhesion to collagen requires interaction between the platelet glycoprotein GPIa-IIa complex and two sites on the α chain of collagen. Three-dimensional conformation appears to be important since microfibrillar collagen is the minimal structural unit bound. Immobilised fibrinogen may bind to GPIIbIIIa without prior platelet activation (Savage & Ruggeri, 1996). Surface coverage by single adherent platelets is typically maximal at shear rates of 50 – 500s^{-1}. This decreases progressively with increasing shear rate and is minimal when rates of 1500 – 2000s^{-1} are attained. In contrast to the interaction of flowing platelets with immobilised VWF, that with immobilised fibrinogen is instantaneously irreversible: less than 10% platelets that first become tethered to immobilised fibrinogen translocate from the initial contact site by a distance greater than their own diameter (Savage & Ruggeri, 1996). The biomechanical properties of the interaction of fibrinogen/fibrin with GPIIbIIIa which preclude platelet adhesion at high shear rates have yet to be elucidated. The limiting factor is likely to be the rate of bond formation.

1.2.4 Platelet activation

Platelet activation is characterised by shape change, granule secretion and specific metabolic reactions. Platelet shape change rapidly follows platelet adhesion to vessel wall components. The normal smooth, biconcave disc shape is lost and platelets become spherical with protuberant pseudopodia. These promote interaction between adjacent platelets. Platelet granules centralise secondary to activation of the cytoskeletal contractile apparatus and secretion follows. Not only are granules forced towards the plasma membrane but so too is the OCS thus facilitating secretion of granule contents. Movement of the OCS may contribute to modifications on the external surface. The cytoplasmic microfibrils appear to depolymerise and reassemble within the pseudopodia. Their subsequent contraction is thought to effect clot retraction and consolidation of the platelet plug.

Secretion of granule contents occurs *in vitro* immediately following adhesion and shape change. The intensity of the reaction varies according to the stimulus. Weak inducers such as low dose ADP or adrenaline primarily promote α granule secretion. Higher concentrations of ADP or adrenaline and low dose collagen result in both α granule and dense body secretion. Release of granule constituents such as serotonin, ADP, VWF, fibrinogen and thrombospondin amplifies this response. Potent platelet agonists such as thrombin or high dose collagen result in the additional release of lysosomal enzymes.

Platelet activation is thus caused by a variety of agonists including thrombin, thromboxane A_2, ADP, collagen and arachidonic acid binding to specific receptors and is represented diagramatically in *Figure 1.1*. The thrombin receptor consists of an extracellular N-terminal domain, seven transmembrane domains and an intracytoplasmic C-domain. Thrombin cleaves its own receptor between arginine 41 and serine 42. The new N-terminal sequence then forms a tethered ligand capable of activating the receptor (Vu *et al*, 1991). The identity of the platelet receptors mediating ADP responses is still unclear. There is evidence for two G protein-coupled ADP receptors and one is likely to be $P2Y_1$ (Jantzen *et al*, 1999;Jin *et al*, 1998;Fagura *et al*, 1998). In addition, a third ADP receptor, similar or identical to the ligand gated ion channel PSX_1 appears to be expressed on platelets and to mediate a very rapid calcium influx (Sage, 1997;Vial *et al*, 1997). Signal transduction is mediated by G proteins and intracellular cyclic 3', 5'-adenosine monophosphate (cAMP): increased cAMP concentrations inhibit platelet adhesion, aggregation and release. In contrast, following platelet activation, adenylate cyclase activity is reduced and cAMP levels therefore fall. Phospholipase C is activated and cleaves phosphatidyl inositol biphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DG). IP_3 acts as an ionophore and calcium is mobilised from the dense tubular system (smooth endoplasmic reticulum). Many calcium dependent reactions follow including phosphorylation of the myosin light chain initiating the contractile reaction of the release mechanism, secretion of platelet granule contents and liberation of arachidonic acid from membrane phosphatidylcholine (see *Figure 1.1*). Arachidonic acid is itself

converted enzymatically to thromboxane A_2 (TXA_2). TXA_2 has a plasma half-life of approximately two minutes. It is a potent vasoconstrictor and like IP_3 mobilises calcium from the dense tubular system thus promoting the release reaction and platelet aggregation. Its importance is demonstrated by the potent anti-platelet effect of aspirin which inhibits cyclo-oxygenase and hence TXA_2 production. Depending on the nature and concentration of the activating agents, concomitant shedding of membrane microvesicles occurs during the process of activation (George *et al*, 1986;Dachary-Prigent *et al*, 1993).

1.2.5 Platelet aggregation

Platelets form aggregates through sequential stages of activation and aggregation. Under physiological conditions, ADP and TXA_2 reach concentrations required to induce platelet aggregation. However this is a transient phenomenon which occurs only at sites of endothelial damage. Signal transduction is thought to be mediated by receptors belonging to the 7-transmembrane domain. The GPIIbIIIa complex is activated and phosphoinositol turnover and prostaglandin metabolism are increased both directly (as above) and indirectly via inhibition of adenylate cyclase. Activation of GPIIbIIIa complex results in conformational change and permits fibrinogen binding primarily through an interaction with the RGD sequence on the fibrinogen α chain. Being a dimer, fibrinogen facilitates the formation of direct bridges between adjacent platelets leading to aggregation. Aggregation itself leads to activation and granule secretion from other platelets. Such positive feedback enhances platelet plug formation at the site of vascular injury.

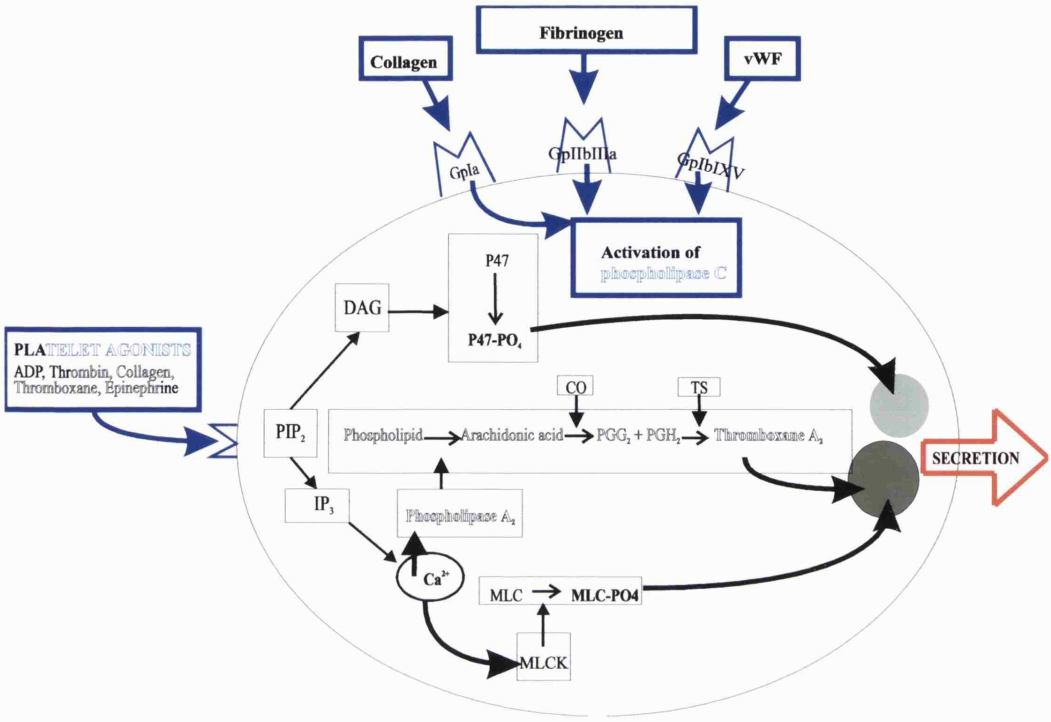


Figure 1.1 Platelet function.

Specific platelet surface glycoprotein (GP) receptors mediate platelet adhesion, aggregation and activation. Platelet adhesion to damaged endothelium is achieved by vWF binding to the GpIb component of the GpIb-IX-V complex. Aggregation follows secondary to bridging of the conformationally altered GpIIbIIIa receptors of adjacent platelets by fibrinogen. Other agonists such as ADP and collagen bind to specific receptors and initiate platelet activation through the activation of phospholipase C. This enzyme catalyzes the cleavage of phosphatidyl inositol biphosphate (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol (DAG). DAG activates protein kinase C whilst PIP_2 mobilizes calcium ions from the dense tubular system and hence activates phospholipase A_2 and myosin light chain kinase (MLCK). The former liberates arachidonic acid from phospholipid and via the actions of cyclooxygenase (CO) and thromboxane synthetase (TS) results in the synthesis of thromboxane A_2. Together with thromboxane A_2, the phosphorylated products of the myosin light chain (MLC) and the intracellular protein P47 stimulate secretion of the contents of the dense alpha and lysosomal granules.

In capillaries, platelet aggregation, together with local vasoconstriction, is usually sufficient to achieve and maintain haemostasis. However in larger vessels this requires recruitment of fibrin into the platelet plug. Coagulation is enhanced by platelet procoagulant functions. During platelet activation, a reorientation ("flip-flop") of the

platelet plasma membrane occurs. Anionic phospholipids such as phosphatidylserine (PS) are exposed on the platelet exterior. Externalisation of these negatively charged phospholipids particularly promotes binding of the vitamin K dependent coagulation factors (factors II, VII, IX and X) whilst specific platelet receptors may exist for factors V, VIII and XI. Assembly of the enzyme-cofactor complexes of the coagulation pathway on the surface of activated platelets not only minimises inactivation of coagulation factors by antithrombin and protein C, but also localises procoagulant activity to the site of endothelial damage.

1.2.6 Laboratory measurement of platelet activation

Many clinical conditions have been reported to be associated with platelet hyperreactivity and/or circulating activated platelets. These include coronary artery disease, stroke, deep vein thrombosis and diabetes mellitus. However the role of platelet activation in clinical settings is controversial in part because the original methods employed to measure platelet activation are subject to major methodological flaws. These include platelet aggregation and immunoassays of plasma platelet factor 4 (PF4) and β-thromboglobulin (βTG) and metabolites of TXA_2. Although platelet aggregation studies may demonstrate changes in platelet reactivity, they cannot determine whether the associated condition directly activates platelets. The technique is also subject to standardisation problems (George & Shattil, 1991). In contrast, although immunoassays of plasma PF4 and βTG may indirectly demonstrate association of a clinical condition with platelet activation, changes in platelet reactivity cannot be assessed. Such immunoassays also require specific plasma separation procedures and are susceptible to artefactual, *in vitro* platelet activation. In addition, none of these techniques allows identification of distinct platelet subpopulations, nor can they measure the extent of individual platelet activation. Some of these methodological limitations have been overcome by the introduction of whole blood flow cytometry. This allows the study of platelet activation under more physiological conditions. Erythrocytes and leucocytes may influence platelet activation (Santos *et al*, 1991;LaRosa *et al*, 1994). A whole blood preparation confers

minimal sample manipulation and consequently both artefactual *in vitro* platelet activation and the potential for loss of platelet subpopulations is reduced. A European Working Group Consensus has been published which outlines procedures for the flow cytometric characterisation of platelet function (Schmitz *et al*, 1998). It recommends the use of whole blood in assays and gives specific recommendations regarding preferred anticoagulant, choice of buffer and fixation. Limitations however still persist. The half-life of activation induced surface markers is undetermined. This will obviously influence results as will clearance of activated platelets from the circulation. Results must therefore be interpreted with caution. Nevertheless, this technique allows identification of individual platelet subpopulations and multiple activation dependent surface receptors. In addition the reactivity of circulating platelets can be measured as discussed below. At present, measurement of no one single activation-dependent antigen or parameter can be recommended above another. The available antibodies monitor different aspects of the activaton process (see *Table 1.2*) and it is certain that additional antibodies will be developed. Since platelets are likely to become activated by different agonists in different clinical situations, the pattern of platelet surface activation may vary. It is prudent therefore to employ a panel of activation-dependent antibodies.

Activation-related surface change	Prototypic antibodies	Antibody specicity	Reference
GPIIbIIIa conformational change	PAC-1	Ligand binding site of GP $IIbIIIa_{active}$	(Shattil *et al*, 1985)
Ligand binding to GPIIbIIIa	Non-inhibitory antibodies to ligand	Epitopes on ligand (eg. Fibrinogen, VWF)	(Schullek *et al*, 1984)
α granule membrane proteins	S12	GMP-140 (P-selectin)	(McEver & Martin, 1984) (Larsen *et al*, 1989)
Lysosomal and dense granule membrane proteins	D495	CD63	(Gerrard *et al*, 1991)
Binding of secreted platelet proteins	TSPI-1	Thrombospondin	(Boukerche & McGregor, 1988)
Development of procoagulant surface	V237	Factor Va light chain	(Sims *et al*, 1988)

Table 1.2 *Platelet activation-dependent monoclonal antibodies*

Thrombin is one of the most important physiological platelet activators (Eidt *et al*, 1989;Kelly *et al*, 1991;Hanson & Harker, 1988). Addition of thrombin to whole blood results in potent platelet aggregation and formation of a fibrin clot. These actions preclude its use as a platelet agonist in a whole blood assay. These methodological problems may be overcome through the use of thrombin in the presence of the synthetic tetrapeptide glycyl-L-prolyl-L-arginyl-L-proline (GPRP). GPRP is an analogue of the polymerisation sites involved in fibrin polymer formation and competitively inhibits fibrin polymerisation (Laudano & Doolittle, 1978). GPRP also inhibits fibrinogen binding to its platelet receptor thereby partially inhibiting platelet

aggregation (Plow & Marguerie, 1982;Adelman *et al*, 1990) but without affecting platelet activation (Michelson *et al*, 1991). Moreover GPRP is stable, resistant to proteolytic agents (including thrombin) and does not suppress thrombin activity (Laudano & Doolittle, 1978). An alternative approach is to employ thrombin receptor agonist peptide (TRAP). This is a peptide fragment of the "tethered ligand" receptor for thrombin (Vu *et al*, 1991). TRAP directly activates platelets without resulting in a fibrin clot. It can therefore be used in the absence of GPRP. However a potential disadvantage is that it may not reflect all aspects of thrombin-induced platelet activation. This is because the "tethered ligand" receptor may not be the only platelet receptor for thrombin (Yamamoto *et al*, 1991).

Expression of novel activation dependent antigens

The two most widely used platelet activation-dependent antigens are the GPIIbIIIa complex and P-selectin. The GPIIbIIIa complex is a receptor for fibrinogen, VWF, fibronectin and vitronectin and is critical for platelet aggregation (Phillips *et al*, 1988). Whereas most monoclonal antibodies directed versus the GP IIbIIIa complex bind to resting platelets, PAC1 is an IgM antibody directed against the fibrinogen binding site exposed by a conformational change in the GPIIbIIIa complex of activated platelets (Shattil *et al*, 1985). It therefore only binds to activated platelets. The specificity of PAC-1 may be due to the fact that one of its hypervariable regions contains an arginine-tyrosine-aspartic acid sequence. This hypervariable region may structurally mimic RGD-containing regions in fibrinogen.

Platelet activation by physiological agonists results in degranulation and incorporation of both granule and lysosomal membranes into the cell surface. Cell surface expression of granule and lysosomal membrane proteins may therefore be used to monitor platelet activation. Such activation dependent antigens include P-selectin, CD63 and the lysosome-associated membrane proteins (LAMP).

P-selectin is also referred to as GMP-140 (granule membrane protein of 140kDa), PADGEM (platelet activation dependent granule external membrane) protein and

CD62p. It is an integral membrane protein found in the α granules of resting platelets and the Weibel-Palade bodies of endothelial cells (McEver, 1991). It is a member of the selectin family of adhesion molecules and recognises carbohydrate ligands related to sialyl Le^x. Both strong and weak platelet agonists induce rapid translocation of CD62p to the cell surface secondary to α granule secretion. It mediates adhesion of activated platelets to neutrophils and monocytes and may also serve to localise leucocytes to the site of vascular injury (Larsen *et al*, 1989). Alternatively CD62p mediated platelet binding to monocyte-macrophages of the reticuloendothelial system may promote the removal of potentially thrombogenic circulating activated platelets (Celi *et al*, 1991). Consequently P-selectin may not be the optimal marker for detection of circulating degranulated platelets. Furthermore it has been demonstrated *in vivo* that P-selectin is rapidly lost from the surface of circulating degranulated platelets although their function is unaffected (Michelson *et al*, 1996).

The CD63 antigen belongs to the tetraspan protein family and is a 30-60kDa integral membrane protein of lysosomal membranes whose function is unknown. Thrombin stimulation results in CD63 expression on the platelet surface where it becomes a major protein constituent. After stimulation its level of expression increases from 650 molecules per unstimulated platelet to 12600 (Nieuwenhuis *et al*, 1987). Granulophysin, the platelet dense granule protein is also present in lysosomal membranes with a similar distribution to CD63. It is now thought that CD63 and granulophysin are the same molecule (Nishibori *et al*, 1992). Like CD63, the lysosome-associated membrane proteins, LAMP-1 and LAMP-2 (CD107a and CD107b respectively) are integral proteins of the lysosomal membrane. In the platelet, both have been shown to be heavily glycosylated proteins of around 110kDa (Silverstein & Febbraio, 1992). They too are expressed following platelet activation by a strong agonist such as thrombin, ADP being ineffective.

Decreased expression of constituent platelet surface antigen

Platelet activation may also result in decreased expression of the constituent platelet surface GPIb-IX as compared to resting platelets (Michelson *et al*, 1991;Michelson &

Barnathan, 1987). This is thought to be the result of translocation of GPIb-IX complexes to the OCS and may be a sensitive marker of platelet activation *in vivo* (Kestin *et al*, 1993).

Platelet-leucocyte aggregates

The role of leucocytes in haemostasis and thrombosis is increasingly appreciated. Activated platelets can bind to leucocytes (Rinder *et al*, 1991b), trigger their activation (Maeda *et al*, 1991;Ruf & Patscheke, 1995) and result in colocalisation of platelets and leucocytes at sites of haemorrhage (Wester *et al*, 1979), atherosclerosis (Joris *et al*, 1983) or inflammation (Issekutz *et al*, 1983). Conversely, factors that are secreted by activated leucocytes can stimulate platelets (Pidard *et al*, 1994;Del Maschio *et al*, 1990). Analysis of platelet-leucocyte aggregates thus represents an additional means of assessing platelet activation.

There are likely to be several mechanisms mediating platelet-leucocyte binding. Of these, P-selectin and its ligand P-selectin glycoprotein ligand-1 (PSGL-1) have been most extensively studied. This interaction mediates not only platelet-leucoyte aggregation formation but also leucocyte-endothelial adhesion (Hamburger & McEver, 1990;Larsen *et al*, 1989;Geng *et al*, 2001) and is calcium dependent (de Bruijne-Admiraal *et al*, 1992;Jungi *et al*, 1986). PSGL-1 is constitutively expressed on the leucocyte surface and does not appear to be modulated as a consequence of cellular activation (Furie & Furie, 1997). The importance of P-selectin binding to PSGL-1 *in vivo* has been demonstrated in animal models. Anti P-selectin antibodies were able to inhibit leucocyte accumulation in experimental thrombi in baboons and attenuate fibrin deposition (Palabrica *et al*, 1992).

Alternative mechanisms of platelet-leucocyte aggregate formation include bridging of CD36 by thrombospondin (Silverstein *et al*, 1989) and fibrinogen bridging of platelet GPIIbIIIa and leucocyte CD11/CD18 (Altieri *et al*, 1988). Fibrinogen-integrin interaction may permit limited binding of unactivated platelets to monocytes and neutrophils (Rinder *et al*, 1991b). Whether leucocyte stimulation promotes or inhibits

the formation of platelet-leucocyte complexes remains unclear. There have been reports of both increased (Rinder *et al*, 1992;Evangelista *et al*, 1996) and decreased (Rinder *et al*, 1994;Lorant *et al*, 1995) complex formation following leucocyte activation.

Microparticles

Among the earliest descriptions of microparticles derived from platelets is that of Wolf who in 1967 used the term "platelet dust" to describe minute particulate material from platelets that were stored for transfusion (Wolf, 1967). These particles possess the coagulant properties of platelet factor 3 (phosphatidylserine) and have a high lipid content. They have an average diameter of approximately 0.2μm and a unilamellar structure on electron microscopy (Zwaal *et al*, 1993). Platelet derived microparticles can apparently be produced by several mechanisms including mechanical disruption of platelets (McGill *et al*, 1987) and activation of platelets secondary to either storage (Solberg *et al*, 1987) or stimulation by agonists (Bode *et al*, 1985). It appears that microparticles generated by different stimuli are distinctive: microparticles induced by C5b-9 do not express fibrinogen receptor whereas those formed by thrombin do (Sims *et al*, 1988). The precise mechanism of microparticle formation is unclear although calcium influx appears to be a prerequisite. One hypothesis is that calpain mediated hydrolysis of actin-binding protein results in disruption of the platelet membrane skeleton and shedding of microparticles (Fox *et al*, 1990). However microparticle gerneration still occurs in ATP depleted platelets after incubation with complement proteins suggesting alternative pathways exist (Wiedmer *et al*, 1990). A haemostatic function for platelet microparticles has been suggested by experiments in which the bleeding time of thrombocytopenic rabbits was shortened following infusion of microparticles (McGill *et al*, 1987). Microvesicles also appear to promote platelet adhesion to exposed endothelium (Owens *et al*, 1992). Electron microscopic studies of the lesions produced to measure bleeding times showed accumulation of microparticles at these sites. However microparticle studies have been hampered by the lack of reproducible quantitative assays. Direct particle counting, quantitative immunoelectrophoresis, platelet factor 3 assay, ^{111}In radiolabelling and more recently,

flow cytometry have all been used. Flow cytometry has the advantage that through relative differences in light scatter, microparticles may be distinguished from platelets in mixed population.

1.3 Von Willebrand factor

1.3.1 VWF synthesis

Von Willebrand factor is a complex multimeric plasma glycoprotein that plays a critical role in the mediation of platelet adhesion to the damaged vascular wall as discussed above. It also functions as a carrier protein for factor VIII. It is synthesised in endothelial cells and megakaryocytes as a precursor polypeptide of 2813 polypeptides (pre-pro-VWF). After cleavage of the 22 residue signal peptide, the subunit consists of a propeptide of 741 residues and a mature protein of 2050 residues. This 270kDa subunit dimerises by disulphide bonding at the carboxyl terminal end and forms multimers of variable length through disulphide bonding at the N-terminus (Ruggeri & Ware, 1993). The resulting ultra large forms of VWF (ULVWF) consist of an increase in number of mature VWF subunits since pro-VWF monomers do not appear to be a constituent (Tsai *et al*, 1991a). The 1:1 stoichiometry of the propeptide and mature VWF may be explained by a noncovalent association promoted by the high calcium concentration and acidic pH found within the trans Golgi network (TGN) and Wiebel-Palade bodies. Analysis of subcellular fractions of endothelial cells metabolically labelled with sulphate has demonstrated that pro-VWF cleavage and covalent multimerisation precedes the formation of Weibel-Palade bodies that bud from the TGN (Vischer & Wagner, 1994). It is within these specialised organelles or the platelet α-granules that high molecular weight multimers are subsequently stored for regulated release. Constitutive secretion is operative only in endothelial cells with directional secretion into both the circulating blood and the subendothelium. The initial proposal that the regulated secretory pathway from Wiebel-Palade bodies is predominantly towards the basolateral compartment (Sporn *et al*, 1989) has been challenged. It has been demonstrated that up to 80% of VWF released from storage

organelles is directed toward the apical side of the cell (Hop *et al*, 1997). One possible explanation for these discrepant results is that *in vivo* the polarity of VWF secretion is modulated by the nature of the release inducing stimulus (Sporn *et al*, 1989). Potent secretagogues include thrombin (Levine *et al*, 1982), fibrin (Ribes *et al*, 1987) and histamine (Hamilton & Sims, 1987). Secretion by the regulated pathway yields multimers of higher molecular mass than those originating from constitutive secretion (Sporn *et al*, 1987). This may have biological relevance since large multimers are haemostatically more effective (see below). VWF release is also influenced by shear stress: laminar shear stress greater than 8dynes/cm^2 acting on a HUVEC monolayer for at least six hours induced a significant increase in the amount of VWF released in culture media (Galbusera *et al*, 1997). This phenomenon appears to be secondary to enhanced secretion rather than *de novo* protein synthesis. Importantly, shear stress also increases VWF deposition in the extracellular matrix (Galbusera *et al*, 1997). Once bound, the molecule is protected from multimeric cleavage and acquires a remarkably high affinity for platelet receptors (Stel *et al*, 1985;Turitto *et al*, 1985).

As important as the polymeric nature of VWF is for establishing multiple bonds with platelets, specific substrate and receptor specificities reside in subunit domains. VWF has a domain structure consisting of repeated A, B, C and D domains. The A repeat has been found in many adhesive proteins, collagens and integrin receptors. The amino-terminal domain within the first 272 residues of the mature subunit forms a complex with factor VIII. The A1 domain binds to heparin-like molecules (Fujimura *et al*, 1987) and therefore possibly to proteoglycans and sulphatides (Christophe *et al*, 1991). It has been shown to play a potentially important role in the immobilisation of VWF onto complex extracellular matrices (Denis *et al*, 1993). Together with flanking regions (residues 449 – 728 of the mature subunit) it also represents the binding site for GPIb in the platelet membrane GPIb-IX-V complex (Girma *et al*, 1995). Collagen binding may be mediated via the A1 domain but occurs more readily at the A3 domain. The site of interaction for αIIbβ3 (GPIIbIIIa), the second platelet receptor binding to VWF is the RGDS sequence located at residues 1744 – 1747 in the

carboxyl-terminal C1 domain. No function has yet been found for the A2 repeat although point mutations identified in von Willebrand disease (VWD) type 2A patients are localised in the area 834-845 of this domain. This is close to a putative cleavage site for a protease (Dent *et al*, 1990;Dent *et al*, 1991) and mutations may cause conformational changes which enhance susceptibility to proteolysis (see below) (Lyons *et al*, 1992). In contrast, a VWF deletion mutant lacking the A2 domain, ΔA2-vWF, was demonstrated to be resistant to proteolysis by VWF-cleaving protease (Lankhof *et al*, 1997).

1.3.2 Post secretory VWF processing

In vitro VWF is secreted from the endothelial cells as an extra large polymer (Sporn *et al*, 1987;Tsai *et al*, 1989a). However, when analysed by SDS agarose gel electrophoresis plasma VWF consists of a series of multimers with molecular sizes ranging from 500kDa to over 12,000kDa (Hoyer & Shainoff, 1980). VWF fragments with mobility corresponding to 189, 176 and 140kDa are consistently detected in normal plasma in addition to the predominant 225kDa subunit (Zimmerman *et al*, 1986;Tsai *et al*, 1991b). The 140kDa fragment is derived from the N-terminal region whereas the 176kDa fragment originates from the C-terminal region of the subunit. Smaller multimers contain a relatively higher proportion of fragments suggesting that proteolysis occurs after multimer assembly (Tsai *et al*, 1991b). That proteolytic cleavage can convert endothelial VWF into the plasma multimers was first indicated in a study using leukocytic serine proteases (Tsai *et al*, 1989b). This proteolytic model of multimer generation is consistent with electron microscopic findings that VWF multimer strands have at least two morphologically distinct ends (Fowler *et al*, 1985). However, neither the leucocytic proteases, plasmin (Berkowitz *et al*, 1987), calpains (Berkowitz *et al*, 1988) or elastase (Berkowitz *et al*, 1988) are responsible for VWF multimer generation *in vivo* since the fragments generated are distinct from those found in normal plasma. Moreover, multimer size distribution is not altered in patients with severe leucopenia (Phillips *et al*, 1991). In contrast, a specific protease generated

a series of multimers from high molecular VWF which were indistinguishable from those found in plasma (Furlan *et al*, 1996;Tsai, 1996).

VWF susceptibility to proteolysis is critically dependent upon its structural conformation. Electron microscopy studies have shown that only a small fraction of VWF multimers (13% VWF molecules) are in the extended form, while 87% are loosely coiled (Slayter *et al*, 1985). Unfolding of VWF may be promoted by both mechanical and chemical factors such as shear stress (Siedlecki *et al*, 1996) and guanidine hydrochloride (Tsai, 1996). Both have been demonstrated to aid VWF proteolysis (Tsai *et al*, 1994;Tsai, 1996). Exposure of tryptophan and/or tyrosine residues may be important because a shift in fluorescence to longer wavelengths is seen after incubation of VWF with guanidine hydrochloride. A similar shift in fluorescence intensity of up to 4nm may also be observed when VWF is exposed to high shear stress.

In vivo, proteolysis generates two predominant VWF fragments of 140 and 176kDa. It has been demonstrated that these originate as a consequence of cleavage of a single peptide bond between residues Tyr-842 and Met-843 of the mature subunit (Dent *et al*, 1990;Dent *et al*, 1991). The 140kDa fragment corresponds to residues 1 to 842 of the VWF subunit and that of 176kDa corresponds to residues 843 to 2050. Since there is no intrachain or interchain disulphide bond across the bond this single event can separate a VWF multimer into two smaller species, each having a different cleaved subunit at the amino or carboxyl terminal end of the polymeric chain (Dent *et al*, 1991). The fact that these two fragments have a combined apparent mass greater than that of the intact subunit must reflect distinctly aberrant individual patterns of electrophoretic mobility. No explanation has been found to date for the origin of the minor subunit fragment of 189kDa. This may possibly reflect heterogeneity resulting from glycosylation or other post-translational processing. The precise mechanism by which VWF proteolysis is achieved remains unclear. Although it has been demonstrated that normal plasma contains a metalloproteinase acting specifically on VWF multimers and cleaving the Tyr842-Met843 bond (Furlan *et al*, 1996;Tsai,

1996), VWF processing post secretion may not be attributable to a single protease. There is evidence *in vitro* for a limited disulphide bond reductase present in both plasma and cryosupernatant which mediates a reversible reduction in ULVWF multimeric size (Phillips *et al*, 1993). Whether this is of physiological significance is unknown.

Nevertheless a novel VWF-cleaving protease activity appears to be of prime importance in post-secretory VWF processing. Degradation of VWF multimers occurs *in vitro* either at low salt concentrations, a process which is promoted by the presence of urea (Furlan *et al*, 1996) or after unfolding of VWF is induced by guanidine hydrochloride (Tsai, 1996). Under both conditions the fragments generated, of molecular weight 140kDa and 170kDa, are consistent with cleavage of the peptide bond between Tyr-842 and Met-843 suggesting that a physiological phenomenon is occurring. Using these models it has been shown that VWF-cleaving protease activity possesses a number of unusual properties. Preincubation with divalent ions results in protease activation in the following order: $Ba^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+}$. Although a metalloenzyme, being inhibited by EDTA and EGTA, it differs from other metalloproteases in that barium ions rather than calcium ions are the most efficient activators. Zinc ions are not required for full activity thus distinguishing it from matrix metalloproteinases. It has a pH optimum of 8 to 10 in contrast to acid lysosomal cathepsins and its activity is unaffected by serine protease inhibitors. The inhibitor profile also differs markedly from that of calpain (Furlan *et al*, 1996). The protease has recently been characterised as a new member of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin type I motif) family, ADAMTS13 (Levy *et al*, 2001;Fujikawa *et al*, 2001;Gerritsen *et al*, 2001). The reported inhibitor profile and metal cation dependency of VWF-CP are consistent with its identity as an ADAMTS.

The ADAMTS family is characterised by the presence of one or more thrombospondin type-1-like (TSP1) domains at the carboxy terminus. These are thought to mediate interactions with the extracellular matrix (Kaushal & Shah, 2000;Hurskainen *et al*, 1999;Tang & Hong, 1999). Members of the ADAMTS family have diverse functions.

Mutations in *ADAMTS2* (procollagen N-proteinase) are associated with Ehlers-Danlos syndrome type VII (Colige *et al*, 1999). Whilst *ADAMTS1* mutations have not been identified in humans, genetically deficient mice exhibit growth retardation, adipose tissue abnormalities and fibrotic changes throughout the genitourinary system, suggesting ADAMTS1 may be central to organogenesis and tissue remodelling (Shindo *et al*, 2000). In contrast, the aggrecanases, ADAMTS4 and ADAMTS5/11, cleave the core protein of the cartilage proteoglycan, aggrecan and may be important in inflammatory joint disease (Tortorella *et al*, 1999).

The *ADAMTS13* gene spans 29 exons and encodes a protein of 1427 amino acids with a predicted non-glycosylated relative molecular mass of 154kDa. This is consistent with a previous estimate of 200kDa for partially purified VWF-CP (Tsai, 1996) but considerably smaller than the 300kDa reported by others (Furlan *et al*, 1996). Subsequent purification of VWF-CP from a factor VIII/VWF concentrate demonstrating trailing of VWF-CP into the major protein peak eluted from a diethylaminoethanol (DEAE) column suggests that VWF-CP binds to large proteins and may explain this anomaly (Fujikawa *et al*, 2001). Complex formation may also explain the unusually long biologic half-life of this protein estimated at 2.1 – 3.3 days (Furlan *et al*, 1999) but a physiologically important interaction has yet to be characterised. Athough α_2-macroglobulin binds and inhibits virtually any protease there was no demonstrable association of VWF-CP with α_2-macroglobulin by immunoaffinity chromatography (Gerritsen *et al*, 2001). In view of the long biologic half-life of VWF-CP this is perhaps to be expected since α_2-macroglobulin-protease complexes are rapidly cleared from the circulation. Following purification of VWF-CP from normal human plasma by affinity chromatography, the protease was demonstrated to migrate in gel filtration as a high-molecular weight complex with clusterin (apolipoprotein J), a 70kDa protein with chaperone-like activity. However VWF-CP in normal plasma does not associate with clusterin suggesting that such complex formation is the consequence of VWF-CP denaturation during the purification procecure (Gerritsen *et al*, 2001).

Genetic analysis suggests several distinct isoforms of ADAMTS13 exist. There is evidence for alternative splicing of exon 17 and an additional potential exon between the current exons 22 and 23. Whether distinct biological functions might be attributed to different isoforms of ADAMTS13 is unknown. Using Northern blot analysis, *ADAMTS13* mRNA was specifically detected in the liver and this is consistent with the observation that VWF-CP activity is reduced in liver failure (Mannucci *et al*, 2001). This was confirmed using RT-PCR to detect the presence of exons 10 – 14 in hepatic tissue. A strong signal was seen in ovarian tissue with weak expression in other tissues including the kidney, pancreas, liver and spleen. Such variable tissue expression is suggestive of potential functions other than VWF proteolysis. As transcripts were undetectable in highly vascular organs such as the heart and lungs it seems unlikely that the vascular endothelium is a primary site of synthesis of ADAMTS13 (Levy *et al*, 2001).

The domain structure suggests that proteolytic processing, either in the TGN or at the cell surface is required for activation. Impaired activation or deficiency of VWF-cleaving protease would be expected to result in persistence of ULVWF within the circulation. This is likely to promote shear induced platelet aggregate formation. At comparative VWF antigen concentrations, ULVWF derived from cultured human umbilical vein endothelial cells is 5 – 15 times more effective than normal circulating plasma VWF multimers at mediating platelet aggregation (Moake *et al*, 1986). Similarly, VWF mediated platelet attachment to a filter device is potentiated by ULVWF as compared to smaller VWF multimers (O'Brien & Etherington, 1997).

1.3.3 VWF-cleaving protease assays

VWF-CP activity results in cleavage of a single peptide bond, 842Tyr-843Met of the mature subunit leading ultimately to the formation of low molecular weight VWF multimers and generation of 140 and 176kDa VWF fragments. To date most assays have utilised these latter properties and are thus semi-quantitative. That described by Furlan in 1997 depends on partial unfolding of VWF by urea and activity is

determined by VWF multimeric analysis using SDS-electrophoresis in 1.4% agarose gels (Furlan *et al*, 1998a). In contrast Tsai utilised guanidine to partially unfold VWF and the generation of dimers of 140 and 176 kDa as the surrogate end-point. These were identified on SDS-polyacrylamide gel electrophoresis as 350 and 200 kDa bands respectively (Tsai & Lian, 1998). Both techniques are cumbersome, extremely labour intensive and allow processing of only a limited number of samples. Several different approaches have subsequently been employed to overcome some of these limiations.

High molecular weight forms of VWF bind preferentially to collagen and this attribute has been utilised in the collagen binding assay. Prior activation with barium ions, overnight incubation and dialysis versus 1.5M urea is still a prerequisite (Gerritsen *et al*, 1999). This method has been further modified to allow patient plasma to be used as the source of the enzyme and substrate, negating the need for purified VWF (Aronson *et al*, 2001). Monoclonal antibodies have also been utilised in a two-site IRMA. In this assay a monoclonal antibody whose epitope is localised on the C-terminal side of the cleavage site is used as a coating antibody and a pool of eight antibodies specific for the N-terminal side subsequently applied. Progressive apparent loss of VWF:Ag is employed as a surrogate marker of proteolysis (Obert *et al*, 1999). Whilst these developments have simplified the determination of protease activity, measurement of VWF-CP still remains essentially semi-quantitive. Recombinant, truncated monomeric VWF has been developed which could be employed in a quantitative assay (Raife *et al*, 2001a). Furthermore the recent identification of VWF-CP as ADAMTS-13 will also permit the development of antigenic in addition to functional assays.

1.4 Endothelium

As discussed above, plasma VWF is primarily derived from the endothelium. Vascular endothelial cells form a continuous monolayer covering the inner surface of the entire vascular endothelium. Despite its apparent simplicity, endothelial structure is highly specialised, reflecting its diverse metabolic functions. Quiescent resting endothelial cells are highly heterogeneous varying not only between organs, but also between

vessels of varying calibre within the same organ. The endothelium may therefore be thought of as a systemically disseminated organ. Some of its structural and functional properties will be discussed below.

1.4.1 Endothelial structure and function

The luminal endothelial cell surface interacts directly with blood and is intrinsically non-thrombogenic: unstimulated platelets adhere minimally to the surface of intact confluent monolayers of endothelial cells either *in vitro* or *in vivo* (Curwen *et al*, 1980;Dejana *et al*, 1980). However, if the endothelium is breached or damaged, the endothelial surface rapidly becomes prothrombotic as a consequence of endothelial activation. Its non-thrombogenic properties are multifactorial. Platelet inhibition is achieved through the synthesis of nitric oxide (NO), prostacyclin (PGI_2), endothelial derived relaxing factor (EDRF) and ATPases. However it is the control of thrombin generation which is central to the balance between the endothelium's natural antithrombotic and induced procoagulant properties. Endothelial expression of tissue factor pathway inhibitor (TFPI) which binds to factor Xa within the tissue factor/VIIa/Xa complex limits thrombin generation. The endothelium also helps contain thrombin activity through the expression of heparin and thrombomodulin. The former is a cofactor for antithrombin which dramatically accelerates the inactivation of thrombin (Busch & Owens, 1982). The latter, an endothelial transmembrane glycoprotein, is responsible for approximately 50-60% of endothelial thrombin binding sites (Maruyama & Majerus, 1985) and functions as a natural anticoagulant. Binding of thrombin to thrombomodulin potentiates the activation of the anticoagulant protein C by thrombin at least 20,000 fold (Esmon & Owen, 1981). It also reduces the ability of thrombin to activate platelets, factor V, factor XIII and fibirinogen whilst promoting endothelial fibrinolytic activity. The relative concentration of thrombomodulin is decreased in larger vessels as compared to the microvasculature due to reduced surface area. This effect is counterbalanced by the constitutive expression of the endothelial protein C receptor (EPCR) predominantly in larger vessels. Binding of protein C to EPCR augments protein C activation by the thrombin-

thrombomodulin complex. As a consequence of all of these properties, little thrombin activity is found in the plasma of healthy subjects (Cines *et al*, 1998).

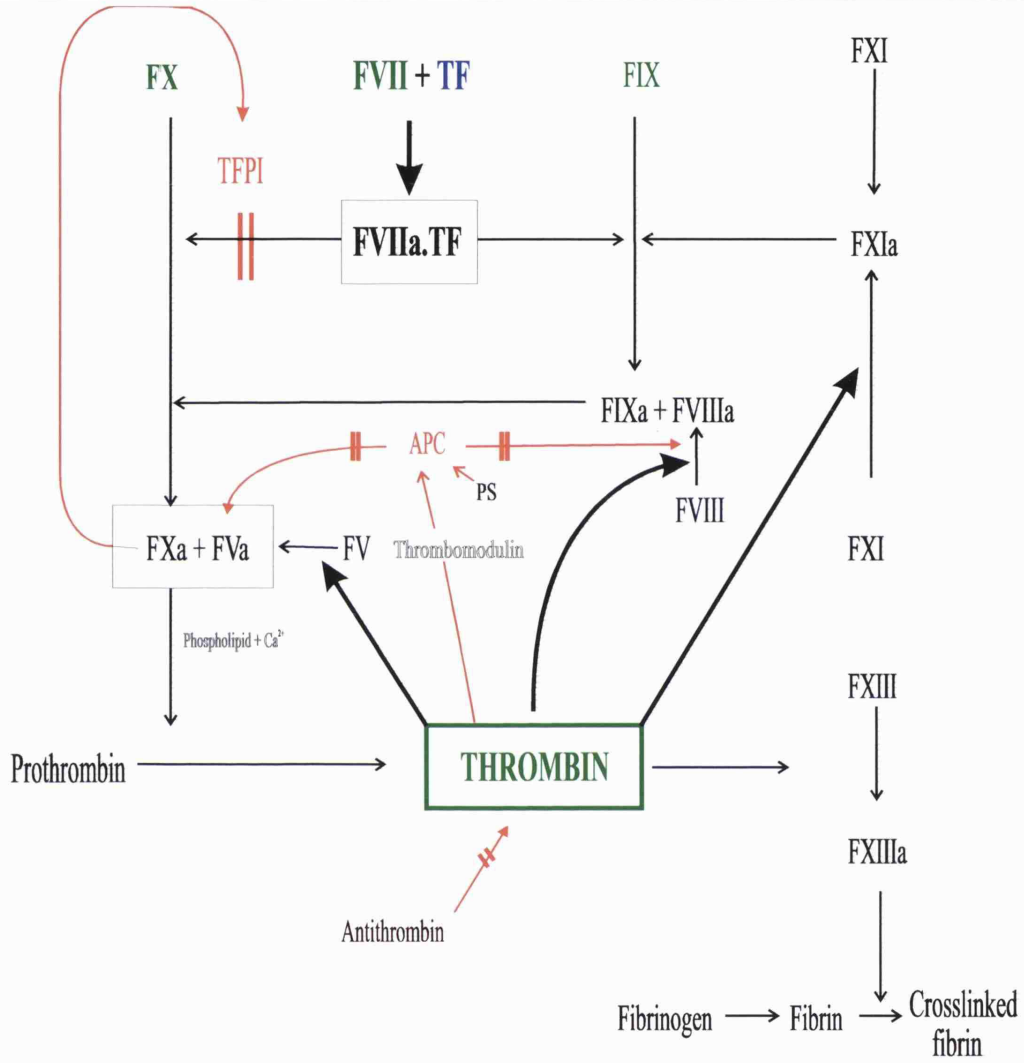


Figure 1.2 *The revised hypothesis of blood coagulation.*

The vitamin K dependent serine proteases II, VII,IX and X are shown in green with the cofactors tissue factor (TF) and factors V and VIII in blue.Tissue factor plays an integral role in the initiation of coagulation through the activation of FVII. Activated factor X (FXa) is generated by the activated factor VII/tissue factor complex (FVIIa/TF). Due to rapid FXa dependent inactivation of FVIIa.TF by tissue factor pathway inhibitor (TFPI) factors IX and XI are required to generate sufficient tenase complex to ensure thrombin generation. The central role of thrombin is clearly shown. Coagulation is limited by the actions of the anticoagulant system: TFPI, activated protein C (APC) and antithrombin shown in red.

Tissue factor (TF), a transmembrane glycoprotein, is the primary cellular initiator of the coagulation cascade. Intact endothelial cells possess essentially no TF activity (Drake *et al*, 1989). However, exposure of endothelial cells to physiological and pathological mediators including thrombin, IL-1, TNF-α, immune complexes or endotoxin, induces TF expression and is the pivotal step in transforming the endothelial membrane from an anticoagulant to a procoagulant surface. Protease-activated receptor (PAR)-1 and PAR-2 are expressed on endothelial cells and serve as macromolecular substrates for active thrombin. Upon proteolytic cleavage of the amino terminal exodomain, a new amino-terminus becomes unmasked which serves as a tethered ligand and induces transmembrane signalling through G-proteins. Unactivated PAR's on endothelial cells cycle slowly between the cell surface and intracellular compartments. Thus cellular responsiveness is retained following irreversible activation by thrombin. Whether the described or novel PAR's can account for the signalling pathways initiated by FVIIa/TF or Factor Xa remains to be established.

Following endothelial perturbation, factor VII or activated factor VII (VIIa) is able to bind to exposed TF resulting in dramatic acceleration of factor VIIa-dependent activation of factors X and IX. This interaction also promotes the ability of trace amounts of activated factor X (Xa) (and less efficiently activated factor IX, IXa) to activate factor VII. Endothelial bound factor IXa is several fold more active than factor IXa in solution (Stern *et al*, 1983). Binding of factors IXa and Xa serves to localise coagulation to the endothelial cell surface and inhibits circulation of activated coagulation factors. The initial Xa produced by this mechanism generates sufficient thrombin to induce local platelet aggregation and activation of the critical cofactors V and VIII resulting in effective haemostasis (see *Figure 1.2*). Fibrinolysis is also localised to the vascular endothelial surface by receptors for plasminogen and plasminogen activators (Plow *et al*, 1995). Endothelial activation results in reduced fibrinolytic potential due to enhanced PAI-1 release.

Thrombin is also a potent stimulator of VWF secretion (Levine *et al*, 1982). As previously discussed, VWF is stored within Wiebel-Palade bodies, structures found in the endothelial cells of most blood vessels but not in any other cell type. These membrane-bound organelles can be up to 4μm long and 0.1μm thick. Using electron microscopy, longitudinally arranged tubular structures that represent highly organised polymers of VWF can be distinguished inside the granule. After stimulation of the endothelial cell, VWF is released into the surrounding blood or basement membrane (Sporn *et al*, 1989). However, little information is known regarding the actual process of Wiebel-Palade body secretion. The signal for secretion is linked to calcium influx (de Groot *et al*, 1984) and the cellular cytoskeleton, specifically the microtubule fraction, is involved in the organelle's generation and translocation to the plasma membrane (Sinha & Wagner, 1987).

Since the environmental milieu of the endothelium is constantly changing the endothelial cell is required to react to multiple intercellular signals. This is achieved by a myriad of cell surface receptors and secondary messenger systems. These include protease activatable receptors (PAR's), endothelium protein C receptor (EPC-R), thrombomodulin and TF. All are associated with noncoated plasmalemmal vesicles called caveolae that are found abundantly on the luminal surface of many endothelia. Caveolae are enriched in lipids and various key signalling molecules, including protein kinase C (PKC), inositol 1,4,5-triphosphate and phospholipase C. They are therefore thought to facilitate signal transduction. For example, PDGF induces protein tyrosine phosphorylation on the luminal surface of cultured endothelial cells preferentially but not exclusively in highly purified caveolae (Liu *et al*, 1996). Caveolae may also play a role in mechanotransduction. The substructural coat of caveolae is formed from polymerised caveolin. Haemodynamic forces impose strain on caveolae and may modulate caveolin conformation sufficiently to permit local translocation and activation events.

Like caveolae, the intermediate junctions (adherens junctions) may play a role in cellular signalling. Vascular endothelial (VE)-cadherin is the major component of the

intermediate junction and has a short cytoplasmic domain linked to the cytoplasmic proteins β-catenin, plakoglobin and p120. Junctional stability is promoted by indirect binding to the actin cytoskeleton through α-catenin which is able to bind to β-catenin and plakoglobin. Since p120 binds neither α-catenin nor actin, a junctional complex in association with p120 is relatively mobile. The VE-cadherin catenin complex is thus dynamic and its compostition may change rapidly in response to the functional state suggesting a role in intracellular signalling (Dejana, 1996). In addition a number of different receptor-like phosphatases, kinases and growth factor receptors are concentrated at intercellular contacts and may facilitate reciprocal interaction. Pathway specificity is suggested by the association of distinct molecules as compared to those associated with tight junctions.

A particular attribute of the endothelium is its ability to respond to mechanical forces such as shear stress. For example, shear stress promotes secretion of VWF and NO and leads to preferential alignment of endothelial cells along its axis. However the endothelium must not only respond to but also withstand high levels of shear stress. Both properties appear to be dependent on stress fibres. Stress fibres are myofibril-like straight filament bundles which are mainly located close to the basal surface and are particularly abundant at sites with levels of high shear stress/wall strain. They are attached by β1 integrins to circumscribed areas of plasma membrane called focal substrate contacts which are tightly attached to components of the extracellular matrix.

The cell-extracellular matrix contact is vital to maintain endothelial structure. Integrins primarily mediate this adhesive contact. All integrins are αβ glycoprotein heterodimers and those belonging to the β_1 and β_3 subclasses are expressed on endothelial cells. Members of the β_1 family include receptors for fibronectin (VLA-5), collagen (VLA-2) and laminin whilst the vitronectin receptor is a member of β_3 family. Integrins also play a role in regulating gene expression and cell growth, differentiation and survival through outside-in and inside-out signalling. Functional diversity is promoted by the large number of integrin cytoplasmic tail binding proteins. Actin binding proteins including talin, vinculin and filamin couple integrins

to the cytoskeleton. In this manner a variety of biochemical signals may be generated including activation of MAP kinases, phosphatidylinositol 3-kinases and protein tyrosine kinases. Integrin signalling is intially focussed at topographically localised regions of the plasma membrane where cell-cell or cell-extracellular matrix contact takes place. This serves to specify anchorage dependent changes in cell shape, polarisation and motility (O'Regan *et al*, 1980). For example, adhesion of endothelial cells to fibronectin results in elevation of cytoplasmic pH and correlates with parallel stimulation of cell spreading and growth (Ingber *et al*, 1990). In addition to such outside-in signalling, ligand binding by integrins may be tightly regulated by cellular signalling molecules. This is referred to as integrin activation or inside-out signalling and reflects either an increase in true affinity of the receptor for the ligand or an increase in avidity.

1.4.2 Endothelial activation

Endothelial perturbation results in five core changes: loss of vascular integrity, expression of leucocyte adhesion molecules, changes in phenotype from antithrombotic to prothrombotic, cytokine production and upregulation of HLA molecules. The prothrombotic changes include loss of surface expressed thrombomodulin and heparan sulphate, enhanced PAI-1 release and expression of TF, E-selectin and P-selectin. Phenotypic modulation of the vascular endothelium by humoral factors such as cytokines and endotoxin constitutes the best studied paradigm of endothelial activation. However, the endothelium may also be activated by dymnamic forces: when cultured endothelial cells are exposed to quasi-physiological levels of fluid mechanical forces *in vitro*, a spectrum of functional and structural changes is observed (Davies, 1995;Resnick & Gimbrone, 1995). Real time visualisation of cultured endothelial monolayer exposed to unidirectional laminar shear stress reveals dynamic remodelling of focal contact sites along its basal aspect (Davies, 1995). Changes are also observed in the phosphorylation state of cytoskeletal proteins associated with these focal adhesion complexes as well as adhesion molecules such as PECAM-1 that are localised to lateral cell-cell junctions (Davies, 1995). In

addition to these structural adaptations, biomechanical forces also stimulate the production in the endothelium of a large and diverse array of potent biological mediators (Davies, 1995;Resnick & Gimbrone, 1995). Blood flow imparts three types of mechanical forces to endothelial cells: shear stress, pressure and tension (stretch). Certain of these effects involve gene regulation at the transcriptional level and are therefore analogous to endothelial activation by humoral factors. The endothelial cell appears able to respond not only to the magnitude of the applied forces but also to their temporal and spatial fluctuations eg. steady versus pulsatile flow, suggesting the existence of primary flow sensors that are coupled via distinct signalling pathways to nuclear events. Positive and negative shear stress responsive elements (SSRE's) have now been identified in the promotors of biomechanically responsive genes and transcription factors that regulated their activation (Resnick & Gimbrone, 1995;Gimbrone *et al*, 1997). For example, shear stress activates two transcriptional factors AP-1 and NF-kB (Shyy *et al*, 1995;Lan *et al*, 1994) which may bind to SSRE (Khachigian *et al*, 1995). When cultured HUVEC are exposed to physiologically relevant shear stress (2.5-46dyn/cm2) of steady unidirectional laminar shear stress there is a time dependent induction of ICAM-1 expression (Nagel *et al*, 1994).

1.5 Clinical features of microangiopathic haemolytic anaemia

Several microangiopathic haemolytic anaemic syndromes including TTP, HUS and DIC may be characterised by similar features. At present there is no pathognomonic assay for TTP and its differentiation from other microangiopathic syndromes relies upon history, clinical examination and routine laboratory assays. Since each feature of the pentad of TTP can occur in HUS and DIC diagnostic uncertainty is not uncommon. In fact as previously stated, the clinical presentation of TTP and HUS may be so similar that this has previously lead to the suggestion that TTP and HUS represent a spectrum of disease. As a consequence older literature refers to HUS-TTP as a single clinical entity. However it is becoming increasingly apparent that the pathophysiology of these two conditions is distinct and thus this explains in part the

wide variation in response to treatment which is seen in clinical practice. A summary of clinical features of the thrombotic microangiopathic syndromes is presented in *Table 1.3* and will be discussed further below.

Diagnosis	TTP	HUS	Pre-eclampsia/ eclampsia	HELLP	DIC
CNS symptoms/signs	+++	+/-	+/-	+/-	+/-
Renal impairment	+/-	+++	+	+	+/-
Fever	+/-	-/+	-	-	+/-
Liver impairment	+/-	+/-	+/-	+++	+/-
Hypertension	-/+	+/-	+++	+/-	-
Haemolysis	+++	++	+	++	+
Thrombocytopenia	+++	++	+/-	++	+++
Coagulopathy	-	-	+/-	+/-	+++

Table 1.3 *Characteristic spectrum of pathophysiological features seen in microangiopathic haemolytic anaemia*

1.5.1 Adult onset TTP

Adult-onset TTP is a relatively rare condition with an approximate incidence of 1 per million although recent figures suggest that this may be closer to 3.7 per million (Torok *et al*, 1995). Whether this represents a true increase in incidence or simply reflects previous under-reporting remains unclear. Women are more commonly affected than men with most series reporting a male: female ratio of 1:2 (Bell *et al*, 1991;Rock *et al*, 1991). The disease can occur at any age although it is most prevalent between the ages of 20-40 years.

TTP is characterised by the classical pentad of thrombocytopenia, microangiopathic haemolytic anaemia (MAHA), fluctuating neurological signs, renal impairment and

fever, often with insidious onset. The range of clinical presentation is broad since neurological manifestations may range from headache to bizarre behaviour, transient sensorimotor deficits (TIA's), seizure and coma. Presence of coma at presentation is a poor prognostic indicator (Sarode *et al*, 1997;Pereira *et al*, 1995). In some patients less common symptoms may be a feature such as gastrointestinal ischaemia (manifest as abdominal pain) (Chang & Gupta, 1998)and serous retinal detachment (Lambert *et al*, 1985). However, up to 35% patients with TTP do not have neurological symptoms or signs at presentation (Rock et al, 1991). Since the triad of acute renal insufficiency, MAHA and thrombocytopenia defines HUS (Gasser *et al*, 1955), diagnostic uncertainty may arise. Moreover, fever and renal impairment are present in only a minority of patients (Rock *et al*, 1991;Rock *et al*, 1998). In practice therefore a diagnosis of TTP may be made in the presence of a microangiopathic haemolytic anaemia and thrombocytopenia in the absence of any other identifiable cause.

1.5.2 Clinical subtypes

The majority of cases of adult onset TTP are idiopathic – in such patients there is no identifiable precipitant and no subsequent recurrence. Single episode TTP is, however, a retrospective diagnosis because it has been estimated that 11-36% patients relapse in the ten years following an index episode (Shumak *et al*, 1995) and a diagnosis of intermittent TTP is then made. In a minority of cases a predisposing factor may be identified and secondary TTP diagnosed. These include include drugs, auto-immune disease, malignancy and infection.

Several drugs have also been implicated in the aetiology of TTP including mitomycin C (Durand & Lefevre, 2001;Moake & Byrnes, 1996), ticlopidine (Bennet *et al*, 1998), clopidogrel (Bennet *et al*, 2000), cyclosporin (Atkinson *et al*, 1983;Dzik *et al*, 1987), contraceptive therapy (Fraser *et al*, 1996;Holdrinet *et al*, 1983;Liang *et al*, 1996) and quinine (Medina *et al*, 2001;Moake & Byrnes, 1996). Perhaps those most closely linked are ticlopidine and cyclosporin. Ticlopidine is an ADP platelet antagonist widely used to prevent rethrombosis post arterial stenting. TTP typically occurs two to four weeks after initiation of therapy with an incidence of one per 1600 to 5000

patients treated (Bennet *et al*, 1998) and is clinically indistinguishable from idiopathic cases. Recently clopidogrel, another thienopyridine compound and ADP antagonist, has been linked to TTP although the frequency is similar to that of idiopathic TTP at only 11 cases per 3 million treated (Bennet *et al*, 2000). Further surveillance will be required to determine whether this represents a true association or not. Cyclosporin is a potent immunosuppressant drug widely used in the post transplant setting when the incidence of TTP is reported to be in the order of 5% (Fuge *et al*, 2001). This is not simply a reflection of cyclosporin usage: TTP post transplantation has also been associated with TBI (Scriber & Herzig, 1997) and GVHD (Holler *et al*, 1989) and may occur in the absence of cyclosporin (Scriber & Herzig, 1997). Similarly whilst mitomycin C has been implicated in TTP, it is recognised that malignancy *per se* and in particular adenocarcinoma are also predisposing factors (Kressel *et al*, 1981).

Infectious precipitants appear to be relatively uncommon since TTP is not associated with seasonal variation and case clustering rarely occurs. Anecdotally TTP appears to be triggered by viral infections but this may represent a prodromal TTP illness since fever is one constituent of the defining pentad of TTP. Nevertheless there have been TTP-like illnesses reported in the context of HIV and *E.Coli* infections. Although *E.Coli* 0157:H7 is more closely linked with HUS there have been cases with typical TTP features (Morrison *et al*, 1986;Kovacs *et al*, 1990). Of note VWF-CP deficiency (see below) was detected in one of 29 children with epidemic HUS, 25 of whom were positive for verotoxin (Hunt *et al*, 2001). In some series, up to 14% TTP episodes have been associated with HIV infection (Ucar *et al*, 1994) although mortality data from the United States for 1988-1991 gives a figure of only 4.4% (Torok *et al*, 1995). Risk appears greatest at CD4 counts of less than 250 x 10^9/l (de Man *et al*, 1997). Of interest, a TTP-like illness in association with Bartonella bacilliformis-like organisms adherent to 0.1% to 2% of circulating erythrocytes has also been reported in five patients (Tarantolo *et al*, 1997).

1.5.3 Laboratory features

TTP is often characterised by severe thrombocytopenia which may be useful in its differentiation from HUS. However in one series, although the mean platelet count was lower in TTP than HUS (18 x 10^9/l versus 36 x 10^9/l) there was a wide range and considerable overlap (Vesely *et al*, 2000). Severe thrombocytopenia at diagnosis (platelet count < 20 x 10^9/l) has been suggested to be a poor prognostic indicator conferring increased mortality (Rock *et al*, 1998) although this is not a uniform observation (Sarode *et al*, 1997). Thrombocytopenia is typically accompanied by overt microangiopathic haemolysis with marked red cell fragmentation and polychromasia. However, shistocytes may be absent from the peripheral blood film in the first 24 to 48 hours following clinical presentation. Routine coagulation profiles are usually normal (Sagripanti *et al*, 1996;Monteagudo *et al*, 1991;Rock *et al*, 1998) although slight increases in D-dimer, fibrin degradation products and thrombin-antithrombin complex (TAT) may be seen (Wada *et al*, 1993;Sagripanti *et al*, 1996;Monteagudo *et al*, 1991). Secondary DIC may however arise from prolonged tissue ischaemia and is an ominous prognostic indicator. Evidence of endothelial damage is demonstrated by increased plasma levels of plasminogen activator inhibitor (PAI-1) (Anthony *et al*, 1998) and thrombomodulin. The latter has also been identified as a poor prognostic factor (Wada *et al*, 1993). Plasma VWF levels are often elevated acutely (Rock *et al*, 1998). Abnormalities of VWF multimers are also common and were identified in 86% of patients either at the onset of or during an acute episode of TTP (Moake & McPherson, 1989). These ranged from the presence of ULVWF multimers in 31% to a relative decrease in the largest plasma VWF forms in 36%. Acute changes in VWF multimeric distribution do not appear to correlate with clinical outcome. However the finding of ULVWF multimers during periods of remission has been associated with intermittent TTP (Moake *et al*, 1982). Renal function is normal in the majority of patients: only 18% patients presenting to the Canadian Apheresis Group had evidence of renal impairment (Shumak *et al*, 1995). Liver function tests often show not only hyperbilirubinaemia but also a raised transaminase level. This is thought to represent hepatic ischaemia.

1.5.4 Congenital TTP

Congenital TTP is extremely rare with fewer than 60 cases reported in the literature. It classically presents during infancy or early childhood with recurrent episodes of haemolysis and thrombocytopenia at predictable intervals (usually every 21 – 28 days) (Chintagumpala *et al*, 1992;Moake *et al*, 1994). In contrast, milder variants `have been described in which patients may present at a later age with intermittent episodes of TTP (Karpman *et al*, 1996). Such episodes are characterised by the classical clinical pentad of microangiopathic haemolytic anaemia, thrombocytopenia, fluctuating neurological signs, renal dysfunction and fever and often appear to be precipitated by febrile illnesses (Karpman *et al*, 1997;Upshaw, 1978). Both presentations are typified by the presence of circulating ULVWF multimers during remission periods consistent with defective post secretory VWF processing (Moake *et al*, 1985;Chintagumpala *et al*, 1992).

1.5.5 Haemolytic uraemic syndrome

Haemolytic uraemic syndrome is characterised by a microangiopathic haemolytic anaemia, thrombocytopenia and renal failure (Gasser *et al*, 1955) but the disease may be associated with more extensive multi-organ disease including enterocolitis, neurological complications, liver dysfunction, pancreatic and cardiac problems. In such cases, particularly when neurological problems are present, there is clinical overlap with TTP (Seigler, 1994). The epidemic form (D+) is associated with a prodromal illness often with bloody diarrhoea in contrast to the rare sporadic or atypical cases (D-) (Milford *et al*, 1990). HUS is seen increasingly following outbreaks of infection with verotoxin producing organisms. It represents a growing public health problem (Dundas & Todd, 1999). *E Coli* 0157:H7 is the most commonly notified verotoxin (VT) producing organism in the United Kingdom. Clinical manifestations may vary from an asymptomatic infection through to bloody diarrhoea, haemorrhagic colitis and HUS. Verotoxin enterococcal (VTEC) associated HUS is seen in up to 20% of cases in recent outbreaks, mainly affecting children and the

elderly (Dundas *et al*, 2001). The organisms are characterised by their ability to produce one or more verocytoxins (or Shiga toxin S1 or S2) (Tesh & O'Brien, 1991).

Haemolysis and red cell fragmentation are usually present at presentation although this rarely develops later in the disease even after improvement in the platelet count. Severe thrombocytopenia is uncommon but prolonged thrombocytopenia for more than 10 days is associated with long term renal sequelae. Poor prognostic features at presentation include a high neutrophil count (Walters *et al*, 1989). Persistent proteinuria after one year may be associated with increased risk of progressive renal dysfunction (Moghal *et al*, 1998). Coagulation studies are usually normal with only mildly raised D-dimer levels in contrast to DIC (Rose & Chant, 1998). The VWF levels are usually markedly raised during the acute illness while multimeric analysis may or may not show ultra large multimers (Rose *et al*, 1984). Factor VIII levels do not correlate with clinical outcome (Milford *et al*, 1991), but high PAI-1 levels correlate with time for platelet recovery and are associated with poor disease outcome (Chant *et al*, 1994). Early stool culture is essential for diagnosis of VTEC associated HUS (Karch *et al*, 1995) because serological diagnosis is unhelpful in the acute phase. Although ELISA IgG assays are reported to be 95% sensitive and 94% specific, current IgM assays are unreliable (Raymond *et al*, 1996).

1.5.6 Pregnancy associated thrombotic microangiopathic haemolysis

Accurate diagnosis of TTP and HUS in pregnancy is complicated by the ability of pre-eclampsia to produce thrombotic microangiopathy with a variety of clinical pictures similar to TTP and HUS. Each feature of the clinical pentad of TTP can occur in a woman with pre-eclampsia. Pre-eclampsia can also produce a unique microangiopathic haemolysis involving the liver: HELLP syndrome (Haemolysis, Elevated Liver function tests, Low Platelets. Reviewing the literature, there are many cases described as TTP that are probably due to pre-eclampsia/HELLP, having resolved quickly post-delivery on ineffective treatment, and *vice versa*, revision of a diagnosis of pre-eclampsia has to be made when it fails to resolve postpartum. A summary of the differentiating presenting features and management of MAHA in pregnancy is presented in *Table 1.4* and will be discussed further below.

TTP in pregnancy is exceptionally rare. However, the distinction of TTP from all other causes of MAHA in pregnancy is vital because the disease is usually fatal in the absence of treatment and delivery has no effect on maternal outcome. Debate continues as to whether it is precipitated by pregnancy, or whether the coincidence of pregnancy and TTP reflects the fact it is most common in women in their third and fourth decade. The prevalence of relapse during subsequent pregnancy suggests that pregnancy may be a precipitating factor (Rose & Eldor, 1987). Weiner reported that more than half the cases presenting in pregnancy occur at or before 24 weeks gestation (Weiner, 1987). The diagnosis becomes difficult when TTP presents in the third trimester or post-partum.

DIAGNOSIS	Classic TTP	Postpartum HUS	HELLP	Pre-eclampsia/ eclampsia
Time of onset	Usually < 24 weeks gestation	Post-partum	Usually > 34 weeks	Usually > 34 weeks
Histopathology	Widespread platelet thrombi	Thrombi in renal glomeruli only	Hepatocyte necrosis and fibrin deposition in periportal sinusoids	Glomerular endothelial hypertrophy and occlusion of placental vessels
Haemolysis	+++	++	++	+
Thrombocytopenia	+++	++	++	++
Coagulopathy	-	-	+/-	+/-
CNS symptoms	+++	+/-	+/-	+/-
Liver disease	+/-	+/-	+++	+/-
Renal disease	+/-	+++	+	+
Hypertension	Rare	+/-	+/-	+++
Effect on fetus	Placental infarct can lead to IUGR and mortality	None, if maternal disease is controlled	Associated with placental ischaemia and increased neonatal mortality	IUGR, occasional mortality
Effect of delivery on disease	None	None	Recovery, but may worsen transiently.	Recovery, but may worsen transiently.
Management	Early plasma exchange is imperative	Supportive +/- plasma exchange	Supportive. consider plasma exchange if persists	Supportive +/- plasma exchange

Table 1.4 *Summary of the differentiating presenting features and management of MAHA in pregnancy*

In 1968 a post-partum HUS was described (Robson *et al*, 1968;Wagoner *et al*, 1968), distinct from antepartum renal failure or renal failure occurring within 48 hours post-partum associated with poor renal perfusion due to obstetric emergencies. It typically presents subacutely with oliguric renal failure and MAHA after an otherwise uncomplicated gestation and delivery. Primiparous are most commonly affected. In one series the mean time of onset was 26 days after delivery with all cases occurring within the first ten weeks (Weiner, 1987). Rarely cases have been described during gestation due to verotoxin-producing *E Coli* (Martinez-Roman *et al*, 1996). It is also more frequently associated with HELLP syndrome than "pure" pre-eclampsia or chronic hypertension (Kahra *et al*, 1998).

Pre-eclampsia affects up to 4% of pregnancies; it most commonly occurs in nulliparous women or multiparous women with new partners. The criteria for diagnosis are new onset hypertension and proteinuria developing in the second half of pregnancy. Both hypertension and proteinuria characteristically regress after delivery. Most women also present with peripheral oedema. Pre-eclampsia is thought to involve and arise from a maternal systemic inflammatory response (Redman *et al*, 1999). Maternal endothelial activation is one part of this inflammatory response and although it occurs in all pregnancies, it is excessive in pre-eclampsia. A variety of putative circulating pre-eclampsia factors of placental origin have been proposed. These include VEGF (Hayman *et al*, 1999), neurokinin B (Page *et al*, 2000), activin A (Muttukrishna *et al*, 1997) or apoptotic debris from the syncytial surface (Redman & Sargent, 2000). One manifestation of endothelial cell activation (ECA) is that the endothelial surface can change from an antithrombotic to prothrombotic phenotype (Hunt & Jurd, 1998). Evidence for this occurring in pre-eclampsia is supported by increased levels of plasminogen activator inhibitor 1 (PAI-1). Other prothrombotic changes include reductions in protein C and antithrombin. Coagulation activation consequently results as demonstrated by increased thrombin-antithrombin complexes (TAT) and D-dimers. Platelet activation also occurs and thrombocytopenia is common. However profound DIC is a rare complication in the absence of placental

abruption. The fall in antithrombin levels seen in pre-eclampsia is not found in TTP (Sagripanti *et al*, 1996) and may therefore be of diagnostic value.

HELLP is diagnosed in the presence of haemolysis, elevated liver enzymes and thrombocytopenia. However at present there is no standardised definition for any of these criteria. HELLP occurs in up to 10% of women with severe pre-eclampsia but severe thrombocytopenia and abnormal liver function can present in the absence of significant hypertension or proteinuria. Exacerbations may occur post partum (Martin *et al*, 1990) and there is a risk of recurrence of approximately 3% in subsequent pregnancies (Sibai *et al*, 1995). Occasionally HELLP can present post-partum, usually within 48 hours of delivery, although it has been reported to occur as late as six days post-partum (Schwartz & Brenner, 1985). Common presenting symptoms include nausea, malaise, epigastric or right upper quadrant abdominal pain and oedema (Sibai & Ramadan, 1993). Fever occurs rarely in HELLP and may be a useful distinguishing feature from TTP and HUS. In one large series HELLP was frequently complicated by DIC (21%), abruption (16%), acute renal failure (8%) and pulmonary oedema (6%) (Sibai *et al*, 1993). Although mild increases in transaminases are present, profound renal failure and hepatic failure are uncommon in the absence of abruption and DIC, findings that help to distinguish HELLP from HUS and acute fatty liver of pregnancy respectively.

Many cases of thrombotic microangiopathy in pregnancy described in the literature are associated with SLE and the antiphospholipid syndrome (APS) (Huang *et al*, 1998;Kincaid-Smith *et al*, 1988). A renal flare of SLE may mimic HUS and/or pre-eclampsia. Correct diagnosis of such patients may be aided by a history of arthralgia or cutaneous manifestations. The propensity for antiphospholipid antibodies to predispose to pre-eclampsia is now recognised in the Sapporo Criteria for definite antiphospholipid syndrome (Wilson *et al*, 1999): severe pre-eclampsia in association with antiphospholipid antibodies is a new defining criterion. Furthermore, antiphospholipid antibodies have also been reported to be associated with the development of TTP and HUS in pregnancy (Kniaz *et al*, 1992).

Acute fatty liver of pregnancy is a rare but potentially fatal cause of liver failure in the third trimester. The clinical picture is dominated by liver failure. Thrombocytopenia is usually mild. Haemolysis is not usually a feature, although pre-eclampsia may be present in half of the cases (Riely, 1987).

1.6 The pathogenesis of TTP

The exact pathogenesis of TTP remains unclear. The probability that published literature regarding TTP represents several heterogeneous disorders is a further complication. Multiple factors have been suggested as playing a role in the pathophysiology of the disease. These include platelet activation, endothelial perturbation, apoptosis, calpain and ULVWF and will be discussed further below.

1.6.1 Platelet activation

The evidence for platelet activation in TTP is conflicting. A platelet aggregating factor in TTP was first reported by Lian and coworkers (Lian *et al*, 1979). Platelet aggregating activity has subsequently been demonstrated in TTP sera and appeared to operate via a GPIIbIIIa dependent pathway (Kelton *et al*, 1987). Platelet aggregation was accompanied by platelet release suggesting platelet activation had occurred. In keeping with this, increased plasma βTG concentrations were documented in three patients with TTP (Katayama *et al*, 1993). Elevated platelet CD62p expression was also detected in nine of 11 patients presenting with TTP (Wright *et al*, 1999). However in a series of seven patients with idiopathic acute TTP no significant difference in platelet surface P-selectin expression was demonstrated using a flow cytometric technique as compared to normal controls. Platelet reactivity to stimulation with thrombin or A23187 ionophore was also maintained (Hoffman *et al*, 1993). Platelet hyperfunction has been reported previously in TTP (Ahn *et al*, 1996) whilst platelet exhaustion has been described in HUS (Kaplan & Fong, 1980;Walters *et al*, 1988;Fong & Kaplan, 1982).

The absence of significant platelet activation in TTP is perhaps surprising in the light of reports that a platelet agglutinating protein may cause activation of normal donor platelets *in vitro* (Li *et al*, 1989). In addition, generation of low levels of thrombin in TTP patients (Takahashi *et al*, 1989) might be expected to activate platelets. However P-selectin may mediate clearance of activated platelets by the reticulo-endothelial system. One study estimated the half-life of activated platelets to be less than an hour *in vivo* (Rinder *et al*, 1991a). An alternative explanation therefore is that local rather than systemic platelet activation is important or that activated free platelets are rapidly consumed. For example, although CD62p positive platelets were not increased in TTP patients compared to ITP controls, there was a greater abundance of activated platelet aggregates (aPA) and micro-aPA suggesting that immediate recruitment to aPA follows platelet activation (Ahn *et al*, 1996). A pathophysiological role was suggested since disease status correlated with percentage aPA and there was a significant reduction in aPA post plasmapheresis. Alternatively incorporation into platelet-leucocyte aggregates may occur. Incubation of TTP plasma with control whole blood caused a significant increase in platelet-leucocyte aggregate formation as compared to control ITP plasma and correlated with CD62p expression suggesting that this is an important mediator of the interaction (Valant *et al*, 1998).

Platelet activation also leads to the generation of platelet microparticles (PMP). Thirteen patients with TTP had significantly elevated whole blood PMP levels at presentation as compared to normal controls. A good reciprocal relationship was found between the number of platelets and percentage of circulating PMPs at diagnosis. However PMP level did not appear to be a simple reflection of thrombocytopenia since PMP levels were only minimally elevated in control patients with chemotherapy-induced bone marrow aplasia. A pathological role was suggested since clinical recovery mirrored the progressive normalisation of PMP levels and platelet count (Galli *et al*, 1996).

Monoclonal antibodies to platelet glycoproteins can cause platelet activation as measured by aggregation and secretion (Horsewood *et al*, 1991). This is thought in

most cases to be the consequence of crosslinking and phosphorylation of the FcγRII receptor (Huang *et al*, 1992). CD36 (GP IV) is a 88kDa glycoprotein expressed on platelets, capillary endothelial cells, reticulocytes and monocytes (Greenwalt *et al*, 1992). It accelerates the initial interaction of platelets with collagen (Tandon *et al*, 1991;Diaz-Ricart *et al*, 1993) and has been proposed as a receptor for thrombospondin (Asch *et al*, 1987;Silverstein *et al*, 1992). Monoclonal antibodies directed against CD36 such as OKM-5 and OKM-8 activate platelets (Aiken *et al*, 1990). Depending on the assay system used, an incidence of 60 – 85% of anti CD36 antibodies in the plasma of TTP patients has been reported (Schultz *et al*, 1998;Tandon *et al*, 1994;Wright *et al*, 1999). However only 50% of these TTP samples induced approximately 70% relese of ^{14}C-serotonin from control platelets and of these about one half failed to induce release from Nak^{a}-negative platelets. Thus platelet activation apparently occurring as a consequence of anti CD36 antibodies was only demonstrated in some 25% cases (Tandon *et al*, 1994).

1.6.2 Endothelial perturbation

Histological examination of tissue derived from patients with TTP fails to demonstrate any evidence of acute inflammation. Nevertheless there is indirect evidence of endothelial perturbation as demonstrated by increased levels of vascular endothelial cell markers in TTP. As discussed previously, endothelial perturbation results in the release of ULVWF multimers, thrombomodulin, tPA and PAI-1. Increased levels of thrombomodulin, tPA and PAI-1 have been documented in patients with TTP (Wada *et al*, 1993;Wada *et al*, 1998;Anthony *et al*, 1998;Kobayashi *et al*, 1995;Zeigler *et al*, 1996). Moreover, mortality was associated with significantly higher plasma levels of tPA and thrombomodulin suggesting that the degree of vascular endothelial injury may correlate with prognosis in TTP (Wada *et al*, 1993;Wada *et al*, 1998;Kobayashi *et al*, 1995). VWF is an acute phase protein and also released as a consequence of endothelial activation or frank endothelial damage. Thus VWF: Ag levels are usually elevated at diagnosis (Zeigler *et al*, 1996;Kelton *et al*, 1984). However in one series mortality was associated with reduced VWF: Ag levels. This may be the consequence

of profound VWF consumption and incorporation into platelet microthrombi (Wada *et al*, 1993). Similarly increased TF expression is associated with endothelial activation. Soluble TF is derived from activated macrophages and vascular endothelial cells. However in a study of 16 patients with TTP, plasma TF antigen levels were slightly decreased rather than increased. This was accompanied by a significant decrease in TFPI levels (Kobayashi *et al*, 1995). TFPI is produced in the liver, macrophages and vascular endothelial cells but the majority of plasma TFPI is thought to derive from the endothelial pool (Sandset *et al*, 1988). It has therefore been suggested that reduced TF and TFPI levels seen in TTP may be the consequence of severe vascular damage.

Plasma P-selectin concentration is increased in TTP (Chong *et al*, 1994;Fijnheer *et al*, 1997;Katayama *et al*, 1993). However the cellular source of the protein remains obscure. It is not the consequence of *in vitro* platelet activation (Katayama *et al*, 1993). Increased concentrations have been found in the context of both elevated βTG (Chong *et al*, 1994) and E-selectin (Fijnheer *et al*, 1997), markers of platelet and endothelial activation respectively. Cloning of endothelial P-selectin predicts three different forms of the protein, including a soluble form that lacks a transmembrane domain (Johnston *et al*, 1989). Both platelets and endothelial cells contain mRNA encoding the soluble form of P-selectin (Johnston *et al*, 1990). An increase in plasma P-selectin concentration may therefore indicate either *in vivo* activation/damage of platelets and/or endothelial cells.

The mechanism of endothelial perturbation in TTP is unclear. Auto-antibodies directed against endothelial cells (AECA) have been described in several diseases in which immune mediated endothelial damage, either primary or secondary to systemic autoimmune processes is present. Some direct evidence for primary vascular injury in TTP comes from immunofluorescent studies of involved vessels by which IgG, IgM and complement were shown to be present in vascular lesions (Weisenburger *et al*, 1976). Anti-endothelial cell antibodies have also been shown to be present in TTP (Wright *et al*, 1999). Specificity was suggested by preferential binding of patient sera to microvascular endothelial cells as compared to HUVEC and inhibition by

heterologous CD 36 antibody. A number of studies have since confirmed the presence of anti CD36 antibodies in TTP (Tandon *et al*, 1994;Schultz *et al*, 1998) whilst antibodies binding a 43kDa protein derived from human microvascular renal endothelial cells have also been detected (Koenig *et al*, 1993). However, whether such AECA have pathogenic potential or are simply an epiphenomenon secondary to inflammatory vascular injury is unknown.

A recent study supports a direct pathogenic role. Affinity purified anti-endothelial cell Fab_2 antibodies from four consecutive patients with TTP bound to microvascular endothelial cells (derived from human bone marrow) (Praprotnik *et al*, 2001). Binding was specific and resulted in enhanced Il-6 and VWF release followed by increased expression of P-selectin, E-selectin and VCAM-1. Increased expression of adhesion molecules was followed by an increase in monocyte adhesion to endothelial cells, another indicator of endothelial activation. Increased production of IL-6 might contribute to adhesion molecule up-regulation since IL-6 has been shown to enhance ICAM-1, VCAM-1 and E-selectin expression on endothelial cells (Reiss *et al*, 1998). Furthermore the level of soluble thrombomodulin also increased in the culture medium and directly correlated with a decrease in cell-associated thrombomodulin (Praprotnik *et al*, 2001). Of note, increased ICAM-1 expression was not demonstrated although stimulation of endothelial cells with TNFα induces ICAM-1 expression. ICAM-1 has been reported as essential for transendothelial migration of leucocytes (Reiss *et al*, 1998). The absence of perivascular inflammation is a characteristic pathologic feature of TTP. Thus in TTP, leucocytes may not migrate into the tissues but may cause direct vessel wall injury by secretion of oxygen radicals, enzymes and other cytotoxic molecules (Savage & Cooke, 1993).

Whether complement mediated endothelial cytotoxicity is important in TTP is unclear. Although complement-dependent antibodies cytotoxic for endothelial cells have been shown in the serum and plasma of TTP patients (Burns & Zucker-Franklin, 1982;Koenig *et al*, 1993), these experiments all utilised UVECs. The lack of macrovascular involvement in TTP questions the relevance of this model. Furthermore

there is no prominent cellular infiltrate or inflammatory reaction in TTP. This would be expected to accompany the type of EC destruction observed in *in vitro* lytic models.

1.6.3 Apoptosis

Cell death may occur by necrosis or apoptosis. Necrosis is usually considered to result from physical injury and is not genetically controlled whereas apoptosis is a deliberate and controlled cellular response to specific developmental and environmental stimuli resulting in cellular disassembly. Cytoplasmic organelle destruction and loss of plasma membrane integrity typify necrosis whereas apoptosis is associated with cytoplasmic blebbing, chromatin condensation and nuclear DNA fragmentation. One major distinction between apoptosis and necrosis *in vivo* is that complete elimination of the apoptotic cell prevents an inflammatory response whereas necrosis does not. Apoptosis has been suggested to play a role in the pathogenesis of TTP for several reasons. Firstly inflammation is strikingly absent in TTP tissue specimens. Instead typical histological appearances include inappropriate endothelial cell proliferation. Cellular proliferation elicited by abnormal signals may precede apoptosis (Oyaizu *et al*, 1993;Alderson *et al*, 1995). Microvascular enlargment of endothelial cells with vacuoles, lysosomes and mitochondria, characteristic of early stages of apoptosis, may also be seen (Wallace *et al*, 1975). Finally circulating endothelial cells ranging from 5-37 cells per ml of blood were detected in one patient with TTP and appeared to correlate with clinical course (Lefevre *et al*, 1993). Although circulating endothelial cells may be a normal finding, much lower numbers are usually detected (average of 2.6 cells per ml of blood) (Solovey *et al*, 1997). Moreover the majority of these cells are apoptotic (Solovey *et al*, 1999). Apoptosis *per se* also promotes endothelial procoagulant properties, some of which have been demonstrated in TTP. For example surface expression of TF is increased whilst that of thrombomodulin, heparan sulphate and TFPI is reduced (Bombeli *et al*, 1997) along with PGI_2 production (Mitra *et al*, 1997).

The evidence supporting the role of apoptosis in the pathogenesis of TTP is limited. Apoptosis of cultured endothelial cells of dermal micovascular but not of umbilical

vein origin was enhanced by plasma from four TTP patients. Plasma from patients with DIC had no effect suggesting specificity (Laurence *et al*, 1996). It was subsequently demonstrated that this phenomenon was restricted to those cell lineages affected *in vivo*: in contrast to dermal, renal and cerebral microvascular cell lines, pulmonary and hepatic microvasculature was unaffected (Mitra *et al*, 1997). Regulation of apoptosis involves a large number of genes including the bcl-2 and interleukin-1β converting enzyme (ICE) families and intermediate genes such as Fas/Fas ligand and p53. In the above experiments apoptosis correlated with the induction of Fas transcripts and resulted in a prothrombotic phenotype as shown by reduced PGI_2 production. However soluble monoclonal Fas antibodies only partially blocked apoptosis suggesting alternative pathways may be important. The stimulus for apoptosis also remains obscure. TNFα and anti CD36 antibodies are two candidates. Apoptosis however was unaffected by surface expression of CD36 or TNFα neutralising antiserum (Laurence *et al*, 1996).

Other studies have not confirmed the above observations. Plasma from a single TTP patient induced apoptosis in a human endothelial cell line of macrovascular origin (Wu *et al*, 1999) whilst 25% TTP plasma samples failed to induce apoptosis of dermal microvasculature above backgound levels (Mitra *et al*, 1997). Apoptosis could also be demonstrated in only 50% of TTP spleens using the TUNEL assay (Dang *et al*, 1999). This technique fails to detect early apoptotic cells and may not identify minor populations of scattered apoptotic cells. It is also possible that microvascular endothelial cell apoptosis does not occur throughout all stages of TTP. However although associated with increased Fas expression, there was no correlation between degree of Fas expression and apoptosis. Moreover annexin II and TF expression, markers of a prothrombotic endothelial phenotype, showed no correlation with the degree of apoptosis induced by TTP plasma in either dermal or pulmonary microvascular endothelial cells (Mitra *et al*, 1997). Further studies are therefore required to investigate the role of apoptosis in the pathophysiology of TTP.

1.6.4 Novel platelet aggregating factor

A platelet aggregating factor has long been postulated to play a role in TTP because of the presence of microvascular platelet thrombi. Moreover in some patients thrombocytopenia precedes all evidence of TTP including shistocytic haemolysis (Kelton *et al*, 1984). These clinical observations, together with pathological observations, suggest that platelet thrombi are the initiating event of the syndrome. Several groups have demonstrated that TTP plasma induces agglutination of autologous and homologous platelets (Ahn *et al*, 1996;Suehiro *et al*, 1999;Kelton *et al*, 1984;Lian *et al*, 1979;Brandt *et al*, 1979;Burns & Zucker-Franklin, 1982) and it is now thought that ULVWF multimers play a vital role in mediating the heightened platelet agglutination seen in TTP. However a number of other factors have previously been postulated including a deficiency of a natural inhibitor of platelet aggregation aggregation (Byrnes & Khurana, 1977;Lian *et al*, 1979), decreased synthesis of PGI_2 by vascular endothelial cells (Remuzzi *et al*, 1978;Hensby *et al*, 1979), decreased PGI_2 stability (Kakishita *et al*, 1996), a 37kDa platelet agglutinating protein (Siddiqui & Lian, 1985), a calcium-dependent cysteine protease (Murphy *et al*, 1987a) and lysosomal cathepsin (Falanga *et al*, 1991).

In 1979 Lian first described a small molecular weight substance demonstrated to have platelet aggregating ability (Lian *et al*, 1979). This was not inhibited by hirudin, heparin in the presence of AT, diisopropyl fluorophosphate, apyrase, aspirin or PGI_2 (Lian *et al*, 1979;Lian & Savaraj, 1981). However it was inhibited by human IgG and Fab fragments purified from adult normal plasma (Lian *et al*, 1984). A 37kDa platelet agglutinating protein was subsequently purified from the plasma of a TTP patient with a similar inhibitor profile but was present in only three of five TTP plasma (Siddiqui & Lian, 1985).

A cysteine protease with platelet agglutinating activity has been reported by two groups (Murphy *et al*, 1987a;Falanga *et al*, 1991). Its precise nature is debated. It has been variously reported as calpain, a calcium-dependent proteinase (Murphy *et al*, 1987a) and a lysosomal cathepsin (cathepsin L) (Consonni *et al*, 1994). Calpain is an

intracellular enzyme found in many tissues including platelets. In the presence of calcium it hydrolyses several proteins at neutral pH including casein, VWF, GPIb and several cytoskeletal proteins. It can also induce platelet activation. Patients with TTP have been reported to have increased calpain activity (Kelton *et al*, 1996) despite the presence of kininogen, a potent inhibitor, in plasma. The association of active calpain with platelet microparticles in plasma from patients with TTP could be an explanation (Kelton *et al*, 1992). It has been demonstrated *in vitro* that calpain proteolytically degrades VWF. However it is generally accepted that the haemostatic properties of VWF correlate with its molecular size. Despite this, proteolysis of VWF by calpain apparently enhanced its binding to activated platelets (Moore *et al*, 1990). It is not clear however whether the presence of this calpain-like protease is due to its release from injured or aggregated platelets or if it functions as an initiation factor that precipitates an acute TTP episode.

It is now thought that VWF plays a central role in the disease process of TTP. Several lines of evidence support this. Immunohistochemical analysis of platelet microthrombi shows the presence of VWF but not fibrinogen (Asada *et al*, 1985) whilst VWF enhances the aggregating effect of TTP sera *in vitro* (Kelton *et al*, 1984). Furthermore abnormal patterns of plasma VWF have been reported in TTP and these abnormalities ususally resolve during convalescence (Kelton *et al*, 1984;Moake *et al*, 1982;Moake & McPherson, 1989;Murphy *et al*, 1987b). UlvWF multimers are a consistent feature during periods of remission in congenital and intermittent TTP (Chintagumpala *et al*, 1992;Moake & McPherson, 1989) and were also identified during 50% cases of acute single episode TTP (Moake & McPherson, 1989). Accordingly platelet rich plasma (PRP) from patients with congenital TTP results in excessive platelet aggregation as compared to adult normal PRP samples when exposed to shear stresses of 90 to 180 dyne/cm^2 (similar to those found in partially occluded microvasculature) (Moake *et al*, 1994). Mixing studies suggest that this is due to a plasma component rather than secondary to a platelet defect. More specifically ULVWF multimers are implicated since not only are they present in TTP plasma but also their addition to normal plasma accentuates platelet aggregation. Similarly, platelet retention, as measured by the

modified Adeplat S test (a glass-bead retention method) at the relatively modest shear stress of 76 dynes/cm^2, is increased in congenital TTP as compared to normal controls (Karpman *et al*, 1997). In this case, loss of ULVWF is associated with normalisation of increased platelet retention again supporting the concept that VWF multimers might play an integral role in TTP (Karpman *et al*, 1996). A pathophysiological role for ULVWF is suggested by the clinical observation that infusion of either FFP or cryosupernatant is effective in controlling or preventing relapse in such patients (Moake *et al*, 1985) and is often accompanied by complete or partial disappearance of ULVWF (Moake *et al*, 1994;Karpman *et al*, 1996;Moake *et al*, 1985;Karpman *et al*, 1997). Moreover *in vitro* studies demonstrate a parallel suppression in shear stress induced platelet aggregation (Moake *et al*, 1994) and platelet retention (Karpman *et al*, 1996). ULVWF multimers are not however a consistent feature of TTP plasma and transiently disappear during relapses of congenital TTP. A relative decrease in the largest plasma VWF forms was also demonstrated in 55% cases of acute TTP (Moake & McPherson, 1989). This may be the consequence of preferential binding of ULVWF multimers to platelets *in vivo*. Certainly flow cytometric techniques have confirmed increased VWF binding to single platelets and platelet aggregates during TTP relapses relative to remission periods (Chow *et al*, 1999). Likewise in an animal model of TTP induced by botrocetin, loss of high and intermediate VWF multimers was observed accompanied by the development of occlusive platelet thrombi. ULVWF multimers appeared transiently within the circulation during the recovery phase (Sanders *et al*, 1995).

1.6.5 VWF-cleaving protease activity

Moake first postulated that defective VWF processing might be central to the pathophysiology of TTP (Moake *et al*, 1985;Frangos *et al*, 1989). The identification of a novel metalloproteinase acting specifically on VWF multimers and cleaving the Tyr842-Met843 bond (Furlan *et al*, 1996;Tsai, 1996), has now permitted investigation of VWF-CP activity in TTP. Initially four patients with CRTTP were shown to lack VWF metalloproteinase activity in the absence of any inhibitor (Furlan *et al*, 1997). Similar findings were reported in six individuals with familial TTP (Furlan *et al*, 1998b). In contrast, of 24 patients with with non-familial TTP, 20 had severe and four moderate protease deficiency. An inhibitor was found in 20 of these cases and was of IgG subclass in five of five cases tested (Furlan *et al*, 1998b). A similar phenomenon has been shown independently by Tsai – 39 samples from 37 patients with acute TTP all had severe deficiency of VWF cleaving protease. Inhibitory activity was detected in 26 of these 39 samples, again of IgG subclass (Tsai & Lian, 1998). In contrast a relative increase in the 176kDa fragment had previously been reported in patients with TTP (Mannucci *et al*, 1989). However, although a relative increase in the proteolytic fragments has been used as evidence for increased proteolysis it can also result as a consequence of preferential depletion of larger multimers (Tsai *et al*, 1991b). It has therefore been proposed that acute single episode TTP may be an autoimmune disorder with a secondarily acquired functional deficiency of VWF-cleaving protease. In contrast, congenital TTP appears to be caused by an absolute deficiency of protease activity that may be due to a constitutional defect in its production, survival or activity. Moreover it appears that assessment of VWF-CP activity may allow accurate differentiation from HUS, supporting the concept that TTP and HUS are distinct pathophysiological entities. Of 23 patients with HUS (familial and sporadic) studied, 21 had normal levels of VWF-cleaving protease activity during the acute episode whilst levels were slightly reduced in two (Furlan *et al*, 1998b). Furthermore the finding that metalloprotease activity was not significantly different from normal controls in eight patients studied with BMT-associated TTP (van der Plas *et al*, 1999) is in accordance with the hypothesis that the current definition of TTP represents a

heterogeneous group of conditions. Certainly, it has long been recognised that contrary to classical TTP, BMT-associated TTP rarely responds to plasma exchange procedures.

These hypotheses may, however, prove to be an oversimplification. A sibling of an index congenital TTP case has been identified with absent protease activity but to date, no TTP or HUS-like syndrome (Furlan *et al*, 1998b). In addition, protease activity levels have not been demonstrated to fully recover in all remission cases of adult onset TTP (Furlan *et al*, 1998b). Whether this is due to an interacting hitherto unrecognised cofactor or because subnormal levels may be efficacious is yet to be elucidated. Indeed the protease does appear to have a uniquely long half-life: calculated as between two to four days in two patients with constitutional protease deficiency (Furlan *et al*, 1999).

1.7 Treatment

1.7.1 Adult onset TTP

1.7.1.1 Plasmapheresis

The mainstay of treatment of acute TTP is daily plasma exchange. Prior to its institution mortality rates were in excess of 90% and have now fallen to 10-30%. Plasma exchange is superior to plasma infusion. A prospective randomised study performed by the Canadian apheresis group assigned a total of 102 patients to receive either plasma exchange or infusion with fresh frozen plasma (FFP) on seven of the first nine days after entry to the trial. Plasma exchange resulted in significantly superior response rates at both the end of the first treatment cycle and at six months (response rates 47% and 78% versus 25% and 49%). Mortality was also reduced at 22% versus 37% (Rock *et al*, 1991). Presumably increased efficacy is secondary to both removal of inhibitory antibody and replacement of plasma product containing normal levels of VWF-CP acitivity. Plasma exchange should be instituted within 24 hours of presentation since delay in treatment initiation may increase treatment failure

(Pereira *et al*, 1995) and as soon as practicable if renal impairment, cardiac failure or coma is present. Reduced level of consciousness has been identified as a poor prognostic factor with an overall survival of 54% (Sarode *et al*, 1997). The duration of plasma exchange therapy required to achieve remission is highly variable. The average number of procedures required for remission in the above study was 15.8 (range 3 – 36).

Although undoubtedly efficacious, the optimal plasma exchange regimen has yet to be determined. In the Canadian apheresis trial, 1.5 x plasma volume exchange was performed on the first three days followed by 1.0 plasma volume exchange thereafter. Whether this intensity is superior to single plasma volume replacement from presentation is unclear. Currently, many centres initiate single volume plasma exchange at presentation, reserving more intensive exchange for resistant cases. Similarly, the optimal duration of plasma exchange is unknown. It is empirically recommended that exchanges should continue for a minimum of two days after complete remission is obtained, defined as normal neurological status, platelet count and LDH with a rising haemoglobin (AABB Extracorporeal Therapy Committee, 1992). Although it is accepted practice to taper the frequency of exchange procedures rather than stopping abruptly in an effort to minimise the risk of early relapse, this is not based on randomised clinical trials.

The optimal replacement fluid administered also remains contentious. Cryosupernatant is at least as efficacious as fresh frozen plasma (FFP). When used in previously untreated patients the response rate was 75% after seven exchanges. Survival was 95% at one month, which compared favourably with historical controls treated with FFP (Rock *et al*, 1996). A randomised controlled trial comparing plasma exchange with FFP versus cryosupernatant is currently being performed in Canada. Possibly of note, cryosupernatant lacks the largest VWF multimers which are present in FFP and cryoprecipitate. So too does solvent-detergent treated (SD) plasma. This has been used as replacement fluid from presentation (Evans *et al*, 1999) but numbers treated are small and there is no published comparative data with FFP or cryosupernatant. Further

clinical experience is required to ascertain the role of SD plasma in the primary treatment of TTP. Likewise although methylene blue (MB) treated plasma has been used in the management of TTP (Martinez *et al*, 2000), clinical experience is extremely limited. It should be noted that high molecular weight VWF multimers are preserved in MB plasma.

1.7.1.2 Adjunctive treatment

Steroids have been widely used in the treatment of TTP although there is scanty evidence documenting their efficacy. Cases of TTP lacking central nervous system abnormalities other than headache, have been shown to respond to steroid treatment alone, although of 54 such patients 44% required plasma exchange due to deterioration or failure to improve (Bell *et al*, 1991). Steroids have also been combined with plasma exchange in initial treatment of TTP. The addition of intravenous methylprednisolone 2mg/kg/day to daily plasma exchange resulted in a complete remission rate of 76% (Bobbio-Pallavicini *et al*, 1997). However, no trial has addressed whether such a combination approach is superior to plasma exchange alone. Not surprisingly there is no consensus regarding dose or mode of administration. Despite the lack of evidence the addition of steroids to plasma exchange as standard therapy is attractive following recent reports that a functional deficiency of a novel VWF-cleaving protease activity secondary to a circulating inhibitory antibody of IgG subtype is of prime importance in the pathogenesis of TTP (Furlan *et al*, 1998b;Tsai & Lian, 1998).

The use of anti-platelet agents in TTP also remains controversial. If shear stress induced VWF mediated platelet aggregation is important, then aspirin therapy would not be expected to be of benefit during the acute phase. Blockade of cyclo-oxygenase mediated thromboxane A_2 production does not inhibit shear induced aggregation *in vitro*. Nevertheless aspirin and dipyridamole have both been used in the initial treatment of TTP. A 78% response rate at six months was achieved when aspirin and dipyridamole were administered in conjunction with plasma exchange (Rock *et al*, 1991). In a prospective randomised trial designed to address the effect of the addition of aspirin and dipyridamole to standard treatment (plasma exchange and steroids) a

similar overall response was obtained in both groups. There was, however, a trend to reduced mortality at 15 days in those treated with anti-platelet agents. No excess haemorrhage was seen in the treatment group (Bobbio-Pallavicini *et al*, 1997) in contrast to the findings of a small retrospective study in which serious bleeding complications occurred in five of 14 patients receiving anti-platelet medication (Rosove *et al*, 1982). It should be noted however that large doses were administered in these last cases (0.9 – 2.7g aspirin daily).

Ticlopidine and its analogue clopidogrel inhibit ADP-platelet interactions and interfere with shear induced aggregation and might therefore be predicted to be of use in TTP. Certainly ticlopidine has been used as maintenance therapy for 12 months after remission of TTP was achieved and appeared to reduce the risk of subsequent relapse from 21.4% to 6.25% although at publication follow-up in this trial was incomplete (Bobbio-Pallavicini *et al*, 1997). Both ticlopidine and clopidogrel have subsequently been associated with TTP. Plasmapheresis is effective treatment in such cases with reported survival rates of 76-91% and is superior to plasma infusion in ticlopidine associated cases (Bennet *et al*, 1998;Bennet *et al*, 2000). This is consistent with the finding that both have been associated with antibody inhibitors to VWF-cleaving protease (Tsai *et al*, 2000;Bennet *et al*, 2000). As a consequence ticlopidine and clopidogrel should be avoided in patients with a previous history of TTP.

Platelet transfusions are contra-indicated in TTP unless there is life-threatening haemorrhage as they have been temporally associated with disease exacerbation (Gordon *et al*, 1987;Harkness *et al*, 1981).

1.7.1.3 Refractory disease

Despite the improvement in survival there remains a subgroup with a slow or incomplete response to plasma exchange ± steroids. Refractory disease may be defined as persistent thrombocytopenia (platelet count < 150 x 10^9/l) or LDH elevation after a total of seven daily plasma exchange procedures. Treatment of this group of patients is difficult and a wide range of additional approaches has been used.

Unfortunately randomised clinical trials have not been performed due to the rarity and heterogeneity of this condition.

There are reports documenting responses to plasma exchange in refractory cases of TTP following the substitution of either cryosupernatant (Molinari *et al*, 1993) or S/D plasma (Harrison *et al*, 1996) for FFP. This may be attributable to the relative lack of the largest plasma VWF multimers discussed above. Intensification of plasma exchange has also been used in cases of refractory TTP with the introduction of either 12 hourly or double volume plasma volume exchanges.

Although vincristine is often used in the treatment of refractory TTP published literature supporting its efficacy comprises only case reports or small retrospective studies. Nevertheless, these suggest that the administration of vincristine in refractory TTP may be temporally associated with platelet recovery (Welborn *et al*, 1990;O'Connor *et al*, 1992;Bobbio-Pallavicini *et al*, 1994). A role for the early administration of vincristine (within three days of presentation) has also been advocated following a small retrospective study (Mazzei *et al*, 1998). The mechanism of action of vincristine remains unclear although a number of hypotheses have been proposed. These include a direct endothelial effect, alteration of platelet surface Gp1b-IX-V and GpIIbIIIa receptor expression and interference with platelet microtubule depolymerisation.

The recent demonstration of autoantibodies with inhibitory VWF-CP activity perhaps explains the reports of TTP responsive to immunosuppressive therapy such as cyclophosphamide and cyclosporin. Cyclosphosphamide has been advocated for the treatment of TTP, particularly those cases characterised by recurrent relapses (severe intermittent TTP) (Bird *et al*, 1990;Udvardy & Rak, 1990;Strutz *et al*, 1998). Both daily dosing and pulsed therapy have been successfully used although reported numbers are extremely low. More recently cyclosporin has been associated with successful treatment of refractory (Hand *et al*, 1998;Kierdorf *et al*, 1993), severe intermittent (Pasquale *et al*, 1998) and post autologous BMT TTP (Van Ojik *et al*,

1997). Although spontaneous resolution cannot be excluded in these cases, clinical and haematological response uniformly occurred seven to 14 days after initiating treatment. The optimal duration of treatment is unknown, with relapses documented after cessation of therapy (Hand *et al*, 1998;Pasquale *et al*, 1998) as is the optimal target therapeutic range. Trough serum levels of 200 – 300μg/l have been used to date.

As discussed previously the advent of VWF-cleaving protease assays is beginning to confirm the clinical suspicion that TTP currently represents a heterogeneous group of conditions. It has long been recognised that plasma exchange is rarely effective in the treatment of BMT-associated TTP suggesting that an alternative pathological process might be involved. Effective treatment for this group of patients is lacking. Cyclosporin and FK-506 have both been linked to TTP and such therapy should be stopped. Whether protein-A column immunoabsorption might be useful in such cases is unclear. Certainly this technique has been successfully employed in the treatment of malignancy associated TTP in which plasma exchange is often found to be ineffective. In one small retrospective series, it was found to be of benefit in seven of ten patients who had been unresponsive to plasma exchange (Gaddis *et al*, 1997).

1.7.1.4 Relapse

Although remission is now attained in over 80% patients, subsequent relapse remains problematic. Data from the Canadian Apheresis Group estimate that over a ten year follow-up period up to 36% TTP cases relapse. Relapse has occurred up to eight years after the index event (Shumak *et al*, 1995). There is no consensus whether there is any effective intervention which might reduce this risk nor is there at present any means of identifying those at risk. Splenectomy has been advocated as a means of reducing relapse. One small retrospective study including six patients showed encouraging results with relapse rates falling from 2.3 ± 2.0 to 0.1 ± 0.1 events per year when the operation was performed during haematological remission (Crowther *et al*, 1996). Anti-platelet medication has also been proposed as a possible alternative. Certainly there was a trend to reduced relapse rate in those patients receiving ticlopidine for 12 months after attaining remission (Bobbio-Pallavicini *et al*, 1997). Since ticlopidine has

itself been associated with TTP (Bennet *et al*, 1998) this is no longer advocated although some centres empirically commence patients on aspirin 75mg daily. Until the mechanism for relapse is understood more appropriate therapeutic interventions cannot be introduced.

1.7.2 Congenital TTP

Successful long term maintenance can be achieved in severe congenital TTP through prophylactic infusion with FFP (Chintagumpala *et al*, 1992;Barbot *et al*, 2001), cryosupernatant (Moake *et al*, 1985) or S/D plasma (Moake *et al*, 1994). Treatment is usually required every three to four weeks (Chintagumpala *et al*, 1992). This is consistent both with the demonstration that congentital TTP is associated with a deficiency of VWF-CP activity in the absence of an inhibitor (Furlan *et al*, 1998b) and with the long half life of plasma VWF-CP acitivity (Furlan *et al*, 1999). Plasma infusion may, however, be reserved for symptomatic episodes in milder cases (Karpman *et al*, 1996). Viral inactivation and the consequent lower risk of transfusion transmitted infection obviously makes S/D plasma an attractive treatment option. It should be remembered, however, that non-lipid viruses are not inactivated by this procedure and furthermore that the product is derived from large donor pools.

1.8 Aims of thesis

The primary objective of this thesis was to investigate the pathophysiology of TTP. This condition is characterised by the formation of platelet microthrombi containing abundant VWF. Consequently it has been hypothesised that TTP may be a disease of primary platelet aggregation mediated by VWF. Two major aspects of this theory were addressed by an investigation of the extent of *in vivo* platelet activation and measurement of VWF-CP activity in patients presenting with TTP. Platelet activation was assessed using dual labeling flow cytometric techniques Thrombin generation, VWF and P-selectin were also measured in an attempt to explore a possible relationship between the parameters.

ULVWF may mediate primary platelet aggregation and may circulate secondary to a deficiency of VWF-CP activity. A VWF-CP assay was therefore developed and utilised to assess protease activity in a variety of microangiopathic haemolytic conditions, in order to ascertain the sensitivity and specificity of protease deficiency for idiopathic TTP. Serial measurements were also undertaken in those diagnosed with idiopathic TTP to determine the correlation, if any, between protease activity and clinical course, particularly with respect to disease severity, the risk of relapse and immunosuppressive therapy. Protease activity was subsequently measured in patients diagnosed with congenital TTP to determine whether disease severity is dependent solely upon protease activity. To assess whether carrier status might be deduced from absolute protease levels, the protease activity of first degree relatives was also measured. To further investigate the role of protease deficiency in congenital TTP, the intrinsic protease activity of plasma and plasma-derived products, including factor VIII and factor IX concentrates was assayed. It was hoped that this would allow identification of alternative plasma derived products which might be clinically efficacious in the management of congenital TTP.

VWF-CP deficiency appeared central to the pathophysiology of adult-onset and congenital TTP. However congenital and adult-onset TTP have distinct clinical

presentations. To determine whether this is secondary to the nature of protease deficiency, correction studies and *in vitro* experiments using IgG purified from selected patients with idiopathic and congenital TTP were performed. It had, however, also been demonstrated that protease deficiency did not invariably lead to clinical sequalae suggesting a multifactorial pathophysiology. One such patient had a concomitant diagnosis of primary antiphospholipid syndrome (PAPS). To investigate the possibility that antiphospholipid antibodies (APA) might be implicated, patients were screened for the presence of APA and protease activity assessed in a group of patients with well documented PAPS and patients with SLE (some of whom also had secondary APS).

2. General Methods

All chemicals were obtained from Merck (BDH) Ltd. or Sigma-Aldrich Chemical Co. Ltd. unless otherwise stated. A list of addresses of companies used to supply reagents, equipment and consumables used in this thesis is given in appendix 1. In this chapter they will be cited by name only.

2.1 Sample collection and separation

2.1.1 Sample collection

Blood was collected from normal, apparently healthy adult subjects as well as from patients with a diagnosis of TTP under the care of the Haematology Department, University College London Hospital, London. All patients gave verbal consent for any additional blood samples collected. All samples used for platelet activation studies were collected from subjects who were rested for approximately 20 minutes prior to venepuncture in order to minimise platelet activation and an atraumatic technique was used. Blood was either collected from a central venous line or from the antecubital vein using a 21G Butterfly® needle and Vacutainer® system with a luer adaptor (Becton Dickinson). The first 4.5ml blood collected was drawn directly into a sterile Vacutainer® containing 0.054 EDTA (K3) to determine a full blood count; the next 4.5ml was collected into a sterile Vacutainer® containing 0.5ml 0.105 M sodium citrate for platelet activation markers and reticulated platelet analysis. Further tubes of citrated and non-anticoagulated blood were then collected for all other tests.

2.1.2 Sample separation

All plasma and serum were separated within two hours of collection. Platelet poor plasma (PPP) was prepared from citrated anticoagulated blood. Samples were initially

centrifuged at 2000g for 15 minutes at room temperature. The top two-thirds plasma was carefully removed with a plastic pasteur pipette and centrifuged again for 15 minutes at 2000g. PPP was removed, aliquoted into polypropylene tubes and frozen at -70°C. Serum was collected from blood which had been allowed to clot at 37°C for 30 minutes before being centrifuged at 2000g for 15 minutes at room temperature. Serum was removed with a plastic pasteur pipette, aliquoted into polypropylene tubes and frozen at -70°C.

2.2 Flow cytometry

2.2.1 General principle

Flow cytometry involves single particles or cells flowing through a laser beam. Particle alignment is achieved by hydrodynamic focussing. The passage of particles through the beam results in scattering of light. Forward scatter (FS) is generated by light deflecting from the surface of the particle whilst side scatter (SS) light results from deflection off internal structures or granules. They reflect particle size and granularity respectively. Dyes may also be bound on the interior or exterior of the particle. These absorb laser light energy and consequently emit fluorescence which may be detected by photomultiplier tubes. In this way information regarding particle characteristics may be collected, converted to electrical pulses and digitalised for computer analysis.

Table 2.1 lists some of the most common fluorochromes used in flow cytometry. In the majority of cases they are conjugated to monoclonal antibodies for the detection of cell surface antigens.

Fluorochrome	Emission wavelength	Fluorescence	Fluorescence detector
Fluorescein isothiocyantate (FITC)	525 nm	Green	FL1
Phycoerythrin (PE)	575 nm	Orange	FL2
R-phycoerythrin-Cy5 (RPE-Cy5)	670 nm	Red	FL4
Thiazole orange (TO)	533 nm	Green	FL1

***Table 2.1** Fluorochromes used in flow cytometry*

Antibody binding can be expressed as mean particle fluorescence intensity (MFI) or the percentage of platelets staining positive for a particular antibody. Advantages and disadvantages exist for both techniques. Expressing results as the percentage of platelets is simpler and, unlike MFI, is independent of variation in signal amplification such as occur with changes in PMT voltage or gain, since the isotypic control signal increases in proportion with the test sample (Michelson, 1996). However it is important to realise tht antibody positive platelets may have very little antigen expressed on their surface.

2.2.2 Quality control

Flow cytometry was performed on a Coulter® Epics® XL-MCL flow cytometer (Beckman Coulter). In order to ensure inter-assay sample reproducibility, a daily verification of the instrument's optical alignment and fluidics system was performed using Flow-Check™ Fluorospheres (Beckman Coulter). This is a suspension of 10μm fluorescent beads with fluorescence emission from 525 to 700nm when excited at 488nm. A half peak coefficient of varation ≤ 1.6% on the relevant histograms was accepted for quality control.

2.2.3 Reticulated platelet measurement

Principle

This two-colour analysis of reticulated platelets utilises the monoclonal antibody CD61-PerCP and a dilution of a thiazole orange solution (TO, Retic-COUNT) to determine the percentage of reticulated platelets in whole blood samples. CD61-PerCP binds to platelet specific surface glycoprotein IIIa (GPIIIa) whilst TO readily crosses the platelet membrane. The platelet cloud is identified by characteristic size (log forward scatter) and granularity (log side scatter) and verified using anti-CD61 which fluoresces in the FL4 channel. Reticulated platelets within the GPIIIa positive population are identified by dual staining with Retic-Count solution which fluoresces in the FL1 channel.

Reagents

- Monoclonal antibody anti-CD61-PerCP (Becton Dickinson)
- Isotype control IgG_1-PerCP (Becton Dickinson)
- Retic-COUNT (Becton Dickinson)
- Isoton® II (Beckman Coulter)

Method

5µl EDTA anticoagulated whole blood was incubated with 5µl test antibody/isotype control for 15 minutes. 1ml Retic-COUNT (diluted 1:8 in Isoton® II) or Isoton ® II was then added to the test and control sample respectively. This was then incubated for 30 minutes in the dark. The tubes were subsequently centrifuged at 800g for five minutes, the supernatants discarded and the pellets gently resuspended in 0.5ml Isoton® II for analysis. Platelets were identified by fluorescence (log FL4) and log side scatter (log SS) whilst reticulated platelets demonstrated positive dual staining for CD61-PerCP and TO. The normal reference range based on geometric mean ± 2 SD of log normalised data from 20 individuals 4.0% - 22.3%. The intra assay CV for measuring percentage reticulated platelets was 7.5%.

2.2.4 Platelet surface markers CD61, CD62p and CD63

Principle

Platelets may be identifed from other circulating blood cells by the use of monoclonal antibodies to platelet-specific antigens such as GPIIIa (CD61). This is of particular importance when there is both thrombocytopenia and marked red cell fragmentation as in TTP. Under these circumstances platelets cannot be identified on the basis of characteristic forward and side scatter since red cell fragments have similar properties. Platelet activation results in degradation and the incorporation of both α granule and lysosomal membrane proteins into the platelet cell surface membrane where their exposure can be used as markers of platelet activation. Such markers include both CD62p and CD63 as previously discussed in the introduction. In addition the reactivity of circulating platelets to exogenous agonists can be assessed.

Reagents

- Monoclonal antibodies anti-CD61-PerCP (Beckton Dickinson), anti-CD62p-PE and anti-CD63-FITC (Immunotech, Beckman Coulter)
- Isotype controls IgG1-PE, IgG1-FITC (Immunotech, Beckman Coulter) and IgG1-PerCP (Becton Dickinson)
- HEPES buffered saline (HBS) – NaCl 0.145 mol/l, KCl 5 mmol/l, $MgSO_4$ 1 mmol/l, HEPES 10 mmol/l, pH 7.4
- Formaldehyde solution – made up to 0.2% in 0.145 mol/l NaCl and filtered with a Sartorius Minisart® 0.2μm syringe filter (Fisher Scientific) prior to use. A fresh aliquot was made daily
- TRAP 400μmol/l (Sigma-Aldrich)
- ADP 10mmol/l

Method

Whole blood (5μl) was incubated with 5μl CD61 and either 5μl test antibody or corresponding isotype control with HBS to give a final total volume of 50μl. To assess platelet reactivity 10μl TRAP stock solution or 5μl ADP diluted 1 in 100 in HBS was added to whole blood and antibodies as above with an appropriate reduction in HBS to give the same final volume of 50μl. After 20 minutes incubation at room temperature

the samples were fixed in 0.5ml formalin saline prior to flow cytometric analysis. All samples were analysed within three hours of venepuncture. Platelets were identified by fluorescence (log FL4) after an analysis marker was set such that 0.5% events were positive on analysis of a whole blood sample incubated with IgG1-PerCP isotype control. CD62p and CD63 binding was expressed at the percentage of platelets staining positive for the particular antibody. If possible a total of 10,000 events per sample were analysed with a maximum acquisition time period of ten minutes. The reference ranges for resting circulating platelets (based on geometric mean ± 2 SD of log normalised data) are 0.23% - 5.9% for CD62p and 1.7% - 10.4% for CD63 with intra-assay CV's of 14% and 8.4% respectively. Reference ranges for platelet reactivity to 80μM TRAP and 10μM ADP are 91.0% - 100% and 35.2% - 100% respectively for CD62p and 76.4% - 94.9% and 13.1% - 43.5% respectively for CD63. These concentrations of TRAP and ADP were studied since they result in maximal expression of these activation markers.

2.2.5 Platelet-leucocyte aggregates

Principle

Platelet-leucocyte aggregates are thought to be another marker of platelet activation. Adhesion of platelets to white cells has been studied previously by quantitating the heterotypic conjugates (rosettes) that form after mixing suspensions of isolated platelets and plolymorphonuclear leucocytes usually by microscopy (Jungi *et al*, 1986). Recently several authors have published methods for the detection of these conjugates using flow cytometric methods (Li *et al*, 1997;Redlich *et al*, 1997). It has been reported that *in vitro* sample manipulations such as sample fixation, erythrocyte lysis and especially repeated centrifugation can cause artefactual platelet-leucocyte aggregates (Li *et al*, 1997).

Reagents

- Monoclonal antibody anti-CD45-RPE-Cy5 (Dako)
- Monoclonal antibody anti-CD42b-PE (Immunotech, Beckman Coulter)
- Isotype control IgG1-PE (Immunotech, Beckman Coulter)

- HEPES buffered saline (HBS) – NaCl 0.145 mol/l, KCl 5 mmol/l, MgSO4 1 mmol/l, HEPES 10 mmol/l pH 7.4
- Hanks balanced saline solution (HBSS) without calcium, magnesium and phenol red (Life Technologies)
- Formaldehyde solution – made up to 10% in distilled water
- Distilled water
- Fixative – made up of 0.5ml 10% formaldehyde solution, 0.6ml HBSS 10% and 0.9ml distilled water

Method

Within five minutes of venepuncture whole blood (25µl) was aliquoted into a tube containing 65µl HBS and 5ml anti-CD45-RPE-Cy5 plus 5ml of either anti-CD42b isotype control or test antibody. After ten minutes incubation at room temperature, the samples were fixed in 84µl fixative and following a further ten minute incubation, 840 µl distilled water was added in order to induce erythrocyte lysis. After a further ten minutes, samples were ready for analysis on the flow cytometer and were stable for at least three hours. A protocol was designed in which only CD45 positive events (i.e. leucocytes) were further analysed. Samples were analysed on a low flow rate to minimise dual events. On the initial scatterplot, log fluorescence (FL4 – RPE-Cy5) was plotted against side scatter and a listmode gate was employed to exclude any red cell debris from being analysed (*Figure 2.1*). Events in the gated region were then analysed further and on a scatterplot of side versus forward scatter, three distinct populations of leucocytes were identified (*Figure 2.1*). Gates were drawn around each of the three regions labelled C (granulocytes), D (monocytes) and E (lymphocytes) and any platelet-specific event (i.e. CD42b positive) occurring within these separate regions were then measured. A minimum of 1,000 monocyte events per sample were analysed. An example of the histograms obtained from a typical PLA analysis is shown in *Figure 2.1*. Platelet events (in association with a leucocyte) are detected by FL2 and as can be seen from the histograms less than 5% of all circulating platelets from normal resting subjects are complexed with platelets. The normal reference range for platelet-leucocyte aggregates was based on the geometric mean ± 2 SD of log normalised data from 20 normal individuals and was 2.6% – 5.3% for platelet-

granulocyte complexes, 3.3% – 7.8% for platelet-monocyte complexes and 2.0% – 5.2% for platelet-lymphocyte complexes. The intra-assay CV's for measuring platelet-leucocyte aggregates in resting samples were 2.1 – 10.5%.

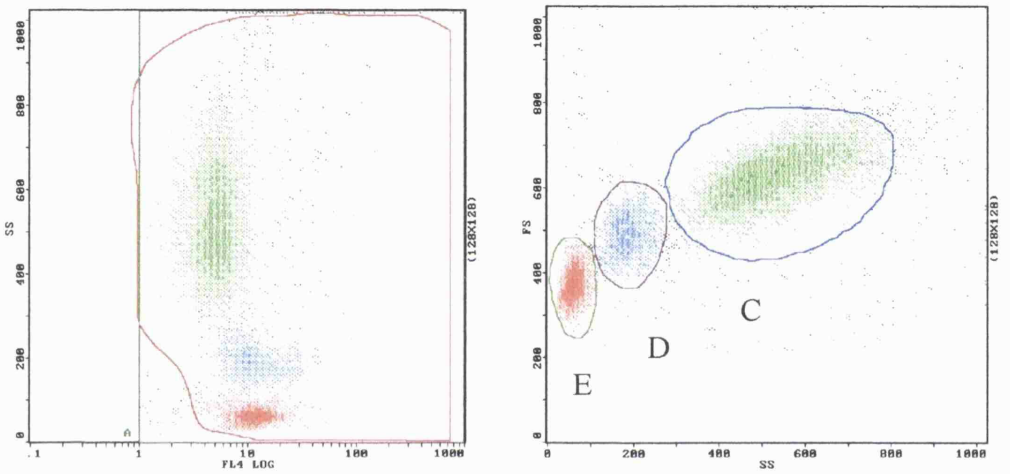


Figure 2.1 *The initial scatterplot is shown on the left in whch log fluorescence (FL4 – RPE-Cy5) is plotted against side scatter and a listmode gate empolyed to exclude any red cell debris from being analysed. Events in the gated regaion were then analysed further and on a scatter plot of side versus forward scatter,shown on the right, three distinct populations of leucocytes were identified. The three regions C, D and E represent granulocytes, monocytes and lymphocytes respectively.*

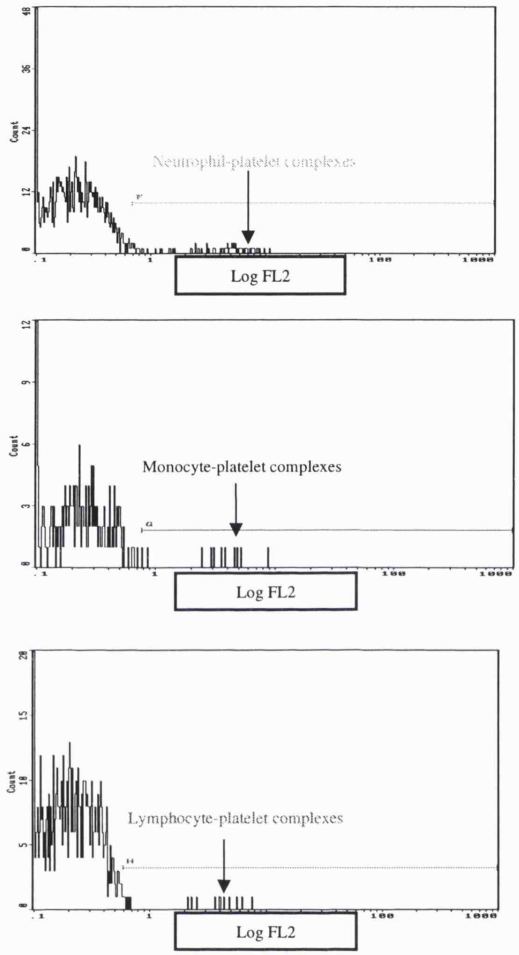


Figure 2.2 *Histograms demonstrating typical fluorescence profiles for platelet-granulocyte (top), platelet-monocyte (middle) and platelet-leucocyte (bottom) complexes where platelets are labelled with CD42b-PE. Count = cell number.*

2.2.6 Platelet-derived microparticles

Principle

Platelet-derived microparticles form when there is vesiculation of the platelet plasma membrane. A number of agonists and mechanical/shear forces (as in freeze/thaw or cardiopulmonary bypass) can lead to the generation of platelet microparticles of varying size. However for the purposes of flow cytometry, some authors have defined microparticles as < 1.0μm diameter (Horstman *et al*, 1994) and they generally express GPIb, IIb and IIIa. These properties make it possible to identify and quantitate microparticles using flow cytometry.

Reagents

- Monoclonal antibody anti-CD41-RPE (Dako)
- Isotype control IgG1-PE (Immunotech, Beckman Coulter)
- Isoton® II (Beckman Coulter)
- Latex Polystyrene beads (Sigma-Aldrich) – 0.8mm diameter

Method

Platelet poor plasma (30μl) was incubated with 5μl monoclonal antibody anti-CD41-RPE or isotype control for 30 minutes at room temperature and diluted in 0.5ml filtered Isoton® II. Each tube was then weighed immediately before flow cytometric analysis. The microparticle gate had been defined previously using Latex polystyrene beads of 0.8μm diameter. Only GPIIbIIIa positive events which fell within this region were classified as microparticles. Each sample was analysed on a high flow rate for two minutes. The dead volume of the flow cytometer was calculated daily by stopping analysis immediately after it had commenced. Following all sample analyses, the tubes were re-weighed and the sample volume calculated according to a sample volume: weight ratio which had previously been determined for that particular sample type. Results were expressed as the number of microparticles per ml plasma. The normal reference range for numbers of platelet microparticles was based on the geometric mean ± 2 SD of log normalised data from 20 normal individuals and was 1.1×10^5 - 1.2×10^6 microparticles per ml plasma. The intra-assay CV for measuring microparticles in resting samples was 2.7%.

2.3 Enzyme Linked Immunosorbent Assays (ELISA)

2.3.1 General principle

The general principle of an ELISA is to bind antibody to the wells of a microtitre plate, expose it to antigen which is then "captured" by the antibody and detect captured antigen by using either the same antibody (single determinant assay) or an antibody reactive to a different epitope of the antigen (double determinant assay). The detecting antibody is conjugated to an enzyme such as peroxidase. Consequently any bound enzyme is then detected by placing substrate in the wells and developing a chromogenic product. The colour produced is measured by a spectrophotometer. By using suitable standards of known concentration, the procedure can be made quantitative. Thus the amount of antigen present is proportional to the amount of colour produced.

2.3.2 Quality control

A quality control (QC) plasma was included in all ELISA assays performed. In the commercial ELISA kits, the QC was provided. If not then clot control F (Hyland Immuno, Baxter AG) was used. Results from assays where the QC fell outside the reference range were not accepted. All assays were performed in duplicate. If the coefficient of variation (CV) was greater than 10%, the results were not accepted.

2.3.3 Thrombin-Antithrombin III (TAT) complex

Principle

The control of thrombin generation and activity are central to maintaining thrombotic homeostasis. Antithrombin represents one mechanism by which excessive thrombin activation is prevented. Antithrombin binds thrombin resulting in an inactive proteinase/inhibitor complex. Such thrombin-antithrombin (TAT) complexes can be measured quantitatively by enzyme immunoassay.

Reagents

All reagents were suppplied by Dade Behring Ltd as part of a kit (Enzygnost® TAT micro) and include:

- 96 well microtitre plate coated with rabbit antibodies against human thrombin
- Rabbit anti-human ATIII, peroxidase conjugated
- Conjugate buffer – Tris buffer solution (50mmol/l) and bovine serum albumin
- TAT standard plasmas – concentrations ranging between 2 to 60µg/l
- TAT control plasma
- Sample buffer – Tris buffer solution (100mmol/l), Tween (10ml/l) and EDTA (37g/l)
- Washing solution – PBS (90mmol/l) containing Tween (18g/l)
- Buffer/substrate – Hydrogen peroxide (0.3g/l) in citrate buffer solution
- Chromogen – o-phenlenediamine dihydrochloride
- Stopping solution – 0.5M sulphuric acid

Method

All determinations were performed in duplicate. Sample buffe (50µl) plus either standard, control or test plasma (50µl) were added to each well of the microtitre plate and after gentle mixing, incubated for 15 minutes at 37°C. Each well was then aspirated and washed three times with 300µl of washing solution. 100µl of conjugate solution was added and incubated for 15 minutes at 37°C. All wells were again aspirated and washed three times before adding 100µl freshly prepared chromogen substrate. Plates were incubated for 30 minutes at room temperature (protected from light) and the reaction was stopped by adding 100µl stopping solution. Absorbance values were measured at a wavelength of 492nm, using an automated plate reader. Mean absorbance values of the standards were used to plot the reference curve (log-log) from which concentrations of test samples were then derived. The intra-assay CV for this method is 4 – 6% and the inter-assay CV is 6 – 9%. The kit reference range for levels of TAT was 1.0 – 4.1µg/l. In our laboratory, the reference range based on the geometric mean ± 2SD of log normalised data from 20 normal individuals was 0.7 – 5.8µg/l.

2.3.4 Prothrombin fragment F 1+2 (F 1.2)

Principle

When prothrombin is converted to thrombin, prothrombin fragment F 1.2 is also generated. Through the use of an enzyme immunoassay to detect F 1.2, it is possible to quantify the amount of thrombin formed.

Reagents

All reagents were supplied by Dade Behring Ltd as part of a kit (Enzygnost® F 1+2 micro) and include:

- 96 microtitre plate coated with rabbit antibodies to human F 1.2
- Rabbit anti-human prothrombin, peroxidase conjugated
- Conjugate buffer – Tris buffer solution containing Tween, bovine serum albumin
- F 1.2 standard plasma – concentrations ranging from 0.04 - 10nmol/l
- F 1.2 control plasma
- Sample buffer – Tris buffer solution containing Tween sodium chloride
- Washing solution – PBS (90mmol/l) containing Tween (18g/l)
- Buffer/substrate – Hydrogen peroxide (0.3g/l) in citrate phosphate buffer solution
- Stopping solution – 0.5M sulphuric acid

Method

All determinations were performed in duplicate. Sample buffer (50µl) plus either standard, control or test plasma (50µl) were added to each well of the microtitre plate and after gentle mixing incubated for 30 minutes at 37°C. Each well was then aspirated and washed three times with 300µl washing solution. 100µl conjugate solution was added and incubated for 15 minutes at 37°C. All wells were again aspirated and washed three times before adding 100µl freshly prepared chromogen substrate. Plates were incubated for 15 minutes at room temperature (protected from light) and the reaction stopped by adding 100µl stopping solution. Absorbance values were measured at a wavelegth of 492nm using an automated plate reader. Mean absorbance values of the standards were used to plot the reference curve (log-log) from which concentrations of test samples were then derived. The intra-assay CV for this method is 5 – 7.5% and the inter-assay CV is 6 –13%. The kit reference range for

levels of F 1.2 was 0.4 – 1.1nmol/l. In our laboratory the reference range based on geometric mean ± 2 SD of log normalised data from 20 normal individuals was 0.3 – 1.8nmol/l.

2.3.5 Soluble P-selectin

Principle

Soluble P-selectin is found in the plasma of normal individuals at ng/ml concentrations and probably arises from both platelets and endothelial cells. Levels of soluble P-selectin may be elevated in a number of pathological conditions and can be measured by a quantitative sandwich immunoassay technique.

Reagents

All reagents were supplied by R&D Systems as part of a kit (Parameter® Human soluble P-selectin immunoassay) and include:

- 96 well microtitre plate coated with murine monoclonal antibody to human sP-selectin
- Sheep anti-human sP-selectin, peroxidase conjugated
- Conjugate diluent
- SP-selectin standards – 6 vials of lyophilised recombinant soluble human P-selectin
- Parameter control – 1 vial of lyophilised human serum containing sP-selectin
- Sample diluent
- Wash buffer concentrate
- Substrate – tetamethylbenzidine
- Stop solution

Method

All determinations were performed in duplicate. Samples and control were diluted either 10 or 20 fold with sample diluent. 100µl standard, diluted sample or diluted control were added to each well of the microtitre plate. Next 100µl diluted anti-sP-selectin conjugate was added and wells were gently mixed. Plates were incubated for one hour at room temperature. Each well was then aspirated and washed three times

with 300µl wash buffer before adding 100µl substrate. After a further 15 minute incubation at room temperature, the reaction was stopped by adding 100µl stop solution. Absorbance values were read at 450nm with a wavelength correction set to either 620 or 650nm using an automated plate reader. Mean absorbance values of the standards were used to plot the reference curve (lin-lin) from which concentrations of test samples were then derived, remembering to multiply by the dilution factor. The intra-assay CV for this method is 4.9 – 5.6% and the inter-assay CV is 7.9 – 9.9%. The kit reference range for levels of sP-selectin is 20 – 44ng/ml. In our laboratory, the reference range based on the geometric mean ± 2 SD of log normalised data from 19 normal individuals was 6.9 – 52.4ng/ml.

2.3.6 Von Willebrand factor antigen

Principle

Microtitre plates are coated with antisera against von Willebrand factor (VWF). Test plasma (diluted in PBS) are applied and specific binding detected using a conjugated are added.

Reagents

- 96 well microtitre plate (Nunc, Polysorp; Life Technologies Ltd.)
- Rabbit anti-human VWF serum (Dako Ltd.)
- Buffer A – 0.01M phosphate, 0.0027M potassium chloride, 0.137M sodium chloride pH 7.4 (Sigma-Aldrich Chemical Co.)
- Buffer B – buffer A containing 0.1% (v/v) Tween 20
- Horse radish peroxidase conjugated rabbit anti-human vWF (Dako Ltd.)
- Citrate phosphate buffer – 0.1 M citrate phosphate buffer pH 5.0
- Substrate – 1,2-ortho phenylenediamine dihydrochloride (orthophenylenediamine, OPD) (Sigma-Aldrich Chemical Co.)
- 3% (v/v) hydrogen peroxide
- 2 M sulphuric acid
- Standard – coagulation reference (Hyland Immuno, Baxter AG)
- Control – clot control F (Hyland Immuno, Baxter AG)

Method

180µl of rabbit anti-human VWF diluted 1:1000 in buffer A was added to each well and incubated at room temperature for one hour. A range (1:10 to 1: 640) of standard solutions were prepared by doubling dilution in buffer B. Test and control samples were diluted in buffer B (range 1 in 20 to 1 in 320). Unbound antibody was removed by washing three times with 200µl buffer B. 180µl standards, tests or control were then added to the microtitre plate in duplicate and incubated at room temperature for one hour. After washing three times with 200µl buffer B, 180µl horse radish peroxide conjugated anti-human VWF diluted 1 in 1000 in buffer B were added to each well for one hour at room temperature. After washing three times with 200µl buffer B 180µl fresh substrate was added and incubated at room temperature until optimum colour development had occurred. The reaction was stopped by adding 50µl 2M sulphuric acid. Absorbance values were read at 490nm using an automated plate reader. The mean absorbance of the blank well was initially subtracted from that of the test wells. Absorbance values of the standards were used to plot the reference curve (linear-log) from which concentrations of test samples were derived. The normal reference range for VWF: Ag concentration is 0.5 – 2.0 IU/ml.

2.3.7 Anticardiolipin antibody

Principle

Microtitre plates are coated with purified cardiolipin. Test serum samples (diluted in adult bovine serum) are applied and specific antibody binding to the cardiolipin is detected using a conjugated anti-human IgG or IgM. Thus both IgG and IgM anticardiolipin antibodies (ACL) may be detected. The assay was standardised using local reference sera cross calibrated against reference sera (a gift from R Malia, Royal Hallamshire Hospital, Sheffield) which in turn had been calibrated against the original Harris standards at an international workshop.

Reagents

All reagents were from Merck Ltd. unless otherwise stated and include

- 96 well microtitre plate (Nunc, Polysorp; Life Technologies Ltd.)
- Cardiolipin from bovine heart (ethanol solution, Sigma-Aldrich Chemical Co.)
- Ethanol – absolute solution
- Phosphate buffered saline (PBS) – 0.01M phosphate, 0.145M sodium chloride adjusted to pH 7.2
- Adult bovine serum (ABS) – 10% (v/v) ABS (Sigma- Aldrich Chemical Co.) in PBS
- Alkaline phosphatase conjugated goat anti-human IgG (γ chain specific) or IgM (μ chain specific) (Sigma- Aldrich Chemical Co.)
- 0.92M diethanolamine buffer – pH 9.8 containing 0.5mM magnesium chloride hexahydrate
- Substrate – 1mg/ml p-nitrophenyl phosphate disodium hexahydrate phosphatase (Sigma- Aldrich Chemical Co.) in diethanolamine buffer
- 3M sodium hydroxide

Method

30μl of a 50μg/ml solution of cardiolipin diluted in ethanol was added to the wells in rows C, D, E and F of the microtitre plate. 30μl ethanol was added to the wells in the remaining rows (blank wells). The plate contents were allowed to evaporate by being left uncovered overnight at 4°C and then washed with PBS. To reduce non-specific binding, plates were blocked with 75μl ABS buffer for one hour at room temperature. After washing once in PBS, 50μl aliquots of doubling dilutions of reference serum (1/50 to 1/1600) and 1/50 dilutions of test samples in ABS buffer were added to both cardiolipin coated and blank wells in duplicate which were then incubated at room temperature for three hours. After washing three times 50μl aliquots alkaline phosphatase conjugated goat anti-human IgG or IgM diluted 1/100 in ABS buffer was added to each well and incubated at room temperature for 90 minutes. After washing, 50μl aliquots of 1mg/ml substrate (*p*-nitrophenyl phosphate disodium hexahydrate phosphatase) in diethanolamine buffer were added to each well and incubated in the dark at 37°C until a suitable colour had developed. The reaction was stopped with 50μl 3M NaOH. Absorbance values were read at 410nm using an automated plate

reader. The mean absorbance of the blank well was initially subtracted from that of the test wells. Absorbance values of the standards were used to plot the reference curve (log-log) from which concentrations of test samples were then derived. The normal reference range for both IgG and IgM ACL was < 5 GPL or MPL units respectively.

2.3.8 Anti beta$_2$glycoprotein I antibody

Principle

A quantitative indirect ELISA on high binding γ-irradiated plates using β_2GPI to capture and immobilise autoantibodies is used. Immobilised antibodies are bound by a conjugated monoclonal anti-human IgG, the presence of which was revealed and quantified when exposed to a suitable substrate.

Method

A partly commercialised method was used which had been validated by Dr S Donohoe (Haemostasis Research Unit, University College London).

Reagents

All reagents were supplied by Axis-Shield Diagnostics Ltd. as part of a kit (Diastat Anti-Beta$_2$ Glycoprotein I) unless stated otherwise and include:

- Microtitre plate pre-coated with β_2GPI
- Alkaline phosphatase conjugated murine monoclonal anti-human IgG in Tris buffer
- Wash buffer concentrate
- Sample diluent concentrate
- Anti-β_2GPI positive control
- Substrate solution (phenolphthalein monophosphate)
- Stop reagent (carbonate buffer containing sodium hydroxide and EDTA)
- An "in house" anti-β_2GPI standard from a well characterised antiphospholipid syndrome patient

Method

A range (1.56 – 100% activity) of standard solutions were prepared by doubling dilution buffer. Test and control samples were diluted 1 in 100 in dilution buffer.

100µl standards, tests or control were then added to the microtitre plate induplicate within a time span of four minutes (limiting drift in results over plate positions) and incubated at room temperature for one hour. Unbound sample was removed by washing three times with 200µl washing solution. 100µl conjugated antibody was added and incubated at room temperature for 30 minutes. After washing a further three times, 100µl substrate solution was added to wells and incubated at rooom temperature for 30 minutes. The reaction was stopped and developed by the addition of 100µl stop reagent. Absorbance values were read at 550nm using an automated plate reader. Mean absorbance values of the standards were used to plot the reference curve (log-log) from which concentrations of test samples were then derived. The cut-off for positivity (>2.88%) was established as 2 SD above the mean on log transformed data of 30 healthy subjects. A plasma with known anti-β_2GPI concentration was assayed on each occasion to ensure reliability of the assay.

2.3.9 Anti-Prothrombin assay

Principle

Microtitre plates are coated with human prothrombin. Test serum samples are applied and specific antibody binding to prothrombin is detected using a conjugated anti-human IgG or IgM. Thus both IgG and IgM antiprothrombin antibodies may be detected. The assay was standardised against a reference plasma with a high anti-prothrombin IgG and IgM activity.

Reagents

All reagents were supplied by Merck Ltd. unless otherwise stated.

- γ-irradiated microtitre plate (Nunc, Maxisorp; Life Technologies Ltd.)
- 10µg/ml human prothrombin in PBS (Enzyme Research Laboratories)
- Buffer A – 0.01 M phosphate, 0.0145 M NaCl pH 7.2 (Sigma-Aldrich Chemical Co.)
- Buffer B – buffer A containing 0.1% (v/v) Tween 20
- Blocking solution/sample diluent – buffer B containing 1% BSA (Sigma-Aldrich Chemical Co.)

- Alkaline phosphatase conjugated goat anti-human IgG (γ chain specific) or IgM (μ chain specific) (Sigma-Aldrich Chemical Co.)
- 0.92 M diethanolamine buffer pH 9.8 containing 0.5 mmol/l magnesium chloride hexachloride
- Substrate – 1mg/ml *p*-nitrophenyl phosphate disodium hexahydrate phosphatase (Sigma-Aldrich Chemical Co.) in diethanolamine buffer
- 3 M sodium hydroxide

Method

A γ-irradiated microtitre plate was coated overnight at 4°C with 80μl 10μg/ml human prothrombin in PBS. Unbound protein was removed by washing three times with 150μl buffer B. All wells were blocked with blocking solution for one hour at room temperature. A standard curve was prepared from a reference plasma with a known high anti-prothrombin IgG and IgM activity (%) by dilution in sample diluent (creating a range of 3.125% to 100% activity). Tests and control samples were diluted 1:50 in sample diluent. 100μl standard, control or test was added to both coated and blank wells in duplicate for 90 minutes at room temperature. After washing three times with 150μl buffer B, 100μl alkaline phosphatase conjugated anti-human IgG or IgM, diluted 1 in 1000 in sample buffer were added to wells for one hour at room temperature. After washing three times with buffer B, 100μl substrate was added and incubated at 37°C for colour development. The reaction was stopped by adding 50μl 3M sodium hydroxide. The absorbance was read at 405nm using an automated plate reader interfaced with ANELISA software. The ANELISA software was programmed to plot the standard curve obtained from the log absorbance against log activity (%). The normal reference range for IgG and IgM aPT was less than 3.9% and 20.1% respectively.

2.4 Von Willebrand factor multimer distribution

Principle

Citrated plasma is diluted in Tris buffer containing SDS and urea. These samples are electrophoresed on agarose gels using the Pharmacia PhastSystem. The gels are

diffusion blotted onto nitrocellulose. Indirect double antibody enzyme linked immuno-localisation allows subsequent visualisation of VWF: Ag multimers.

Reagents

- Casting frame for agarose PhastGels
- Gelbond 60 x 60mm (Pharmacia Biotech)
- Running gel – 4g HGT Seakem Agarose (FMC Corporation) 0.5 M Tris buffer with 0.1% SDS pH 8.8
- Stacking gel – 4g HGT Seaken Agarose (FMC Corporation) 0.125M Tris buffer with 0.1% SDS pH 6.8
- Sample buffer – 1.211g Tris 20g SDS 480.38mg Urea 0.375mg disodium EDTA 0.05g bromophenol blue pH 8.0
- PhastGel SDS buffer strips (Pharmacia)
- PhastGel sample applicator 8/1 (Pharmacia)
- Nitrocellulose BA 85 0.45Mm (Scheider & Schnell) (Anderman & Co Ltd.)
- Chromatographic paper (Whatman 3MM)
- 0.02 M borate buffered solution pH 7.2
- Blocking solution – 5% (v/v) dried powdered milk in distilled water
- Rabbit anti-human VWF (Dako Ltd.)
- Alkaline phosphatase conjugated swine anti-rabbit IgG (Dako Ltd.)
- Ethanolamine buffer 0.1 M pH 9.6
- Nitroblue tetrazolium 1g/L in ethanolamine buffer
- Substrate stock solution – 10ml methanol 5ml acetone 60mg 5-bromo-4-chloro-3-indolyl phosphate (disodium salt)
- Magnesium chloride 1 M
- Alkaline phosphatase staining solution – 50ml ethanolamine buffer 5ml nitroblue tetrazolium 0.75ml substate stock solution 0.2ml magnesium chloride 1 M

Method

GelBond was attached hydrophobic side up to the backing plate of a casting casette before fixing the U-frame in place. The assembled casette was placed at 56°C for 20 minutes before the running gel was poured into the casting frame. After gelling at

room temperature for five minutes there was a further ten minute incubation at 4°C. The two halves of the casting casette were separated and the running gel cut to size. The GelBond was re-attached hydrophilic side uppermost and the casting casette realigned. Stacking gel was poured into the casette and refrigerated at 4°C for one hour after gelling at room temperature. The gel and GelBond were then removed from the casting casette and placed on the electophoretic bed beneath a PhastGel buffer strip holder containing buffer strips. Test samples had been previously diluted to VWF concentrations of approximately 0.1IU/ml in VWF multimer buffer and were applied to the gel using a PhastGel sample applicator. Electophoresis was then commenced (see *Table 2.2* for programming)

Program stage	Voltage (V)	Current (mA)	Watts (W)	Temp (°C)	Volt hours (Vh)
1.1	250	10.0	2.0	15	001
1.2	100	1.0	3.0	15	002
1.3	100	3.0	3.0	15	035
1.4	100	0.1	1.0	15	010

***Table 2.2** Programming for VWF multimer electrophoresis*

After electrophoresis the gel was diffusion blotted onto moistened nitrocellulose membrane at 55°C for 25 minutes. The membrane was placed in blocking solution for one hour before washing three times in BBS. 25ml of rabbit anti-human VWF antibody diluted 1:750 in BBS was poured over nitrocellulose membrane and incubated at room temperature overnight. After washing three times in BBS, the membrane was incubated at room temperature for two hours with gentle mixing with 25ml alkaline phosphatase conjugated swine anti-rabbit IgG diluted 1 in 750 in BBS. After washing twice in BBS and once in ethanolamine buffer, alkaline phosphatase staining solution was poured onto the nitrocellulose and mixed gently. After visualisation, the membrane was washed in distilled water and allowed to dry. A

normal platelet poor plasma was included in each gel for quality control. Approximately 25 bands should be visualised in normal plasma.

2.5 Assessment of von Willebrand Factor cleaving protease activity

Principle

A specific von Willebrand factor cleaving protease (VWF-CP) activity is thought to be central to post secretory VWF processing. VWF is initially secreted as ultra large VWF polymers (ULVWF) by both the constitutive and regulated pathways. However VWF circulates as a series of smaller multimers of molecular weight 500 – 20,000kDa. Certain VWF fragments are also consistently found in plasma: those of 140kDa and 176kDa correspond to cleavage of a single peptide bond, the tyrosine 842 – methionine 843 of the mature subunit. Generation of VWF fragments or proteolysis of VWF as shown by loss of high and intermediate VWF multimers can therefore be used for *in vitro* determination of VWF-CP activity.

Reagents

- Sample buffer - 10mM Tris (hydroxymethyl)methylamine 0.015M NaCl pH 8.0
- VWF substrate – facteur von Willebrand de coagulation (Laboratoire Français du Fractionnement et des Biotechnologies)
- 6 M urea
- 100 mM $BaCl_2$
- Reagents for VWF multimer analysis as above

Final method

Test and control plasmas were diluted 1 in 5 in sample buffer. 10µl $BaCl_2$ 100 mM was added to a 90µl aliquot of test or control plasma diluted 1 in 5 in sample buffer. This was incubated at 37°C for five minutes. 50µl VWF substrate diluted 1 in 20 in sample buffer was added to each sample before the final addition of 50µl 6 M urea. The samples were incubated at 37°C overnight before performing standard VWF multimeric analysis according to the method described above. All test and control plasmas were diluted 1 in 5 in VWF multimer sample buffer before electrophoresis. A positive and negative control (pooled normal plasma (PNP) in the absence or presence

of EDTA) was included in each assay. This allowed expression of VWF cleavage, shown as loss of high and intermediate molecular weight multimers, as a relative ratio of PNP activity. The linear extent of each sample's multimeric profile was measured and subtracted from that of the negative control. This value was expressed as a ratio of that of PNP activity. PNP activity was semi-quantitatively expressed as the difference in linear multimeric profiles of the negative and positive control.

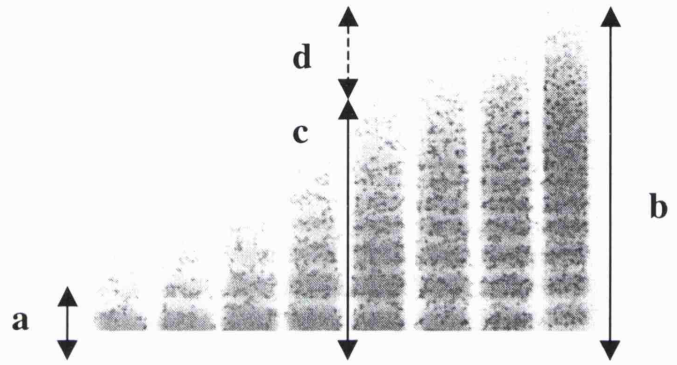


Figure 2.3 *Calculation of VWF-cleaving protease activity. A positive and negative control run in lanes 1 and 2. For any sample the degree of proteolysis is expressed as a ratio of that of the positive control. VWF-CP activity ratio = (b – c)/(b – a)*

(a = positive control, b = negative, c = control sample, d = distance)

The protease activity of PNP was arbitrarily assigned as 100%. To allow semi-quantitation of a sample's intrinsic protease activity, this was also represented as the difference in the extent of linear migration of the negative control (b) and the positive control (a) measured to the nearest 0.5mm, as shown in the above calibration curve. This distance represents the extent of loss of intermediate and high molecular weight multimers which would be seen if a sample possessed VWF-CP activity comparable to that of PNP. Since VWF cleavage is shown as loss of high and intermediate molecular weight multimers, the relative loss of such multimers for a test plasma may be represented by the difference in linear multimeric profile between the negative control

(b) and the sample (c), represented by the distance (d) in *Figure 2.3*. Semi-quantitation of the assay was achieved by expressing this value as a ratio of that of PNP:

$$\text{VWF-CP activity ratio} = (b - c)/(b-a)$$

By definition, the activity ratio of PNP is equal to 1.0 whilst that of the negative control is zero. A negative value for a test plasma is highly suggestive of the presence of ultra large VWF multimers in the sample.

The normal reference range for plasma VWF-CP activity (based on geometric mean ± 2 SD of log normalised data) was 0.80 – 1.25 for plasma and for serum 0.93 – 1.27. The inter assay CV and intra-assay CV for measuring VWF-CP is 9.8% and 9.2% respectively. A positive and negative control (pooled normal plasma (PNP) in the absence or presence of EDTA) was included in each assay.

Method development

A number of assays have now been described for measuring VWF-CP activity. At the time this study was commenced two methods had been described. That published by Tsai depended on the generation from purified vWF of dimers of 176kDa and 140kDa fragments which appear as 350kDa and 200kDa bands respectively on sodium dodecyl sulphate (SDS) polyacrylamide electrophoresis (Tsai & Lian, 1998). The other reported by Furlan relied on the extent of degradation of VWF multimers as assessed by SDS electrophoresis on 1.4% agarose gels (Furlan *et al*, 1997). This used VWF purified by gel filtration on a Sepharose-2B column from a cryoprecipitate of normal human plasma and eluted with 0.13 mol/l NaCl 0.01 M citrate 0.01 M TRIS-HCl pH 7.4. Plasma samples were diluted in a solution of 0.15M NaCl and 10mmol TRIS (pH 7.4) containing 1mmol/l serine protease inhibitor (Pefabloc SC). These were activated with Ba^{2+} prior to incubation with purified VWF and the reaction mixture was then dialysed for 24 hours at 37°C against 1.5M urea and 5 mM TRIS-hydrochloric acid pH 8.0 before multimer analysis. This latter method was modified and the results of these experiments are summarised below.

Modification of sample buffer

VWF was initially purified according to the above technique whilst plasma was diluted in the following sample buffer: 0.15 M NaCl 10 mM TRIS pH 8.0. Despite pre-activation with Ba^{2+}, when 75µl diluted normal plasma was incubated with 75µl purified VWF in the presence of 1.5 M urea (final concentration), VWF degradation was not achieved. Since direct incubation was used rather than dialysis it was initially considered that this might be secondary to differences in pH since it had been demonstrated that protease activity was highly pH dependent. However dialysis overnight of the purified VWF substrate against the sample buffer (0.15M NaCl 10mM TRIS pH 8.0) had no effect on subsequent proteolysis. In addition to pH, proteolytic activity had also been demonstrated to be highly sensitive to ionic strength. The experiment was therefore repeated with sample buffers either omitting or containing 0.15M or 0.015M NaCl in addition to 10mM TRIS pH 8.0. Partial degradation was obtained as shown below in *Figure 2.4.*

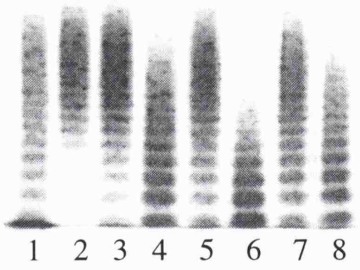


Figure 2.4 *Effect of sample buffer ionic strength.*
Normal plasma and VWF substrate are shown in lanes 1 and 2. Thereafter 75µl normal plasma diluted 1:20 in varying sample buffers (below) was incubated with 75µl VWF substrate and 1.5M urea. Lanes 4,6 and 8 were preactivated with Ba^{2+}.
Lanes 3, 4: 0.15M NaCl 10mM TRIS pH 8. Lanes 5,6: 0.015M NaCl 10mM TRIS pH 8. Lanes 7,8: 10mM TRIS pH 8

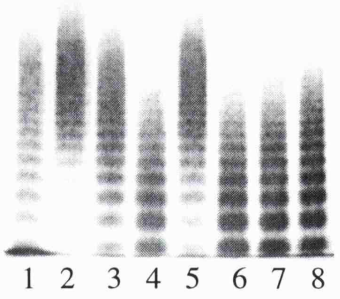
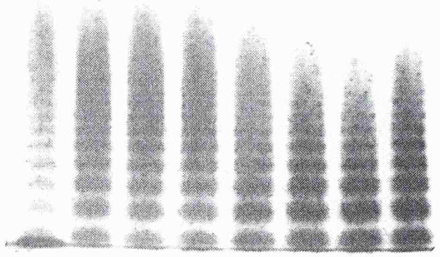
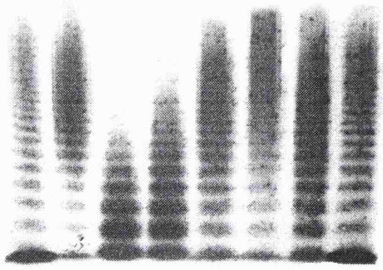


Figure 2.5 *Effect of pH .*
Normal plasma and VWF substrate were run in lanes 1 and 2. Lanes 3 to 8 show 75µl normal plasma diluted 1:20 in 0.015M NaCl 10mmol TRIS of pH 6, 7, 8, 9 or 10 respectively and incubated with 75µl vWF and 1.5M urea after preincubation with Ba^{2+} *Lane 3 is an unactivated sample (pH 8)*



1 2 3 4 5 6 7 8

Figure 2.5 *Incubation time*
Lanes 1 and 2 show the multimeric distribution of a normal plasma and VWF substrate. Lanes 3 to 8 represent 75µl normal plasma diluted 1:20 in 0.015M NaCl 10mmol TRIS pH 8.0 preactivated with Ba2+ and incubated with 75µl VWF substrate in the presence of 1.5M urea for 2, 4, 8,12, 16 and 24 hours respectively.

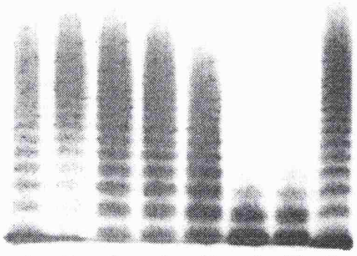


1 2 3 4 5 6 7 8

Figure 2.6 *Optimal activation by divalent metal ions.*
A PNP sample is shown in lanes 1 and 8. Lanes 3 to 7 show 75µl normal plasma diluted 1:20 in 0.015M NaCl 10mmol TRIS pH 8.0 and incubated with 75µl VWF and 1.5M urea after preincubation with 10mmol Ba^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+} or Cu^{2+} respectively. A negative control is shown in lane 2 (normal plasma activated with $Ba^{2+}$and incubated as above in the presence of EDTA)

Optimisation of assay conditions

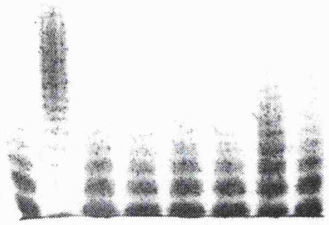
A series of experiments were performed to optimise VWF proteolysis. Although degradation was achieved under the above conditions it was less extensive than that demonstrated by Furlan. However neither manipulation of sample buffer pH (*Figure 2.5*), prolonged incubation (*Figure 2.6*) or use of alternative divalent metal ions as activators (*Figure 2.7*) resulted in enhanced proteolysis. Cleavage was subsequently maximised by increasing the concentration of test plasma relative to VWF substrate (*Figure 2.8*).



1 2 3 4 5 6 7 8

Figure 2.7 *Optimisation of test:substrate ratio.*

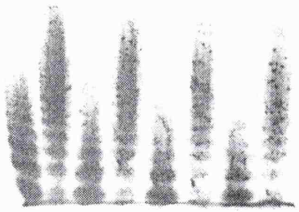
PNP is shown in lanes 1 and 8. Lanes 3 to 7 show 25, 50, 75, 100 and 125µl normal plasma respectively diluted 1:20 in 0.015M NaCl 10mmol TRIS pH 8.0 and incubated with 1.5M urea and 125, 100, 75, 50, 25µl VWF respectively after preincubation with 10mmol Ba^{2+}. A negative control is shown in lane 2 (normal plasma activated with $Ba^{2+}$and incubated as above in the presence of EDTA).



1 2 3 4 5 6 7 8

Figure 2.8 *Effect of protease inhibitors.*

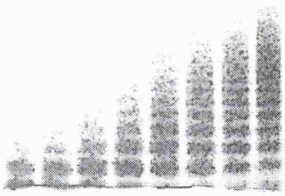
Lanes 3 to 7 show 100µl PNP diluted 1:20 in 0.015M NaCl 10mmol TRIS pH 8.0, preactivated with 10mmol Ba^{2+} and incubated with 50µl vWF and 1.5M urea with 0.01mM aprotinin, 1mM PMSF, 1mM bezaminidine,10mM thiol protease inhibitor and 10mM Pefabloc SC respectively. A positive control runs in lanes 1 and 8 and a negative in lane 2 (100µl PNP diluted and activated as above and incubated with 50µl VWF and 1.5M urea + EDTA).



1 2 3 4 5 6 7 8

Figure 2.9 *Alternative VWF substrate.*

100µl PNP diluted 1:20 in 0.015M NaCl 10mmol TRIS pH 8.0, preactivated with Ba^{2+} and incubated with 1.5M urea and 50µl LFB VWF concentrate. Concentrate was diluted 1:5, 1:10, 1:20 and 1:40 in lanes 1-2, 3-4, 5-6 and 7-8 respectively. Lanes 2, 4, 6 and 8 acted as negative controls, being incubated with EDTA..



1 2 3 4 5 6 7 8

Figure 2.10 *Dilution curve*

Serial double dilutions of PNP in 0.015M NaCl 10mmol TRIS pH 8.0, preactivated with Ba^{2+} and incubated with 1.5M urea and 50µl LFB VWF concentrate. Double dilutions from 1:5 to 1:320 are shown in lanes 1- 7. Lane 8 is a negative control (1:5 dilution incubated in presence of EDTA).

VWF substrate was shown to lack endogenous VWF-CP activity and protease activity in PNP was effectively inhibited by EDTA. There was no significant inhibition with aprotinin, PMSF, benzamidine or a thiol protease inhibitor (chloroacetyl-HOLeu-Ala-Gly-NH2) as reported by Furlan and Tsai although Pefabloc SC resulted in minimal inhibition (*Figure 2.9*). Initial screening showed degradation of vWF substrate by normal controls but not with plasma from a patient presenting with acute TTP.

Preliminary method

Test plasma was diluted 1 in 20 in sample buffer (0.015M NaCl 10mM TRIS pH 8.0). 10µl $BaCl_2$ 100 mM was added to a 90µl aliquot of diluted plasma. This was incubated at 37°C for five minutes. 50µl purified VWF was added to each sample before the final addition of 50µl 6 M urea. The samples were incubated at 37°C overnight before performing standard VWF multimeric analysis according to the method described above.

Method modification

This method was hampered by the procedure of VWF purification. On occasion poor yields of purified vWF were obtained requiring repeated application of cryoprecipitate and finally contamination of the purified substrate with vWF proteolytic actvity occurred. A search was therefore made for an alternative vWF substrate. Facteur von Willebrand de coagulation (Laboratoire Français du Fractionnement et des Biotechnologies) is a high purity VWF concentrate (50IU/ml). When substituted for cryoprecipitate derived VWF substrate there was initially minimal degradation. To promote the sensitivity of the assay for detection of reduced VWF-CP activity, test samples were diluted 1 in 5 and VWF concentrate 1 in 20 in sample buffer (*Figure 2.10*). Under these conditions there was no loss of high molecular weight multimers when VWF concentrate was substituted for PNP as the test sample. Progressive loss of high and intermediate size VWF multimers was proportional to VWF-CP activity as shown by serial doubling dilution of PNP in sample buffer (*Figure 2.11*). This method was adopted as it reduced assay variability by ensuring a uniform VWF substrate composition.

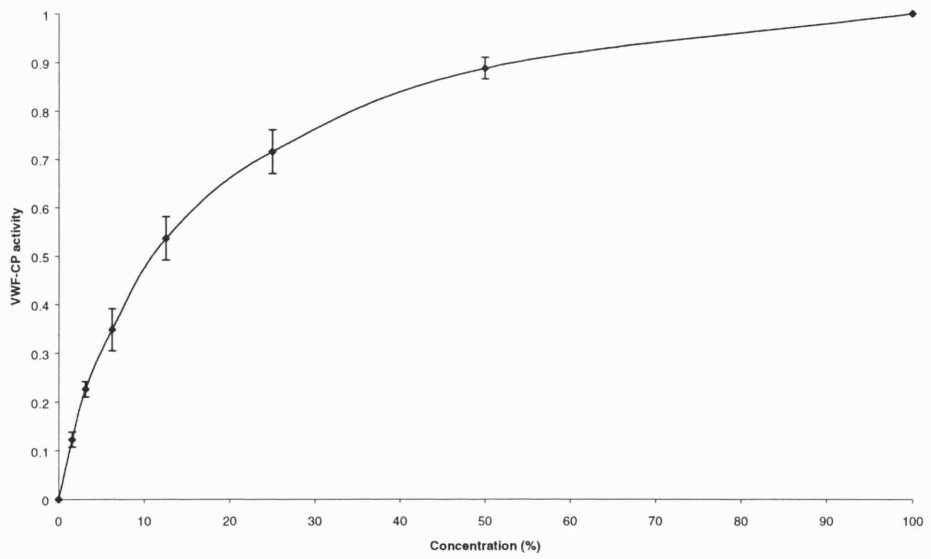


Figure 2.12 *VWF-CP assay: calibration curve*

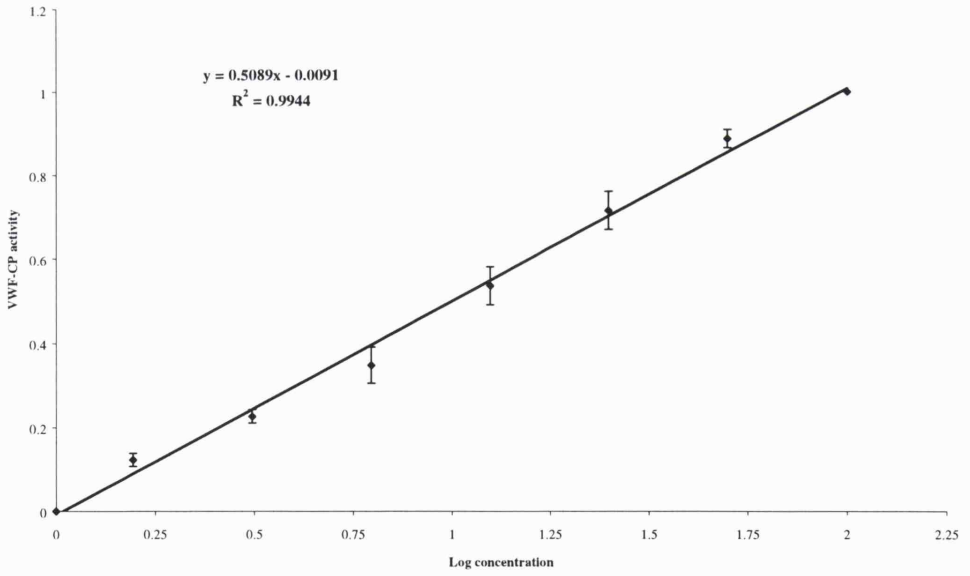


Figure 2.13 *VWF-CP assay: log-linear calibration curve*

Alternative methods for the measurement of VWF-CP activity have recently been published. One, an ELISA technique relies on the progressive loss of VWF collagen binding ability with reducing VWF multimer size (Gerritsen *et al*, 1999) whilst the other, uses IRMA methodology (Obert *et al*, 1999). These assays may allow more accurate quantitation of VWF-CP activity and increase testing capacity but it should be stressed that as yet no comparative studies of the differing techniques has been performed.

2.5.1 Correction studies

Principle

Reduced VWF-CP activity may be the consequence of an absolute decrease in protease activity or or a circulating inhibitor. Correction studies may aid in differentiation. Correction is seen on a 1:1 mix with PNP if there is an absolute deficiency whereas there is no or incomplete correction in the presence of an inhibitor.

Reagents

- Pooled normal plasma (PNP)
- Sample buffer - 10mM Tris (hydroxymethyl)methylamine 0.015M NaCl pH 8.0
- VWF substrate – facteur von Willebrand de coagulation (Laboratoire Français du Fractionnement et des Biotechnologies)
- 6 M urea
- 100 mM $BaCl_2$
- Reagents for VWF multimer analysis as above

Method

A 1:1 mix of sample and PNP was incubated at 37°C for one hour. This was subsequently diluted in sample buffer to give a final 1:5 dilution of PNP before proceeding with the standard VWF-CP assay as detailed above. Positive and negative controls used PNP that had been incubated similarly for one hour at 37°C.

Method development

To investigate possible time dependence of any circulating inhibitory VWF-CP activity, a 1:1 mix of patient plasma and PNP was incubated at 37°C for either 10, 30,

60, 120, 240 or 360 minutes. An aliquot was then diluted in sample buffer to give a final 1 in 5 dilution of PNP. This was preactivated with Ba^{2+} and incubated overnight with VWF substrate in the presence of urea at 37°C as described above. To compensate for any loss of activity of normal plasma, control PNP was also incubated for six hours at 37°C before proceeding with the assay. Nine plasma samples from patients with TTP known to have a severe deficiency of VWF-CP activity were screened in this manner. Results are shown in *Figure 2.14*. As no significant time dependent inhibition of VWF-CP activity was detected, a one hour incubation period was adopted.

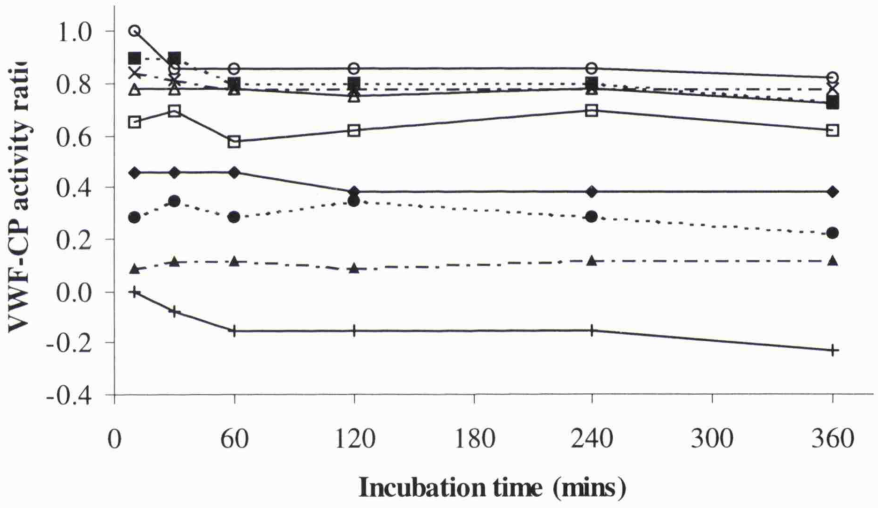


Figure 2.14 *Investigation of effect of incubation time on protease activity of 1:1 mix of patient plasma with known protease deficiency and PNP (n = 9). The 1:1 mix was incubated for either 10, 30, 60, 120, 240 or 360 minutes before activation with Ba^{2+} and overnight incubation with VWF substrate. No significant time dependent inhibition was detected.*

2.6 Immunoglobulin G isolation

The IgG fraction was isolated by affinity purification on a protein G column.

Reagents

All reagents were from from Merck Ltd. unless otherwise stated.

- Hi-Trap Protein G Sepharose (Pharmacia Biotech Ltd.) packed in a 1 x 12 cm column
- PBS azide - 0.01 M phosphate, 0.145 M sodium chloride pH 7.2 containing 0.2g/l azide
- Glycine azide buffer – 0.1 M glycine pH 2.8 containing 0.2g/l azide
- 1.0 M Tris pH 9.0
- VWF-cleaving protease sample buffer - 0.015M NaCl 10mmol TRIS pH 8.0

Method

Plasma or serum samples were diluted 1 in 5 in PBS azide and applied to the Protein G sepharose column which had been pre-equilibrated with PBS azide. The column was washed with PBS azide until the flow through absorbance $A280_{nm}$ had returned to baseline. Bound protein was then eluted with 0.1M glycine buffer and collected in 4ml fractions into tubes containing 0.4ml of 1 M Tris. Protein containing fractions were pooled and the protein dialysed to vWF-cleaving protease sample buffer and concentrated to 2mg/ml using an Amicon concentrator and PM30 membrane (Amicon Ltd.). Protein concentration was determined by measuring the absorbance at 280nm with a 1cm light path and extinction coefficient $E^{1\%,1cm} = 13.6$ (Johnstone & Thorpe 1987 Immunochemistry in Practice Oxford: Blackwell Scientific Publications) and the protein was stored at -70°C.

3. Platelet activation in TTP

3.1 Introduction

TTP is characterised by the formation of microvascular thrombi composed primarily of platelets. The thrombi formed contain abundant von Willebrand factor (VWF) but scanty fibrin and fibrinogen. This is in direct contrast to those thrombi formed in DIC when the reverse is seen (Asada *et al*, 1985). It has therefore been suggested that TTP may be a disease of primary platelet activation and aggregation (Harkness *et al*, 1981). This is supported by histological appearances since neither significant perivascular inflammation, obvious endothelial cell desquamation or subendothelial exposure have been demonstrated in TTP. Experimental evidence supporting this hypothesis has been provided by work using a cone and plate viscometer. This minimises platelet-surface interactions and allows direct platelet aggregation under elevated shear fluid stresses. Using this model it has been demonstrated that platelet rich plasma (PRP) from patients with congenital TTP results in excessive platelet aggregation as compared to adult normal PRP samples when exposed to shear stresses of 90 to 180 dyne/cm^2 (comparable to those found in partially occluded microvasculature) (Moake *et al*, 1994). Similarly, platelet retention, as measured by the modified Adeplat S test (a glass-bead retention method) at the relatively modest shear stress of 76 dynes/cm^2, is increased in congenital TTP as compared to normal controls (Karpman *et al*, 1997).

Platelets may form aggregates through sequential stages of adhesion, activation and aggregation. It therefore appears reasonable to hypothesise that platelet activation may play a role in the pathogenesis of TTP. Some studies have reported increased expression of platelet CD62p expression (Wright *et al*, 1999) or platelet aggregate formation in TTP (Ahn *et al*, 1996) although these are not uniform findings (Hoffman *et al*, 1993). The purpose of this investigation was to look for evidence of *in vitro* platelet activation in patients with TTP both at presentation and serially throughout a haemolytic episode using a panel of flow cytometric methods. Platelet activation is a

complex process. It is therefore unlikely that a single parameter detects all instances of platelet activation. In addition to flow cytometric methods an ELISA technique was used to measure markers of thrombin generation and soluble P-selectin.

3.2 Methods

3.2.1 Patients

Eight patients with adult onset TTP were studied serially during an episode of TTP. Although one patient was HIV positive, severe deficiency of VWF-CP activity was later confirmed (see chapter 4). The remaining patients all had idiopathic disease.

3.2.2 Blood collection

Patients and controls were rested for approximately 20 minutes prior to venepuncture in order to minimise platelet activation. A vacutainer system® was used to obtain blood samples and plasma was prepared for specific assays as outlined in section 2.1.

3.2.3 Flow cytometric studies of platelet activation

Resting and ADP and TRAP induced platelet surface expression of CD62p and CD63 were measured as described in section 2.2.4. Platelet reticulocytes, platelet-leucocyte aggregates and platelet derived microparticles were measured according to sections 2.2.3, 2.2.5 and 2.2.6 respectively. All samples for platelet activation studies were analysed within three hours of venepuncture.

3.2.4 Enzyme Linked Immunosorbent Assays (ELISA)

P-selectin, TAT and F1.2 were measured as described in section 2.3.

3.3 Results

Patient characteristics

The characteristics of the eight patients studied are shown in *Table 3.1*. There was a male: female ratio of 1:3 and a median age of 36 (range 26 – 50). Neurological features were absent in only one patient who had a previous history of TTP who presented relatively early with a platelet count of 82 x 10^9/l. The median platelet count at presentation was 17.5 x 10^9/l. Predisposing factors were identified in a single patient (patient number 5) who was HIV positive with a CD4 count of 50 x 10^9/l.

Twenty healthy volunteers were used as control subjects for the platelet studies. There were 11 females and 9 males and their ages ranged between 20 and 44 years (median age 33). None of the control subjects was thrombocytopenic. Three patients, all female, with chronic idiopathic thrombocytopenic purpura (ITP) were therefore included in the study (median age 57, range 30 – 69, median platelet count 35 x 10^9/l, range 12 x 10^9/l – 81 x 10^9/l).

Expression of platelet degranulation markers

Results will be discussed in terms of platelet surface CD62p and CD63 expression. However to be included in analysis, dual expression of a platelet specific marker, CD61 and an activation marker was mandatory. A total of 69 samples were serially analysed from eight patients during a symptomatic episode of TTP. Results for the percentage of platelets expressing CD62p and CD63 are shown in *Figures 3.1* and *3.2* respectively. At presentation the median value for CD62p expression was 4.8% as compared to 1.3% in healthy controls (p value = 0.001). Percentage CD62p positive platelets was extremely high at 22% in one subject, patient number 8. This did not appear to predict subsequent clinical course since only seven plasmaphereses were required to attain durable remission in this case. In contrast, two patients, numbers 6 and 7, died within 48 hours of relapse but only 4.76% and 3.21% platelets respectively were positive for CD62p, both values within the normal range. The remaining six patients were followed to remission. Fourteen samples were analysed once a durable remission had been attained. Despite apparent disease resolution, platelet surface

CD62p expression remained elevated as compared to healthy controls with a median value of 2.24% (p value = 0.01). The overall median value for CD62p was significantly higher in patients diagnosed with TTP than healthy controls (2.3% versus 1.3%, p = 0.0009).

Patient Number	Sex	Age	Presenting platelet count (x 10^9/l)	Creatinine (μmol/l)	CNS symptoms	Fever	Antecedent history of TTP
1	F	38	19	61	+	-	-
2	M	50	82		-	-	+
3	M	35	14	84	+	+	-
4	F	26	16	119	+	+	-
5	F	32	6	189	+	+	-
6	F	37	19	83	+	-	-
7	F	26	28	83	+	Not documented	-
8	F	40	9	67	+	-	+

Table 3.1 *Clinical and laboratory features of eight TTP patients*
F = female; M = male.

Similarly the overall median value for platelet percentage positive CD63 expression was significantly higher in patients with TTP than healthy controls (7.5% versus 3.82%, $p < 0.0001$). Likewise, CD63 expression was elevated at presentation with a median value of 8.2% (p value = 0.01). However once again percentage CD63 positivity at presentation did not appear to predict clinical course: the two patients with a fatal outcome had values of only 8.33% and 6.06%, both within the normal range. Like CD62p expression, despite attainment of durable remission, platelet CD63 positivity remained significantly higher than healthy controls with a median value of 5.62% (p value = 0.04).

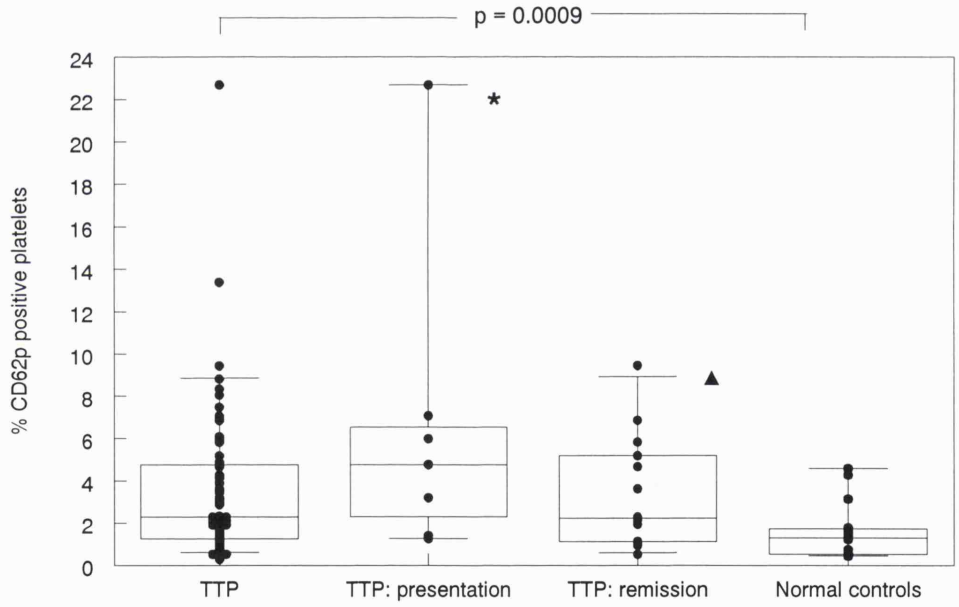


Figure 3.1 *Percentage platelets expressing CD62p in TTP samples (n =69) and controls (n=20). Presentation (n = 8) and remission (n=14) samples are also shown.*
Horizontal line indicates mean, box the 25th to 75th percentile and bars the 5th and 95th percentile. ★ p = 0.001 (presentation vs control), ▲ p = 0.01 (remission vs control).

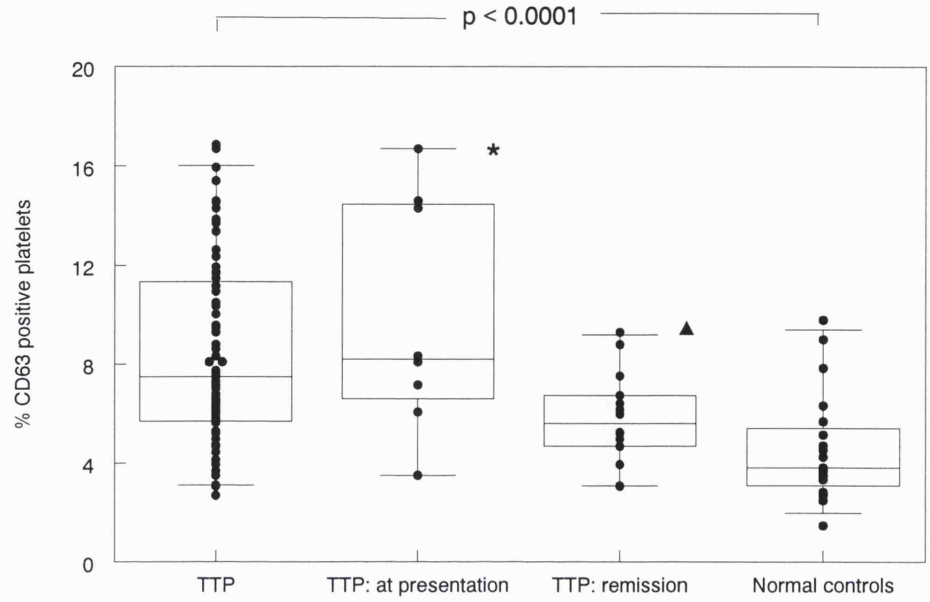


Figure 3.2 *Percentage platelets expressing CD63 in TTP samples (n =69) and controls (n=20). Presentation (n = 8) and remission (n=14) samples are also shown.*

Horizontal line indicates mean, box the 25th to 75th percentile and bars the 5th and 95th percentile. ★ p = 0.01 (presentation vs control), ▲ p = 0.04 (remission vs control).

Within an individual, serial analysis demonstrated an apparent reciprocal relationship between both platelet CD62p and CD63 expression and platelet count (*Figure 3.3*). Overall there was a significant negative correlation between both CD62p and CD63 expression and platelet count (*Figures 3.4* and *3.5*) but there was no significant correlation between CD62p and CD63 expression (data not shown). As a consequence CD62p and CD63 expression was studied in three patients with chronic immune thrombocytopenia (ITP). Median platelet count at the time of study was 35 x 10^9/l (range 12 x 10^9 – 81 x 10^9/l). CD63 expression was elevated above the normal range in all three cases (median value 13.3%, range 13.0 – 13.5%). In contrast CD62p expression remained within the normal range (median value 3.3%, range 1.0 – 4.6%).

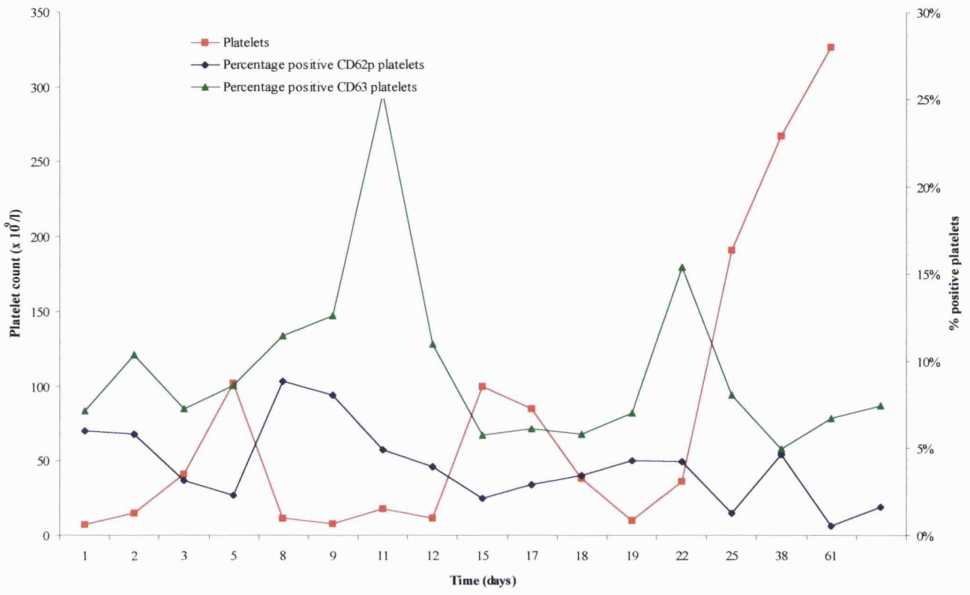


Figure 3.3 *Serial platelet count and percentage positive CD62p and CD63 platelets as illustrated by patient 3 who attained a durable remission on day 35 after two earlier exacerbations.*

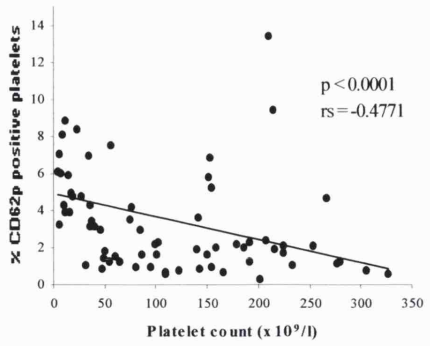


Figure 3.4 *Correlation between percentage positive CD62p platelets and platelet count.*

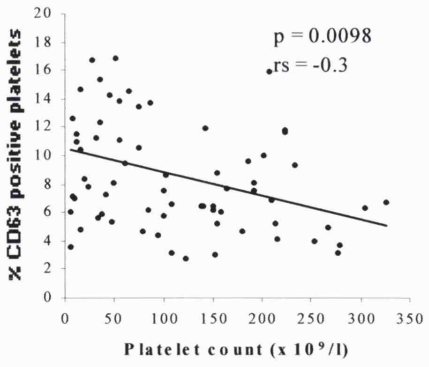


Figure 3.5 *Correlation between percentage positive CD63 platelets and platelet count.*

Platelet reactivity

TRAP induced platelet expression of activation markers CD62p and CD63

Agonist induced platelet surface expression of CD62p and CD63 was used as a surrogate marker of platelet function. At presentation median values for TRAP induced CD62p and CD63 expression were 43.7% and 38.5% as compared to 96.5% and 85.8% in healthy controls (p values < 0.0001) (*Figures 3.6* and *3.7*). TRAP induced CD62p expression was only 23.3% and 24.9% at presentation/relapse of the two patients who subsequently died. Two further patients had values within this range, each on a single occasion, both during a severe exacerbation when the platelet count was 10 x 10^9/l and 16 x 10^9/l respectively.

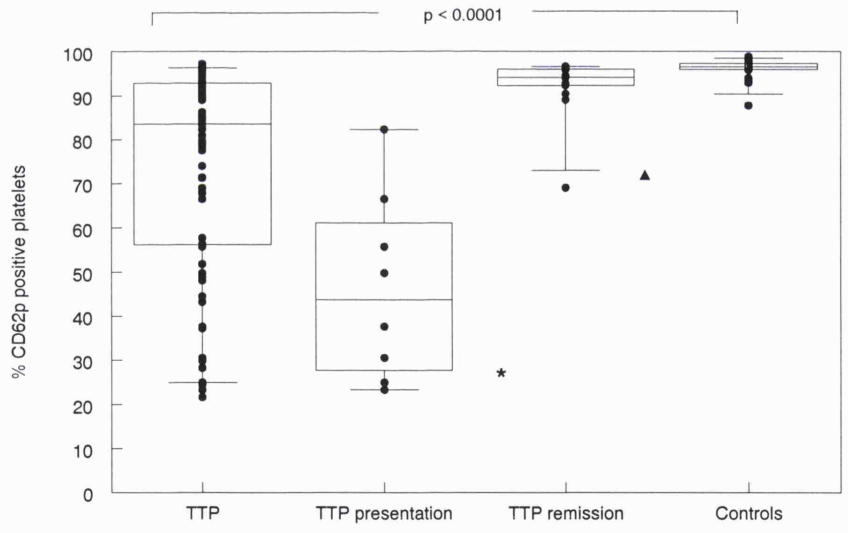


Figure 3.6 *TRAP induced percentage platelets expressing CD62p in TTP samples (n =69) and controls (n=20). Overall, presentation (n = 8) and remission (n=14) TTP samples are shown.*

Horizontal line indicates mean, box the 25th to 75th percentile and bars the 5th and 95th percentile. ★ p < 0.0001 (presentation vs control), ▲ p = 0.005 (remission vs control).

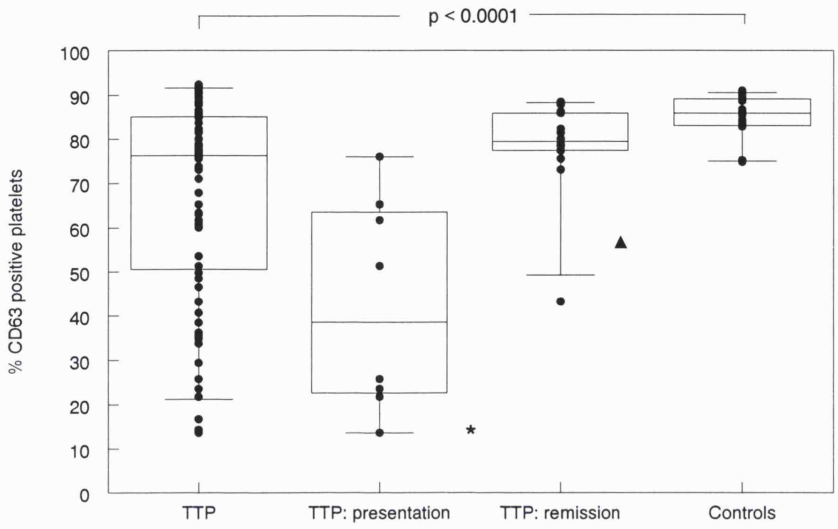


Figure 3.7 *TRAP induced percentage platelets expressing CD63 in TTP samples (n =69) and controls (n=20). Overall, presentation (n = 8) and remission (n=14) TTP samples are shown.*

Horizontal line indicates mean, box the 25th to 75th percentile and bars the 5th and 95th percentile. ★ $p < 0.0001$ (presentation vs control), ▲ $p = 0.006$ (remission vs control)

Serial studies suggested that changes in TRAP induced CD62p and CD63 expression predicted subsequent platelet response (see *Figure 3.8*). Furthermore in those ITP samples tested there was a variable reduction in TRAP induced CD62p and CD63 expression, (median values 81.1% and 78.11%, ranges 80.2 – 91.4% and 67.1 – 89% respectively) suggesting impaired platelet reactivity may be secondary to thrombocytopenia. Indeed there was a significant positive correlation between platelet count and both TRAP induced platelet CD62p expression ($p < 0.0001$, $rs = 0.84$) and platelet CD63 expression ($p < 0.0001$, $rs = 0.71$) in TTP samples (*Figure 3. 9*). There was also a significant positive correlation between percentage reticulated platelets and both TRAP induced platelet CD62p expression and platelet CD63 expression ($p = 0.02$, $rs = 0.35$ and $p = 0.006$, $rs = 0.4$ respectively) (*Figure 3.10*). Nevertheless TRAP induced CD62p and CD63 expression remained significantly reduced after attainment

of haematological remission with median values of 94.2% and 79.4% respectively (p values 0.005 and 0.006).

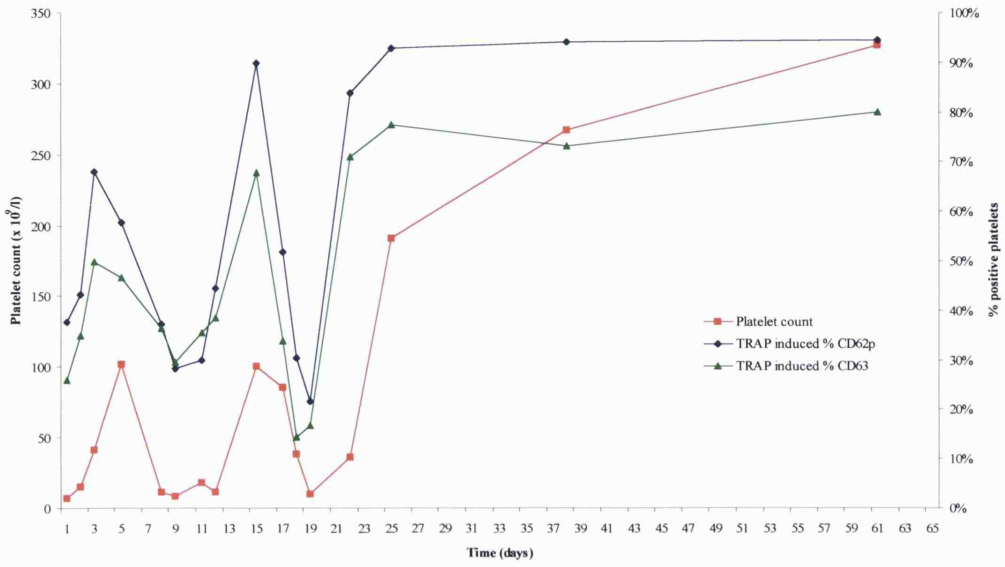


Figure 3.8 *Serial platelet count and TRAP induced percentage positive CD62p and CD63 platelets as illustrated by patient 3 who attained a durable remission on day 35 after two earlier exacerbations.*

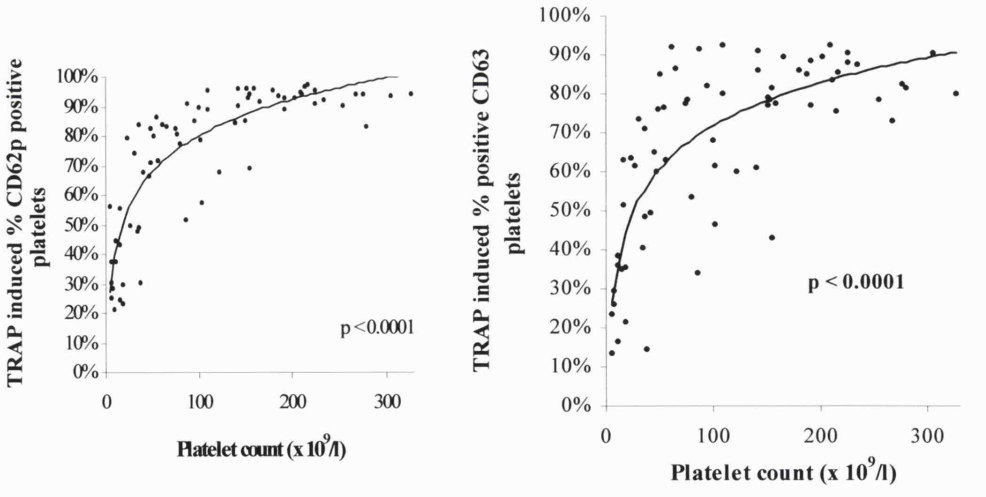


Figure 3.9 *Correlation between TRAP induced percentage CD62p and CD63 positive platelets and platelet count.*

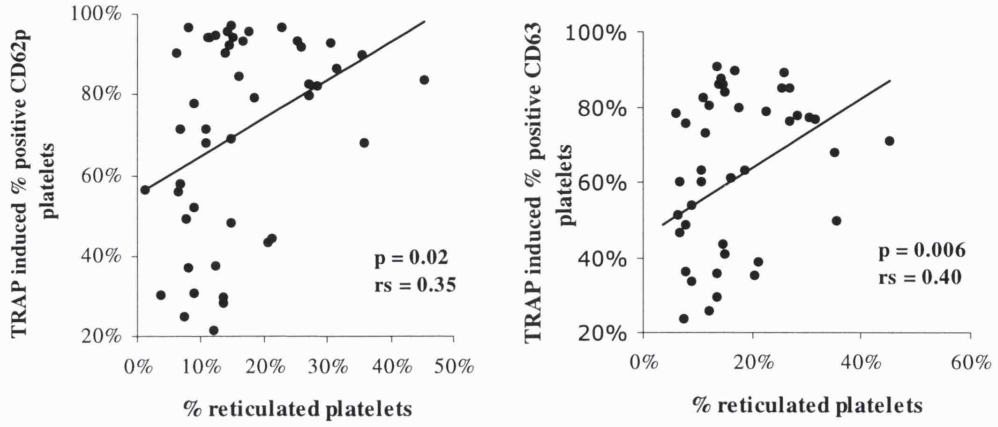


Figure 3.10 *Correlation beween TRAP induced percentage CD62p and CD63 positive platelets and percentage reticulated platelets.*

ADP induced platelet expression of activation markers CD62p and CD63

At presentation, median values for ADP induced CD62p and CD63 expression were significantly reduced as compared to healthy normal controls (p values 0.0005 and 0.002 respectively). The lowest percentage CD62p positive platelets resulting from ADP stimulation (4.8%) was seen in one of the fatal cases. Unfortunately the assay failed in the other fatal case. No other samples generated percentage positive platelets of less than 14%. Whilst ADP induced CD63 expression was less than 11% at presentation/exacerbation of those two patients who died, three of the surviving six patients also had values within this range on at least one occasion. Overall median values for ADP stimulated percentage positive CD62p and CD63 platelets were 16.3% and 11.7% as compared to 62.5% and 25.9% in normal controls respectively (p values < 0.0001 and $= 0.008$ respectively). However platelet reactivity normalised once haematological remission was attained (*Figures 3.11* and *3.12*).

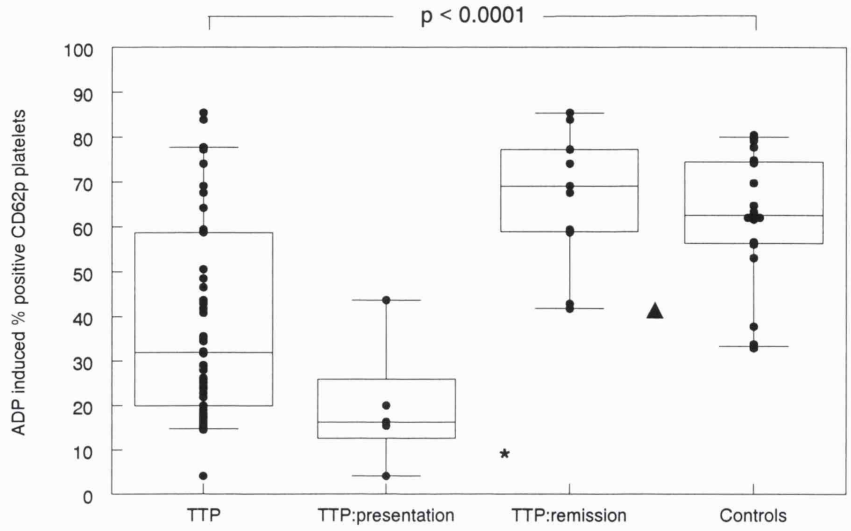


Figure 3.11 *ADP induced percentage CD62p positive platelets in TTP samples (n =50) and controls (n=20). Presentation (n = 6) and remission (n=11) samples are also shown.*

Horizontal line indicates mean, box the 25th to 75th percentile and bars the 5th and 95th percentile. ★ p = 0.0005 (presentation vs control), ▲ p = ns (remission vs control).

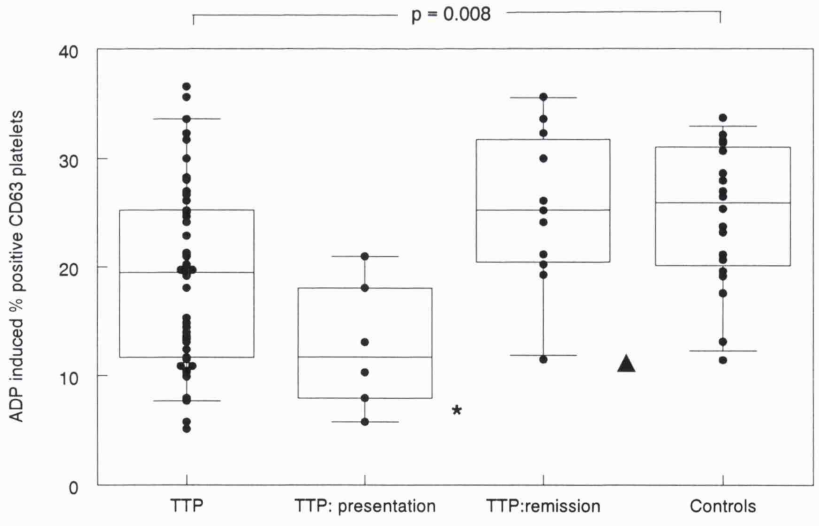


Figure 3.12 *ADP induced percentage CD63 positive platelets in TTP samples (n =50) and controls (n=20). Presentation (n = 6) and remission (n=11) samples are also shown.*

Horizontal line indicates mean, box the 25th to 75th percentile and bars the 5th and 95th percentile. ★ p = 0.002 (presentation vs control), ▲ p = ns (remission vs control).

Serial studies showed fluctuations in ADP induced CD62p and CD63 expression which appeared to predict changes in the absolute platelet count (*Figure 3.13*).

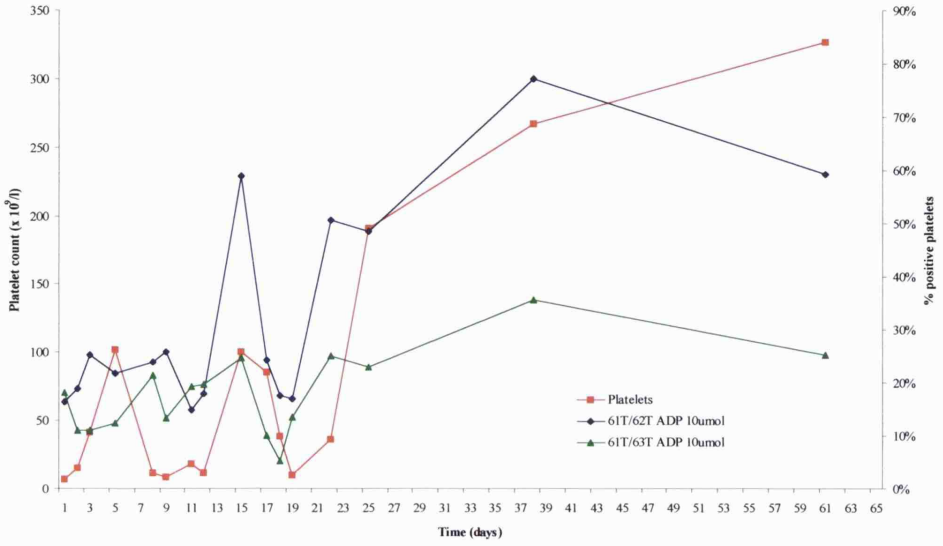


Figure 3.13 *Serial platelet count and ADP stimulated percentage positive CD62p and CD63 platelets as illustrated by patient 3 who attained a durable remission on day 35 after two earlier exacerbations.*

There was a significant positive correlation between platelet count and ADP induced platelet CD62p and CD63 expression (*Figure 3.14*). However in contrast to TRAP induced platelet activation, there was no significant correlation between ADP induced platelet reactivity and percentage reticulated platelets (data not shown). Subsequently ADP induced CD62p and CD63 expression was assessed in three ITP samples. Two of the three samples had values for CD62p expression that fell within the normal range whilst the abnormally low value (26.7%) was seen in the sample with the highest platelet count (81 x 10^9/l) and 29% reticulated platelets. ADP induced platelet CD63 expression was within the normal range in all three cases.

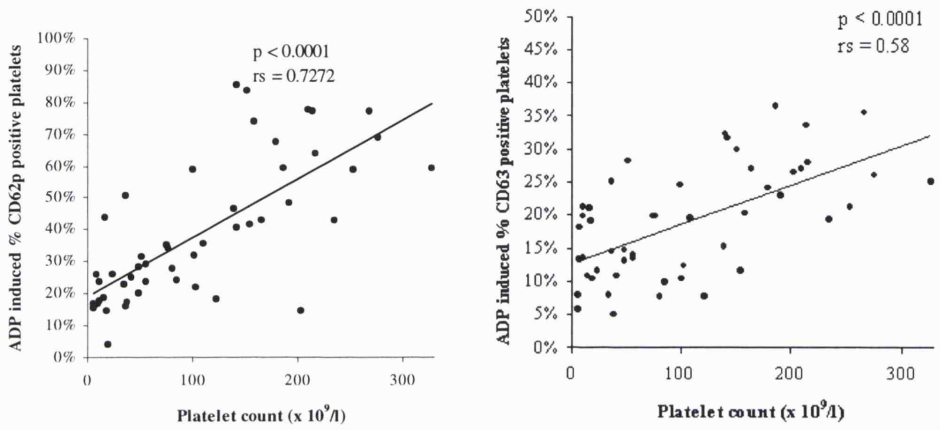


Figure 3.14 *Correlation between ADP induced percentage CD62p and CD63 positive platelets and platelet count.*

Platelet microparticles

Results for numbers of microparticles/ml plasma are shown in *Figure 3.15*. The median values for numbers of microparticles were 0.008 x 10^6 at presentation (p = 0.002) and 0.305 x 10^6 at remission (p = ns) with an overall median value of 0.225 x 10^6 (p = 0.004). The median value for control samples was 0.352 x 10^6. Serial analysis showed recovery of microparticle count with disease resolution and one individual example is shown in *Figure 3.16*. In keeping with this there was a positive correlation between the number of microparticles/ml plasma and absolute platelet count as shown in *Figure 3.17*. Although there was a significant negative correlation between CD63 expression and microparticle number this was not the case for CD62p expression.

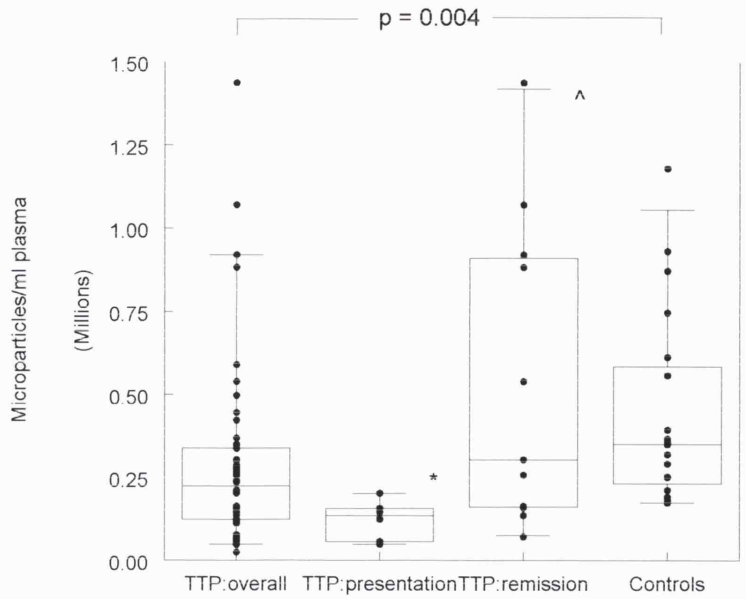


Figure 3.15 *Microparticle/ml plasma in TTP samples (n = 50) and controls (n = 20). Presentation (n = 6) and remission (n = 11) samples are also shown.* Horizontal line indicates mean, box the 25th to 75th percentile and bars the 5th and 95th percentile. ★ p = 0.002 (presentation vs control), ▲ p = ns (remission vs control).

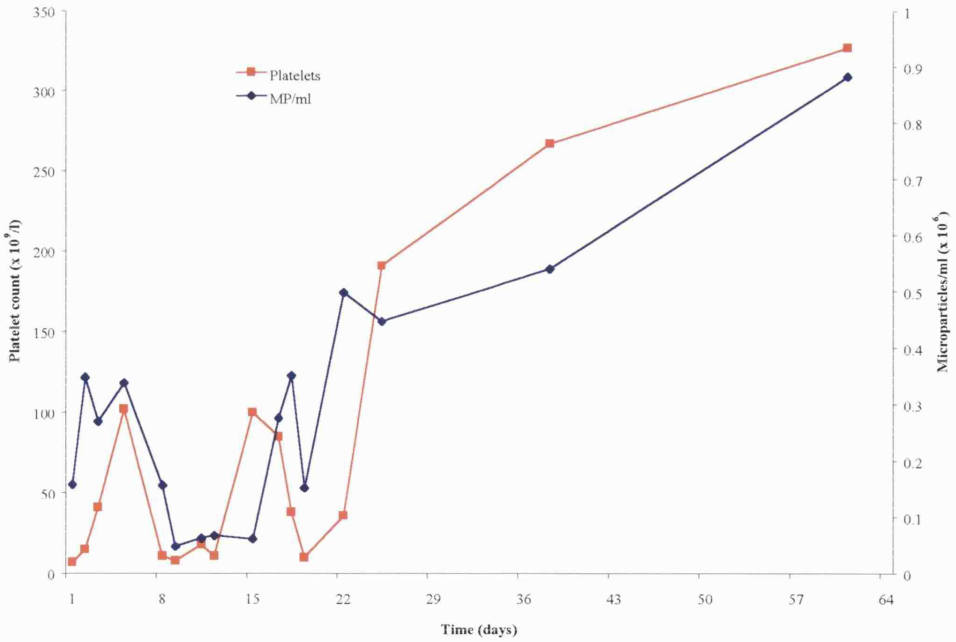


Figure 3.16 *Serial platelet count and microparticle number per ml plasma as illustrated by patient 3 who attained a durable remission on day 35 after two earlier exacerbations.*

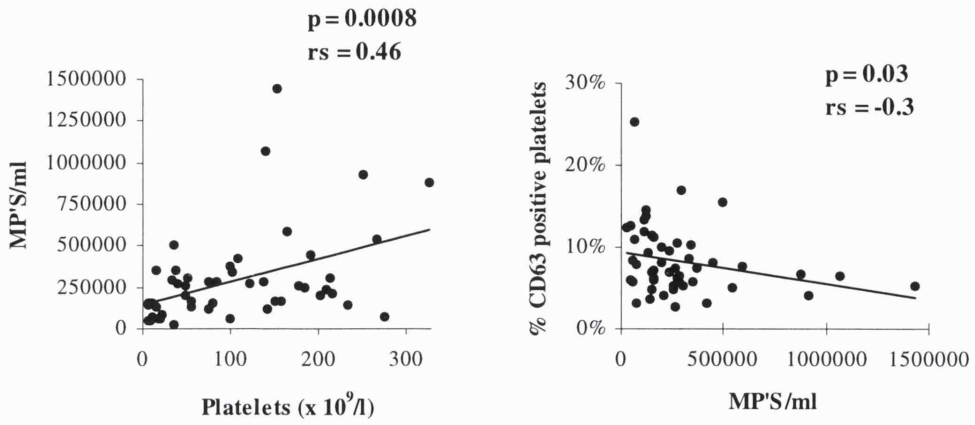


Figure 3.17 *Correlation between platelet count and percentage CD63 positive platelets and number of platelet microparticles per ml plasma.*

Platelet-leucocycyte complexes

Results for the percentage of platelet-granulocyte complexes, platelet-monocyte complexes and platelet-lymphocyte complexes are illustrated in *Figures 3.18, 3.19* and *3.20.* Overall there was a significantly higher percentage of platelet-monocyte complexes in TTP samples as compared to healthy controls (median 6.4% versus 5.0%, p = 0.01), but there was no significent increase in platelet-monocyte complexes, platelet-granulocyte complexes or platelet-lymphocyte complexes at initial presentation. There was no significant correlation with platelet-monoycte complexes and either percentage platelets positive for CD62p or absolute platelet count.

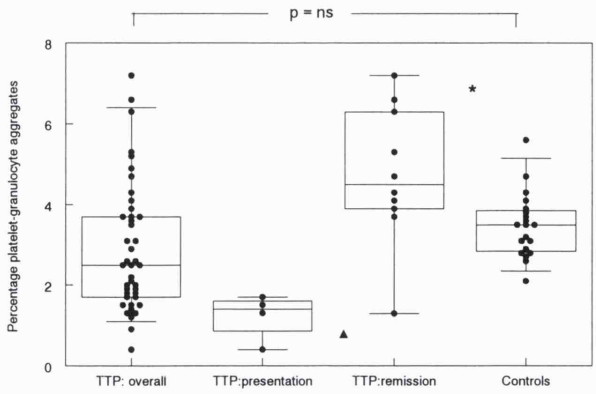


Figure 3.18 *Percentage platelet-granulocyte aggregates in TTP samples (n = 43) and controls (n = 20). Presentation (n = 4) and remission (n = 10) samples are also shown.*
Horizontal line indicates mean, box the 25th to 75th percentile and bars the 5th and 95th percentile. ▲ p = 0.009 (presentation vs control), ★ p = 0.009 (remission vs control).

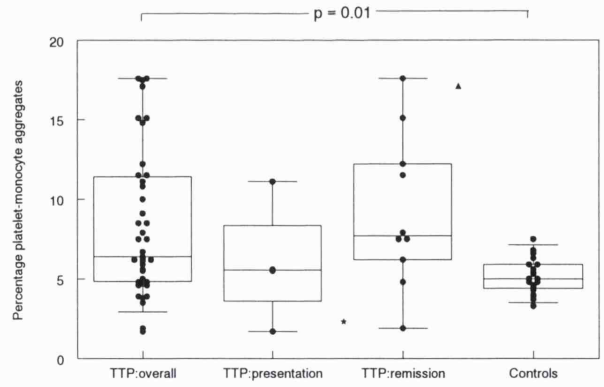


Figure 3.19 *Percentage platelet-monocyte aggregates in TTP samples (n = 43) and controls (n = 20). Presentation (n = 4) and remission (n = 10) samples are also shown.*
Horizontal line indicates mean, box the 25th to 75th percentile and bars the 5th and 95th percentile. ★ p = ns (presentation vs control), ▲ p = 0.007 (remission vs control)

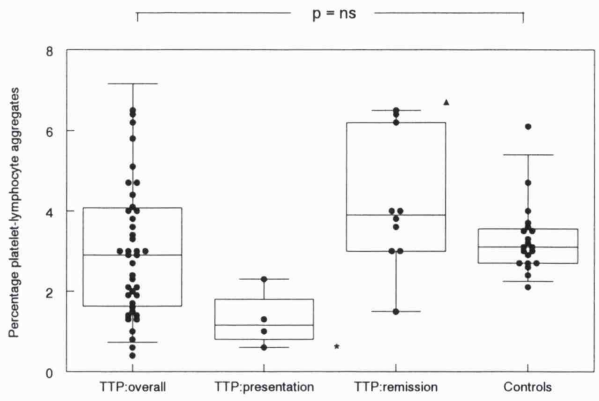


Figure 3.20 *Percentage platelet-lymphocyte aggregates in TTP samples (n = 43) and controls (n = 20). Presentation (n = 4) and remission (n = 10) samples are also shown.*
Horizontal line indicates mean, box the 25th to 75th percentile and bars the 5th and 95th percentile. ★ p = 0.008 (presentation vs control). ▲ p = ns (remission vs control)

Soluble P-selectin

Soluble P-selectin was measured in 61 of the 69 samples. The median value at presentation was 65.05ng/ml with six of the eight samples falling above the normal range (6.9 – 52.4ng/ml). Soluble P-selectin concentration was positively correlated with percentage positive CD62p platelets (rs = 0.45, p = 0.0003) and also showed a significant negative correlation with absolute platelet count (rs = -0.5, p < 0.0001) (*Figure 3.21*). There was however no correlation between P-selectin and VWF:Ag concentration. Values for soluble P-selectin normalised with attainment of remission.

Thrombin generation markers

Results for the levels of TAT and F1.2 from 59 of the TTP samples are shown in *Table 3.2*. These included five samples from presentation, all of which had elevated concentrations of TAT with a median value of 10.04μg/ml (range 7.8 – 29.5μg/ml) Four also had elevated F 1.2 levels (median value 2.7nmol/l, range 1.2-3.9nmol/l). There was a significant negative correlation between TAT levels and absolute platelet count (Spearman Rank Correlation Coefficient, rs = -0.79, p < 0.0001) whilst that

between F 1.2 and absolute platelet count was of borderline significance. Of note TAT levels and percentage CD62p positive platelets also showed a significant positive correlation (rs = 0.39, p = 0.01). There was no significant correlation between TAT levels and percentage CD63 positive platelets.

Parameter	Controls	TTP
TAT (μg/ml)	2.4 (1.1, 4.8)	5.7 (1.8,19.7)
F 1.2 (nmol/l)	0.8 (0.4, 1.6)	1.4 (0.3, 3.3)

Table 3.2 *Median values (with 5th and 95th percentiles) for TAT and F 1.2 for controls and TTP patients.*

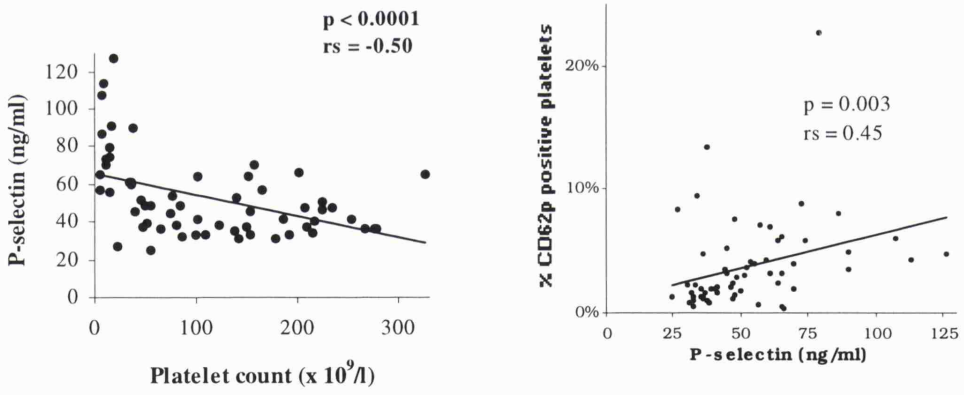


Figure 3.21 *Correlation between platelet count and percentage CD62p positive platelets and plasma P-selectin concentration.*

3.4 Discussion

The precise mechanisms responsible for microvascular thrombosis in patients with TTP remains unclear and it is perhaps unlikely that any one single mechanism is responsible. It is widely accepted that platelets form aggregates through sequential stages of adhesion, activation and aggregation. Furthermore there is evidence for activation of coagulation in acute TTP as shown by increased concentrations of TAT and plasmin-antiplasmin complexes (Takahashi *et al*, 1989;Wada *et al*, 1993). Since platelets are easily activated by small amounts of thrombin or membrane perturbations such as mechanical trauma this suggests that platelet activation would be a prominent feature of TTP. Despite this there has been limited experimental evidence for platelet activation in TTP. This may be partly explained by technical limitations owing to extreme thrombocytopenia at presentation of the condition. Some of these difficulties may be overcome by the use of flow cytometric techniques. In this study dual labelling was used to allow accurate identification of the platelet population and concomitant expression of platelet degranulation markers. At presentation of acute TTP percentage positive CD62p and CD63 platelets were elevated as compared to normal controls. However on an individual basis only three (38%) had values above the normal range (0.2 – 5.9%). In contrast in a previous report of 12 patients studied prior to institution of plasmapheresis, nine (82%) had elevated platelet surface expression of CD62p (Wright *et al*, 1999). However this is not a uniform finding. In an earlier study published by Hoffman *et al*, seven patients were studied but no significant difference in platelet surface CD62p expression was found (Hoffman *et al*, 1993). There are a number of reasons for these conflicting results. Differences in technique may be implicated. In the latter report platelet surface CD62p expression was measured in platelet rich plasma. Centrifugation may have resulted in loss of surface P-selectin. In contrast when sample manipulation was minimised through the use of whole blood assays, as in this investigation and that reported by Wright *et al*, a significant increase in circulating CD62p positive platelets was seen. However platelet surface P-selectin may not be an ideal marker for the detection of circulating degranulated platelets. Circulating degranulated platelets rapidly lose platelet surface P-selectin to the plasma

pool but continue to circulate and function (Michelson *et al*, 1996;Berger *et al*, 1998). Furthermore P-selectin expressed on activated platelets mediates their recruitment to platelet-leucocyte aggregates (Palabrica *et al*, 1992), platelet aggregates (Ahn *et al*, 1996) and thrombi. Platelets may also undergo activation locally and once activated may be preferentially removed from the circulation. The half-life of circulating activated platelets has been calculated to be one hour or less (Rinder *et al*, 1991a). The failure of elevated percentage positive CD62p platelets to predict clinical course may be a consequence of these multiply interacting factors.

Platelet count recovery was used as a surrogate marker of clinical remission. Despite resolution of clinical symptoms and normalisation of standard markers of disease activity, percentage positive CD62p platelets remained elevated as compared to normal controls. It is now recognised that clinical remission may occur in the context of persistently depressed VWF-CP activity (see chapter 4). Subclinical platelet activation could be a sequalae of this phenomenon. However there was no significant difference between values of CD62p expression in those patients in whom VWF-CP was within the normal range as compared to those in whom it remained reduced, nor was there any significant correlation between platelet CD62p expression or VWF-CP activity (data not shown). Another possible explanation is that patients continued on intermittent plasmapheresis for several days after attainment of clinical remission to reduce relapse risk. Plasmapheresis per se may result in platelet activation and could account for this phenomenon (Wun *et al*, 1994).

Since platelet surface expression of CD62p may not be an ideal marker of platelet degranulation, surface expression of CD63, a marker of lysosomal degranulation, was also measured. Similar results were obtained: a significantly higher median value for platelet surface CD63 expression was seen at presentation as compared to controls which remained elevated despite attainment of haematological remission. There was a significant negative correlation between both CD62p and CD63 expression and absolute platelet count. It is possible that the finding of apparent platelet activation in association with TTP may simply be a phenomenon of thrombocytopenia. In an

attempt to address this issue, platelet surface expression of CD62p was measured in three cases of chronic ITP with a median platelet count of 35 x 10^9/l. Values lay within the normal range in all three cases. Published literature supports this finding: in a study of ten patients with ITP with a median platelet count of 21 x 10^9/l, basal CD62p expression was not elevated (Rinder *et al*, 1998). Similarly in a further study of 27 ITP patients with a median platelet count of 56 x 10^9/l, the number of free activated platelets was not significantly increased as compared to healthy controls (Ahn *et al*, 1996). In contrast platelet expression of CD63 was increased in all three ITP samples. This is inconsistent with the CD62p findings because it is accepted that increased platelet activation is required for lysosomal as compared to α granule degranulation (Kaplan, 1994). This result may be the consequence of preferential loss of CD62p positive platelets as discussed above. However platelet-activating antibodies have also been reported in ITP (Yanabu *et al*, 1992). These are heterogeneous in nature and various target antigens have been reported. Platelet activation may occur via distinct pathways and lead to unique activation profiles. It is possible that selective CD63 expression may result as a consequence. This could also explain the lack of correlation between percentage CD62p and CD63 positive platelets seen here in TTP when both are markers of platelet activation.

Despite severe thrombocytopenia, TTP is rarely associated with haemorrhagic phenomena. This suggests that platelet function is maintained. Platelet aggregation is the standard technique for assessment of platelet function. However this technique requires platelet rich plasma and cannot be easily performed in acute TTP. TRAP and ADP induced platelet surface expression of CD62p and CD63 was therefore used as a surrogate marker of platelet function. In all instances agonist induced expression of platelet degranulation markers was significantly reduced at presentation. Previous publications have reported impaired aggregation and secretion of platelets from patients with HUS and suggested that platelets were exhausted by prior *in vivo* activation (Walters *et al*, 1988;Fong & Kaplan, 1982;Kaplan & Fong, 1980). In contrast, platelets from seven patients were activated normally by thrombin and calcium ionophore (Hoffman *et al*, 1993). In this study, despite apparent *in vitro*

evidence of impaired platelet activation by ADP and TRAP, none of the patients had clinical symptoms or signs of a bleeding diathesis. This may be attributable to the relatively high percentage of circulating reticulocytes. Overall percentage reticulated platelets were significantly increased as compared to controls (median 14.0%, range 1.1 – 45.4% versus 9.9%, range 3.3 – 18.2% $p < 0.0001$). *In vitro* studies suggest that younger platelets have superior functional capabilites compared to older circulating platelets (Johnson *et al*, 1971;Karpatkin, 1969;Shulman *et al*, 1968). Certainly using density gradients, isolation of younger platelet subsets showed greater adhesiveness to collagen and increased platelet aggregation (Karpatkin, 1969). However this technique has several limitations not least that platelet granule release results in reduced platelet density. Nevertheless biotinylation studies in animals have demonstrated decreasing platelet function as platelets age within the circulation (Peng *et al*, 1994) whilst flow cytometric techniques have confirmed the heterogeneity of platelet response to thrombin (Johnston *et al*, 1987) and TRAP (Rinder *et al*, 1998). For example in ITP all platelets (both reticulated and older platelets) have an enhanced α-granule threshold response to TRAP as compared with controls. In addition 3μM TRAP results in a two-fold increased expression of CD62p by reticulated platelets as compared to older platelets whether originally derived from ITP or control samples (Rinder *et al*, 1998). The significant positive correlation between TRAP induced percentage positive CD62p and CD63 platelets and percentage reticulated platelets seen here is consistent with these reports. However at presentation there was no significant elevation of percentage reticulated platelets as compared to healthy controls. This suggests that during acute disease haemostasis may be promoted by alternative means. P-selectin has been proposed to play an important procoagulant role and this will be discussed further below.

ADP platelet reactivity normalised once haematological remission was attained whilst TRAP reactivity remained reduced. The cause of this discrepancy is unclear but may be attributable to the fact that TRAP is a potent platelet agonist whereas ADP results in only moderate platelet activation and hence degranulation. Despite the attainment of haematological remission, platelet surface expression of the degranulation markers

CD62p and CD63 was increased as previously discussed. Reduced platelet reactivity may result from depletion of platelet intragranular reserves. It is possible that since ADP results in only partial degranulation as compared to TRAP, reduced platelet reactivity is not observed with ADP activation but is with TRAP.

In addition to surface expression of degranulation markers, platelet activation also results in the release of platelet microparticles (PMP's). Elevated circulating PMP's have previously been reported in TTP with serial assessment showing that improvement of platelet count was accompanied by a parallel decrease of circulating PMP's (Galli *et al*, 1996). Paradoxically PMP's were reduced at presentation in this study. These conflicting results may be the consequence of differences in assay techniques. The method employed here allows quantitation of the absolute number of PMP's per ml platelet poor plasma but may result in underestimation of PMP's due to loss during centrifugation. In contrast, in that reported by Galli *et al*, a whole blood technique was used and both platelets and PMP's identified using platelet specific monoclonal antibodies (CD42b or CD61a). Circulating PMP's were expressed as the percentage of the total fluorescent events. In the context of thrombocytopenia percentage PMP's may be increased despite a reduction in absolute numbers. In support of this hypothesis, platlelet microparticles (expressed as u/ml) were not elevated in three patients diagnosed with TTP when an ELISA technique was employed (Osumi *et al*, 2001). Nevertheless when washed platelets are exposed to high shear stress (108dynes/cm^2), comparable to partially occlued microvasculature, platelet microparticles are released (Miyazaki *et al*, 1996). In the same series of experiments it was shown that the number of microparticles generated was lower in whole blood suggesting that in a physiological milieu there may be significant consumption of microparticles. Activated platelet microparticles generated by stimulation with collagen may be absorbed by a 0.8μ
m filter (Osumi *et al*, 2001). Furthermore platelet microparticles can mediate leucocyte-leucocyte interactions and lead to the accumulation of leucocytes on P-selectin surfaces (Forlow *et al*, 2000). Thus the apparent reduction in platelet

microparticles during the acute phase of TTP demonstrated here may be secondary to their prefential localisation to platelet thrombi and role in leucocyte recruitment.

Circulating platelet-monocyte aggregates have been suggested to be a more sensitive marker of *in vivo* platelet surface activation than platelet surface P-selectin (Michelson *et al*, 2001). Platelet adhesion to monocytes and neutrophils is mediated by the platelet surface expression of P-selectin (Larsen *et al*, 1989;Hamburger & McEver, 1990) which binds to P-selectin glycoprotein ligand-1 on leucocytes (Moore *et al*, 1992;Sako *et al*, 1993;Moore *et al*, 1995). However there was no significant increase in platelet-monocyte aggregates or platelet-granulocyte aggregates at presentation in this study although relatively few samples (n = 5) were available for analysis. This was an unexpected finding particularly as TTP plasma has been shown to promote platelet-leucocyte aggregate formation *in vitro* (Valant *et al*, 1998). It may be explained by coexistent thrombocytopenia which effectively reduces the availability of platelets for binding: in the above investigation platelet counts were within the normal range. Alternatively this observation may be attributable to the relatively short half-lives of such aggregates: in a baboon model the *in vivo* half-life of platelet-monocyte aggregates was approximately 30 minutes whereas that of platelet-granulocytes was only five minutes (Michelson *et al*, 2001). Platelet-leucocyte interactions are also subject to complex dynamics – platelet activation *in vitro* resulted in preferential platelet-platelet adhesion with a simultaneous decrease in platelet-leucocyte aggregates that subsequently showed a time-dependent recovery following spontaneous platelet aggregate dissociation (Rinder *et al*, 1991c). Furthermore leucocyte activation can reduce P-selectin dependent binding of platelets to neutrophils (Rinder *et al*, 1994). It is also likely that endothelial perturbation and dynamic flow *in vivo* further modulate cellular interaction. These observations may in part explain why no significant positive correlation was found between percentage positive CD62p platelets and percentage platelet-monocyte aggregates. Loss of platelet surface P-selectin to the plasma pool could also contribute.

P-selectin is an integral membrane glycoprotein localised to the α-granules of platelets and Weibel-Palade bodies of endothelial cells (Berman *et al*, 1986;Wagner, 1993). Consequently an increase in plasma sP-selectin concentration may follow either platelet or endothelial activation. Elevated plasma sP-selectin concentration has been reported previously in TTP (Chong *et al*, 1994;Katayama *et al*, 1993;Fijnheer *et al*, 1997) and it has been suggested that endothelial cells are the likely source (Fijnheer *et al*, 1997). P-selectin is transiently expressed at the endothelial cell surface following fusion of Weibel-Palade bodies with the plasma membrane. Since several alternatively spliced P-selectin transcripts exist, those lacking the transmembrane domain (Johnston *et al*, 1989;Johnston *et al*, 1990) may be shed, contributing to the plasma pool (Dunlop *et al*, 1992). The endothelium could also contribute to the plasma pool by alternative pathways such as proteolysis or transcription regulation or induction of the alternatively spliced P-selectin (Johnston *et al*, 1990). Analysis of P-selectin mRNA expression *in vivo* and *in vitro* show it is transiently induced by TNF-α, endotoxin and LPS, leading to P-selectin expression 2-4 hours later (Gotsch *et al*, 1994;Bischoff & Brasel, 1995;Sanders *et al*, 1992).

In this study, although P-selectin was elevated in six of the eight patients, there was no correlation with VWF:Ag concentrations, which were also significantly increased. This is surprising if endothelium is the prime source of sP-selectin because VWF is the main constituent of Weibel-Palade bodies, but may be explained by alternative secretory pathways discussed above. However in healthy individuals, degranulation of Weibel-Palade bodies by desmopressin failed to increase plasma sP-selectin concentration (Jilma *et al*, 1996). This may be the consequence of rapid internalisation that also contributes to transient P-selectin expression on the endothelial cell surface (Subramaniam *et al*, 1993). Whilst there was no correlation with VWF:Ag concentrations, there was a significant positive correlation between plasma sP-selectin and percentage positive CD62p platelets suggesting in fact that activated platelets were the major source of P-selectin in TTP.

P-selectin has long been considered a marker of inflammation or platelet activation. However a recent study has proposed that it has pro-coagulant activity. Mice were genetically engineered to express P-selectin without the cytoplasmic tail and a constitutive increase in sP-selectin followed. In comparison to wild type and P-selectin knockout mice, there was increased fibrin deposition in an *ex vivo* perfusion chamber at the site of platelet thrombus formation and amelioration of haemorrhage produced by local Schwartzman reaction *in vivo* (André *et al*, 2000). P-selectin was elevated at presentation in five of the eight cases. The highest value was seen in one of the patients who died, the sample being taken two hours prior to death. Disseminated microthrombi were observed at post-mortem. Whether increased sP-selectin concentration is a poor prognostic marker in TTP secondary to its procoagulant activity warrants further investigation. Of note however, plasma sP-selectin concentrations were significantly positively correlated with TAT and F1.2 concentrations ($rs = 0.64$, $p < 0.0001$ and $rs = 0.53$, $p = 0.0002$ respectively).

In summary, there is evidence for platelet activation in TTP: increased percentage CD62p and CD63 positive platelets are a feature and show a significant negative correlation with disease activity (platelet count). However a generalised increase in all platelet activation parameters is not seen and probably reflects the complex dynamics of cellular recruitment to microvascular thrombi under flow conditions. Whether such platelet activation is a primary feature of TTP or secondary to endothelial perturbation is unclear. Plasma concentrations of sP-selectin correlate with percentage CD62p positive platelets suggesting platelets are the major source of circulating sP-selectin. Recent studies suggest that sP-selectin may be procoagulant. It is possible that sP-selectin concentration and hence the degree of platelet activation may be of prognostic importance in TTP and requires further study.

4. An investigation of vWF-cleaving protease activity in acute TTP

4.1 Introduction

Platelet microthrombi containing abundant VWF but little fibrin and fibrinogen are the major histological abnormality found in TTP (Asada *et al*, 1985). VWF is initially synthesised as an ultra large polymer (ULVWF) which is haemostatically more effective than the smaller plasma VWF multimers (Moake *et al*, 1986;Moake *et al*, 1994). Although ULVWF may be detected during an episode of acute TTP (Moake & McPherson, 1989;Veyradier *et al*, 2001a) it is normally absent from the circulation. Instead, circulating VWF consists of a series of multimers of molecular size 500-20,000kDa. VWF fragments of 140 and 176kDa are also a consistent finding (Zimmerman *et al*, 1986;Tsai, 1996). These are generated by cleavage of a single peptide bond, the Tyr 842-Met 843 bond (Dent *et al*, 1991). As discussed in section 1.6.5 it has long been suggested that abnormal VWF processing may be an integral component of the pathophysiology of TTP. The strong association between reduced VWF-CP activity and acute idiopathic TTP was simultaneously first reported independently by Tsai and Furlan in 1998 (Furlan *et al*, 1998b;Tsai & Lian, 1998). It also appears that VWF-CP activity may finally allow accurate differentiation of TTP from HUS. Whether TTP and HUS represent distinct pathophysiological entities or extremes of a single condition (so called TTP-HUS) remains contentious. Furlan has presented data from a total of 23 patients with a clinical diagnosis of HUS. Of these, 10 had familial and 13 sporadic disease. VWF-CP activity was normal in 21 cases and slightly decreased in the remaining two patients. This is in contrast to TTP where 26 of 30 patients had a severe deficiency (< 5% normal activity) and the remainder a moderate deficiency of protease activity (Furlan *et al*, 1998b).

The current definition of TTP includes several different clinical variants such as single episode, secondary and intermittent TTP. Whether all are characterised by reduced

VWF-CP activity remains to be documented: only six cases of secondary TTP were included in a total of 61 cases in the above preliminary reports (Furlan *et al*, 1998b;Tsai & Lian, 1998). Furthermore, over a ten year follow-up, up to 36% patients presenting with idiopathic adult onset TTP will relapse (Shumak *et al*, 1995). As yet there is no means of accurately identifying those at risk although the presence of ULVWF multimers during clinical remission has been associated with a relapsing course (Moake *et al*, 1982). This suggests that in some cases there may be a persistent abnormality of VWF processing and those thus affected are subject to future relapse. Of note, in one series of 15 patients studied serially, VWF-CP activity correlated with normalisation of platelet count in only five cases. In the remaining ten cases, recovery of VWF-CP was either achieved only after several months of clinical and haematological remission or was still persistently reduced at follow-up (Furlan *et al*, 1998b). In contrast, of the five patients studied serially by Tsai, all demonstrated parallel recovery of VWF-CP activity and platelet count with disease resolution (Tsai & Lian, 1998).

The purpose of this investigation was two-fold. Firstly VWF-CP activity was measured in adult onset idiopathic and secondary TTP in addition to other related microangiopathic syndromes such as HUS and pre-eclampsia to verify the association and specificity of protease deficiency for TTP. Secondly patients presenting with TTP were followed serially to investigate the relationship between VWF-CP activity, therapeutic intervention and clinical status.

4.2 Methods

4.2.1 Patients

A total of 66 patients with a diagnosis of microngiopathic haemolysis were studied. This included 24 patients with acute idiopathic TTP, 14 with secondary TTP, ten with HUS and six pregnancy associated cases. A further ten cases designated as "miscellaneous" were also studied. These patients presented with thrombocytopenia and haemolysis in the context of underlying sepsis or malignancy and clinically were

not considered to have TTP although it entered the differential diagnosis. Control subjects for VWF-CP activity were 25 healthy volunteers.

4.2.2 Blood collection

A vacutainer system® was used to obtain blood samples and plasma was prepared for specific assays as outlined in section 2.1.

4.2.3 Von Willebrand factor antigen

VWF antigen levels were measured as described in section 2.3.6.

4.2.4 Von Willebrand factor multimer analysis

Electrophoretic VWF multimer analysis using 0.8% agarose gels was performed using the PhastSystem as described in section 2.4.

4.2.5 Von Willebrand factor-cleaving protease activity

VWF-CP activity was assessed according to the final method described in section 2.5.

4.3 Results

4.3.1 Verification of reduced VWF-cleaving protease activity in idiopathic adult-onset acute TTP

Patient characteristics

The characteristics of 24 patients presenting with idiopathic adult onset TTP are shown in Table 4.1. The male: female ratio was 1:2 with a median age of 38 years (range 22 – 62). Six of the patients had a previous history of TTP and during the study period (March 1998 – May 2001) a further three patients relapsed following their index episode. Nine patients (38%) therefore received a diagnosis of intermittent TTP. Where appropriate, details of any subsequent relapse are included. This allowed a total of 32 episodes to be studied. A diagnosis of TTP was made in the presence of microangiopathic haemolysis and and thrombocytopenia in the absence of any other

apparent underlying cause. All patients had severe thrombocytopenia (platelet count < 50 x 10^9/l) at first presentation (range 4 – 48 x 10^9/l, median 14 x 10^9/l). A significantly higher median platelet count was seen in those presenting in relapse (range 3 – 101 x 10^9/l, median 27 x 10^9/l, $p < 0.0001$). Neurological dysfunction and fever were present in 60% and 44% respectively. The latter is an under-estimate since temperature was not documented in several instances or could not be ascertained from referral centres. Significant renal impairment (definition creatinine > 177μmol/l = 2mg/ml) was never documented.

Twenty-five healthy volunteers were used as control subjects for the protease studies. There were 11 non-pregnant females and 14 males with an age range of 22 to 51 years.

Patient	Sex	Age	Platelet (x 10^9/l)	Creatinine (μmol/l)	CNS dysfunction	Fever	Past history of TTP
1	F	38	19	61	+	-	-
2	M	50	8	150	+	+	-
			82		-	-	
3	M	47	7	88	+	+	+
			12	98	-	-	
4	F	37	19	83	+	-	-
5	F	26	28	83	+	Not documented	-
6	F	26	16	119	+	+	-
			92		-	-	
7	F	40	9	67	+	-	+
8	M	35	14	84	+	+	-
			101	72	-	-	
			32	86	-	-	
9	M	37	7	102	+	+	+
10	F	24	7	88	+	+	-
11	F	54	14	127	+	-	-
12	F	54	4	113	+	+	-
13	F	24	9	69	+	+	-
14	F	45	17	71	-	+	+
			8	110	-	Not documented	
			3	72	-	+	
15	F	37	27	86	+	Not documented	+
16	F	29	48	73	-	?	-
17	F	51	13	83	+	?	-
18	M	38	40	126	-	?	+
19	F	22	11	74	+	+	-
20	M	49	9	135	+	+	-
			47	106	-	+	
21	F	32	43	69	-	-	-
22	F	59	36	86	+	?	-
23	M	62	41	107	+	+	-
24	M	47	54	93	-	-	+

Table 4.1 *Clinical and laboratory features of 24 patients with idiopathic TTP.* F = female; M = male.

VWF-CP activity

A total of 32 samples from 24 patients with acute idiopathic TTP were assayed for VWF-CP activity. Results are shown in *Figure 4.1* and expressed as a ratio compared to PNP. A negative value is consistent with the presence of ULVWF. VWF-CP activity was significantly reduced in acute idiopathic TTP as compared to normal controls (median 0.0, range –0.29 – 0.79 versus 1.0, range 0.69 – 1.17, $p < 0.0001$).

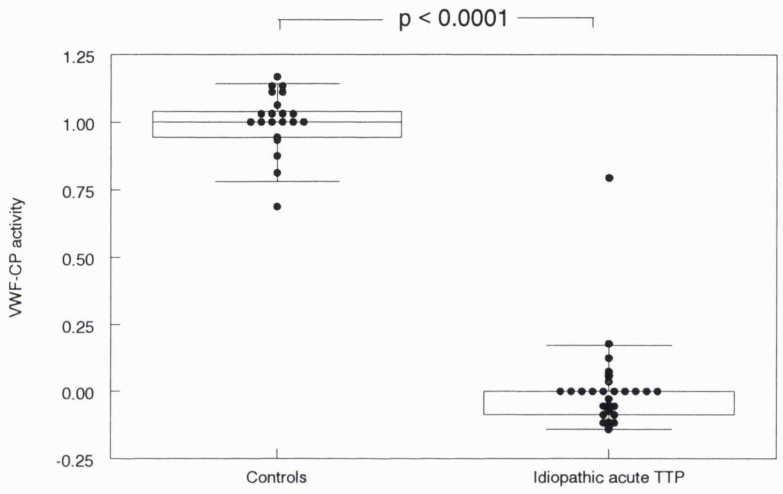


Figure 4.1 *Plasma levels of VWF-CP activity in controls (n=25) and acute idiopathic TTP at presentation (n=32)*
Horizontal line indicates median, box the 25th to 75th percentile and bars the 5th and 95th percentile.

There was one distinct outlier in this group of patients clinically diagnosed with idiopathic acute TTP. The patient (number 23), a 62 year old man, presented with a three day history of confusion, lethargy and dizziness. On examination he was febrile and jaundiced. Investigation revealed micrangiopathic haemolytic anaemia (Hb 5.1g/dl) and thrombocytopenia (platelet count 41 x 10^9/l) in the presence of normal renal function (creatinine 107μmol/l). Four components of the classical TTP pentad were therefore present. However there were also some atypical features. Routine coagulation profiles are usually normal in TTP (Sagripanti *et al*, 1996;Monteagudo *et al*, 1991;Rock *et al*, 1998) although slight increases in D-dimer, fibrin degradation

products and thrombin-antithrombin complex (TAT) may be seen (Wada *et al*, 1998;Sagripanti *et al*, 1996;Monteagudo *et al*, 1991). However in this case there was a prolonged prothrombin time (PT 14.4 seconds, normal range 10 – 13 seconds) and a grossly reduced Clauss fibrinogen (fibrinogen <0.5g/l) in addition to mild elevation of D-dimers (D-dimer 4-8mg/l). VWF multimeric profile was normal. Deranged liver function tests: hyperbilirubinaemia and a raised transaminase level, are also common in TTP. Here the major abnormality of liver function tests was a raised alkaline phosphatase (412 U/l, range 45-122) whilst 4cm firm hepatomegaly was noted on clinical examination. An underlying neoplasm was therefore considered. The patient failed to respond to plasma exchange and suffered a cardiopulmonary arrest after two single volume procedures had been completed. A post mortem was declined and thus an underlying malignancy could not be excluded.

VWF multimer profile

ULVWF multimers were identified in 22 episodes (69%) at presentation. Of the remaining episodes, loss of normal high molecular weight multimers and a normal multimeric profile were seen in one and nine samples respectively. As compared to those samples in which a normal VWF multimeric profile was preserved, an abnormal VWF multimeric distribution was associated with a significantly lower VWF-CP activity ratio (–0.05, range –0.29 – 0.07 versus 0, range –0.05 – 0.18) ($p = 0.02$) (see *Figure 4.2*). Patient 23 was excluded from this analysis.

VWF:Ag

The median value for VWF:Ag at presentation of acute idiopathic TTP was 1.79 IU/ml (range 0.59 – 5.74 IU/ml). There was no significant negative correlation between VWF-CP activity at presentation and VWF:Ag concentration (see *Figure 4.3*).

Platelet count

The median value for platelet count at presentation was 17 x 10^9/l. A platelet count of less than 20 x 10^9/l has previously been associated with a worse prognosis (Rock *et al*,

1998). However there was no correlation between VWF-CP activity and platelet count at presentation nor any significant difference in protease activity between those patients presenting with a platelet count less than or greater than 20 x 10^9/l.

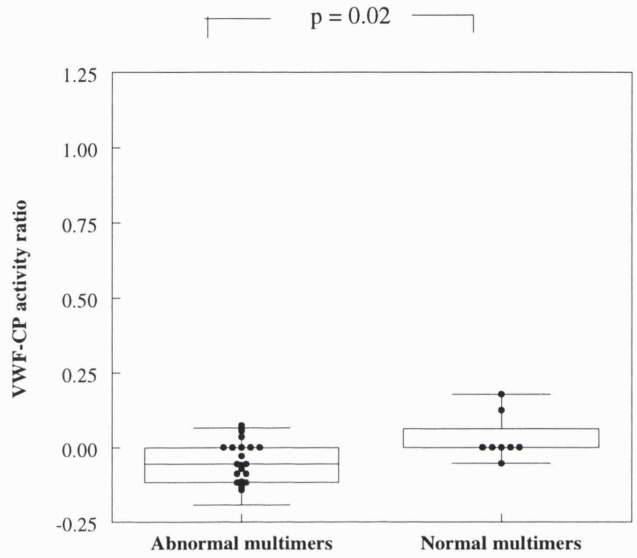


Figure 4.2 *Plasma levels of VWF-CP activity in acute TTP presenting with abnormal (n=23) or normal multimeric profile (n=9)*
Horizontal line indicates median, box the 25th to 75th percentile and bars the 5th and 95th percentile.

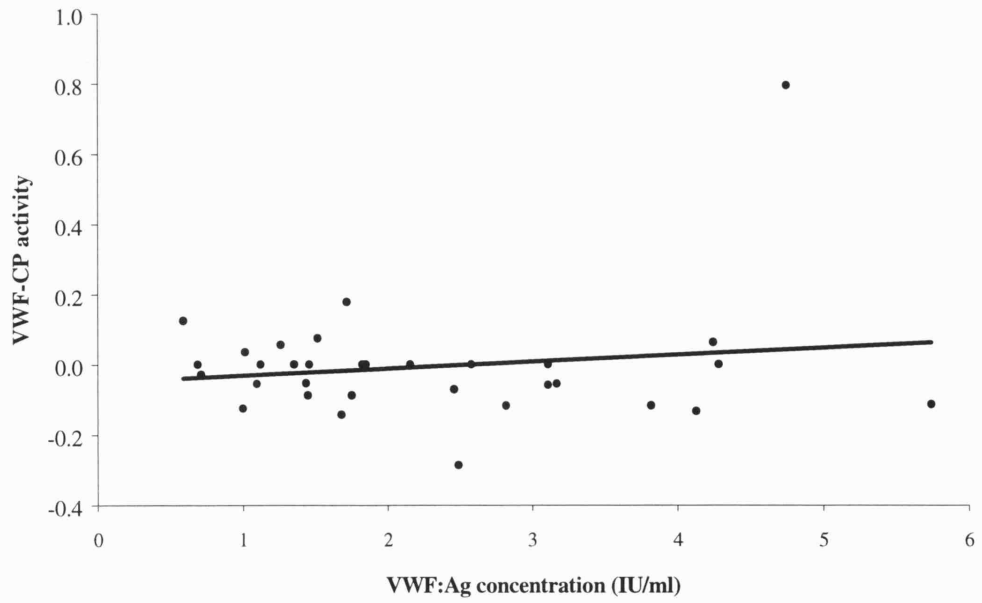


Figure 4.3 *Correlation between VWF-CP activity and VWF:Ag concentration in patients presenting with acute TTP*

4.3.2 VWF-cleaving protease activity in secondary TTP

Patient characteristics

The characteristics of the 14 patients presenting with secondary TTP are shown in *Table 4.2*. Nine had received an autologous or allogeneic bone marrow transplant (BMT) procedure, three were HIV positive, one had been diagnosed with SLE 13 years previously but still had active disease and the other had recently received mitomycin C. All the HIV positive cases were characterised by marked immunosuppression (CD4 count less than 200 x 10^9/l). Although compared to those patients presenting with idiopathic TTP there was a greater range of platelet counts (range 1 – 71 x 10^9/l, median 22.5 x 10^9/l), this did not reach statistical significance. Significant renal impairment (creatinine > 177μmol/l = 2mg/ml) was present in 43%. Thus the clinical picture more closely resembled HUS than TTP.

VWF-CP activity

Secondary TTP was characterised by greater heterogeneity in VWF-CP activity than acute idiopathic TTP (*Figure 4.4*). VWF-CP activity was significantly reduced in the post -BMT setting as compared to normal controls (median VWF activity ratio 0.91, range 0.59 – 1 versus median 1.0, range 0.69 – 1.17, p = 0.003) but did not differ significantly from HUS. Only three HIV positive cases were available for study. Of these, one had typical features of TTP presenting with thrombocytopenia and MAHA in the context of fluctuating neurology. VWF-CP activity was unrecordable in this patient. The second had typical TTP features but had received prednisolone 60mg daily prior to transfer. The platelet count had risen from 5 x 10^9/l at presentation to 53 x 10^9/l during this time period. The failure to detect severe protease deficiency may therefore have been a consequence of partial treatment. In contrast the remaining patient had significant renal impairment, absent neurology and only moderate protease deficiency in the absence of any prior treatment specific for TTP. Similarly a moderate reduction in protease activity were seen in the one of the two remaining cases (SLE associated) whilst normal protease activity was maintained in the single mitomycin C associated case studied.

Patient	Sex	Age	Predisposing factor	Platelet count (x 10^9/l)	Creatinine (μmol/l)	Bilirubin (μmol/l)	LDH (U/l)	CyA level (μg/l)	Tacrolimus level (μg/l)
1	M	19	Post BMT	58	324	19	333	858	-
2	M	55	Post BMT	6	222	45	2179	860	-
3	M	18	Post BMT	1	154	33	1141	-	15.9
4	M	44	Post BMT	13	185	35	4296	231	-
5	M	41	Post BMT	9	146	177	860	-	>30
6	F	32	Post BMT	27	88	77	1633	451	-
7	F	30	Post BMT	9	169	109	-	141	-
8	M	16	Post BMT	18	110	41	1443	-	-
9	F	50	Post BMT	50	308	99	984	151	
10	F	32	HIV	6	189	13	3569	-	-
11	F	37	HIV	53	80	7	1234	-	-
12	F	41	HIV	71	426	5	1631	-	-
13	F	44	SLE	63	62	17	2864		-
14	F	67	Mitomycin	41	106	27	1020	-	-

Table 4.2 *Clinical and laboratory characteristics of 14 patients with secondary TTP.*
F = female; M = male.

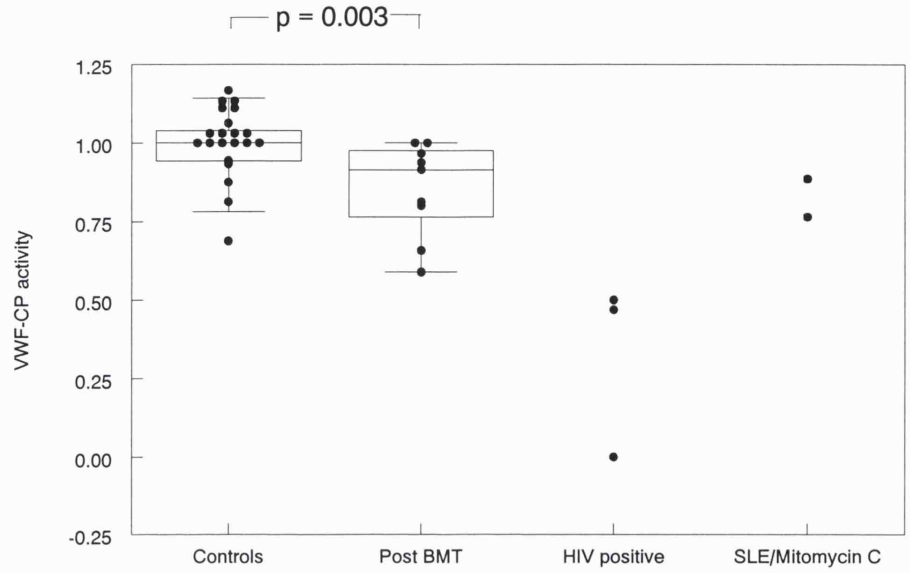


Figure 4.4 *Plasma levels of VWF-CP activity in controls (n=25), post BMT associated TTP (n= 9), HIV associated TTP (n=3) and miscellaneous secondary TTP (n=2)*
Horizontal line indicates median, box the 25th to 75th percentile and bars the 5th and 95th percentile.

4.3.3 VWF-cleaving protease activity in HUS

The characteristics of ten patients with HUS are presented in *Table 4.3*. There were six females and four males, whose ages ranged from six months to 65 years (median 21 years). Patients number 4 and 7 had documented *E coli 0157* infection and a previously affected sibling respectively. The median platelet count at presentation was 59 x 10^9/l (range 14 – 200 x 10^9/l) as compared to a median value of 14 x 10^9/l (range 3 – 101 x 10^9/l) at first presentation of idiopathic TTP cases (p = 0.004). Of note, patient 2 presented with sudden onset of nephrotic syndrome and on biopsy had thrombotic microangiopathy that was thought consistent with HUS despite the absence of thrombocytopenia. All patients had features of renal impairment as shown by raised creatinine or proteinuria. Median creatinine at the time of study was 334μmol/l (range 87 – 1800μmol/l). Fever and neurological symptoms were seen in only 10% and 20% respectively.

Patient	Sex	Age	Associated factors	Platelet count (x 10^9/l)	Creatinine (µmol/l)	CNS dysfunction	Fever
1	F	0.5		142	**87** (58 upper)	-	-
2	F	59	APS	200	157	-	-
3	F	12		31	534	-	-
4	F	1	*E coli* 0157	67	283	-	-
5	F	25		14	275	-	-
6	M	19		17	217	-	-
7	M	23	Family history	71	1800	-	-
8	M	43		16	499	+	+
9	M	9		125	1087	+	-
10	F	65		51	385	-	-

Table 4.3 *Clinical and laboratory characteristics of ten HUS patients.*
F = female; M = male; APS = antiphospholipid syndrome.

VWF-CP activity

VWF-CP activity in acute HUS was significantly greater than that in idiopathic TTP (median value 0.88, range 0.0 – 1.0 versus 0.0, range -0.29 – 0.79, $p < 0.0001$) whilst it was also significantly reduced with respect to normal controls (median value 0.88 range 0.0 – 1.0 versus 1.0, range 0.69 – 1.17, $p = 0.002$). However VWF-CP activity of only three patients fell outside the normal range. Two of these appeared to be outliers with a VWF-CP activity consistent with those results obtained in idiopathic TTP. However the outliers, patients 4 and 5, both had classical features of HUS with renal impairment and no neurological features. Moreover, patient 4, a female infant, presented with renal failure following a diarrhoeal prodrome and subsequently was confirmed to be positive for *E coli* 0157 serotype. This finding could not be attributable to age since patient 1, aged only six months, had protease activity within the normal range. Furthermore, convalescent samples were subsequently assayed and in both cases there was complete resolution of protease deficiency.

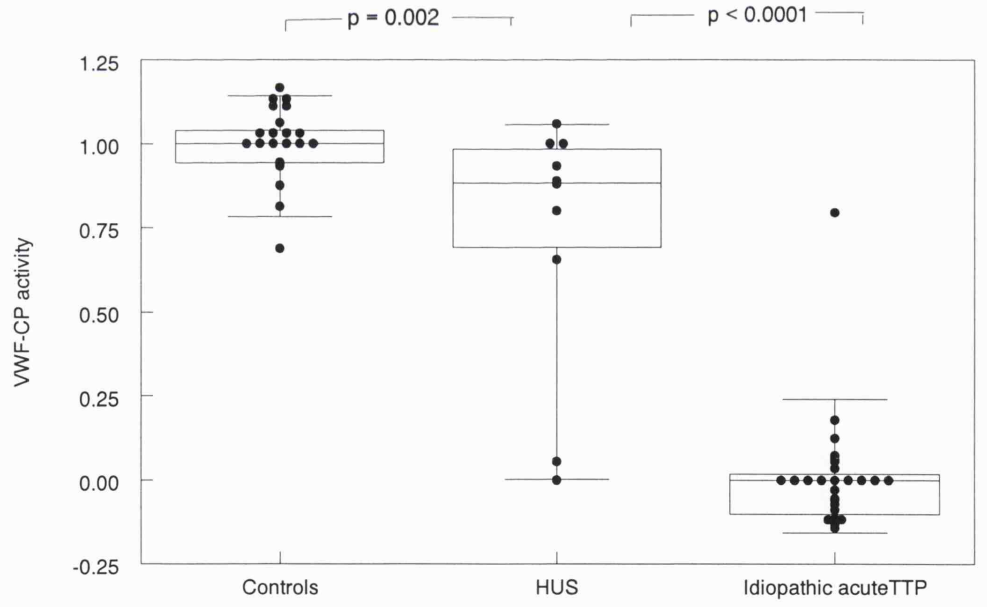


Figure 4.5 *Plasma levels of VWF-CP activity in controls (n=25), HUS (n = 10) and idiopathic TTP (n= 32).*
Horizontal line indicates median, box the 25th to 75th percentile and bars the 5th and 95th percentile.

4.3.4 VWF-cleaving protease activity in pregnancy associated thrombotic microangiopathy

Patient characteristics

The characteristics of six patients presenting with pregnancy associated thrombotic microangiopathy are presented in *Table 4.4*. In this small series four presented peripartum and two in the second trimester. Patients 1 and 2 fulfilled criteria for the HELLP syndrome whilst patient 6 had a clinical diagnosis of DIC. Patients 4 and 5 were thought to have TTP and were plasma exchanged. Patient 3 was a diagnostic conundrum. She had previously presented with gestational thrombocytopenia and had developed a mild HELLP picture peri-partum. In this pregnancy red cell fragmentation and thrombocytopenia developed at 25 weeks and severe fetal ascites was documented simultaneously. The pregnancy terminated one week later with a still birth in the absence of neurological symptoms, significant hypertension, proteinuria or abnormal liver function tests.

VWF-CP activity

Accurate diagnosis of thrombotic microangiopathy in pregnancy is complicated by the plethora of conditions resulting in similar clinical presentations. Here two patients had a clinical diagnosis of TTP, one of whom had a previous history of TTP. Both had severely reduced VWF-CP activity consistent with their diagnosis. The remaining four patients with diagnoses ranging from DIC to HELLP had VWF-CP activity within or just below the normal range (median 0.87, range 0.75 – 1.1) which was not significantly reduced as compared to normal controls (*Figure 4.6*).

Patient	Age	Presentation	Previous history	New onset hyper-tension	Platelet count (x 10^9/l)	Creatinine (μmol/l)	Clotting profile PT 10 – 13 s APTT 22 – 36 s TT 12 – 15 s Fibrinogen 1.5-4g/l	LDH (211-423) (U/l)	ALT (7-49) (U/l)	Diagnosis
1	27	24 hours post partum	-	-	28	172	PT 11.2 APTT 36 TT 14	1916	264	HELLP
2	23	35 weeks gestation	-	+	26	67	PT 10.1 APTT 38 TT 14 Fibrinogen 3.27	2101	464	HELLP
3	46	26 weeks gestation	Gestational thrombo-cytopenia; mild HELLP	-	25	66	PT 9.9 APTT 31.1 TT 14.2 Fibrinogen 2.15	493	107	-
4	21	Primip 22 weeks gestation	-	+	7	74	INR 0.98 APTTR 0.61 Fibrinogen 1.69	3203	14	TTP
5	37	38 weeks gestation	TTP age 24 (non-gestational)	-	17	99	PT 10.7 APTT 24.8 Fibrinogen 2.67	880	14	TTP
6	19	Post partum	-	+	136	287	PT 18.9 APTT 53 TT 21 Fibrinogen 0.69	-	88	DIC secondary to sepsis

Table 4.4 *Clinical and laboratory characteristics of six patients with gestational thrombotic microangiopathic haemolysis*

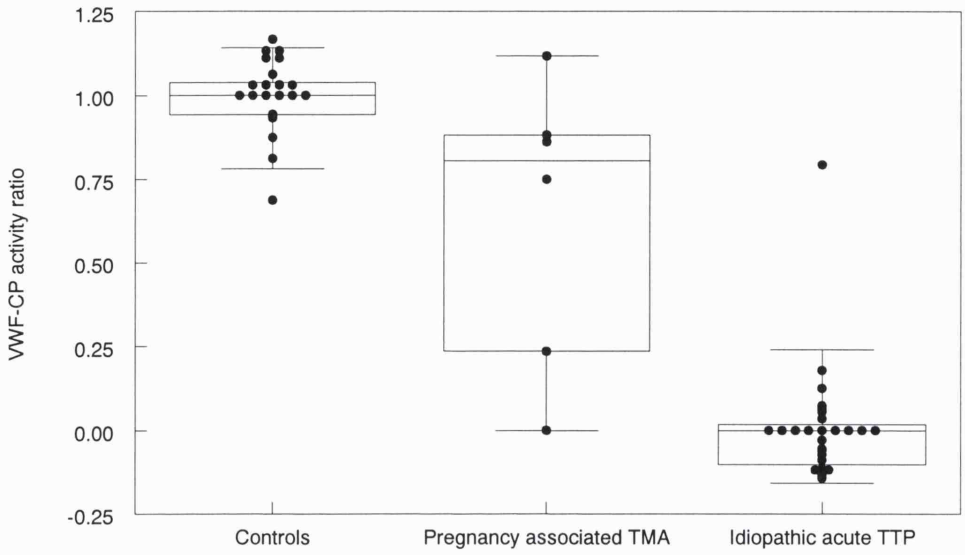


Figure 4.6 *Plasma levels of VWF-CP activity in controls (n=25), pregnancy associated TMA (n=6) and idiopathic TTP (n= 32).*
Horizontal line indicates median, box the 25th to 75th percentile and bars the 5th and 95th percentile.

4.3.5 VWF-cleaving protease activity in atypical haemolytic/thrombocytopenic syndromes

Patient characteristics

Patient characteristics of ten patients presenting with atypical haemolytic/thrombocytopenic syndromes are shown in *Table 4.5*. There were two males and eight females with an age range of 10 to 80 years (median 64.5 years). In the majority of these cases the patients had either underlying sepsis or malignancy (suspected or confirmed). Thrombocytopenia was variable with a median platelet count of 60 x 10^9/l (range 5 – 148 x 10^9/l). Median creatinine was 70μmol/l (range 34 – 202μmol/l) with significant renal impairment documented in only one case. The classical TTP pentad was never present. Instead the majority of patients were incidentally found to have microangiopathic changes and thrombocytopenia on blood film examination during routine investigation.

Patient	Sex	Age	Associated medical conditions	Hb (g/dl)	Platelet count (x 10^9/l)	Creatinine (µmol/l)	Bilirubin (µmol/l)	LDH (IU/l)
1	F	56	Probable malignancy (CA-125: 245)	8.8	89	87	19	-
2	F	24	-		120	60	-	-
3	F	26	Extensive tuberculosis	9.1	25	76	14	742
4	M	10	Recurrent thrombocytopenia ?cause	13.1	40	44	3	639
5	F	71	Sepsis, low grade non Hodgkins lymphoma	9.8	5	34	-	-
6	M	80	-	8.6	148	98	94	888
7	F	81	Sepsis secondary to psoas abscess	8.1	19	202	75	-
8	F	63	Breast cancer	5.8	57	84	45	2651
9	F	66	-	10.9	118	51	10	309
10	F	67	Metastatic breast cancer	11.3	63	64	26	783

Table 4.5 *Clinical and laboratory characteristics of ten patients with miscellaneous haemolytic/thrombocytopenic conditions.*
F = female; M = male.

VWF-CP activity

VWF-CP activity was significantly reduced in miscellaneous microangiopathic states as compared to normal controls (median 0.76, range 0.63 – 1.07 versus 1.0, range 0.69 – 1.13, p = 0.01). It was also significantly higher as compared to idiopathic TTP (p < 0.0001). However there was no significant difference as compared to those patients presenting with HUS.

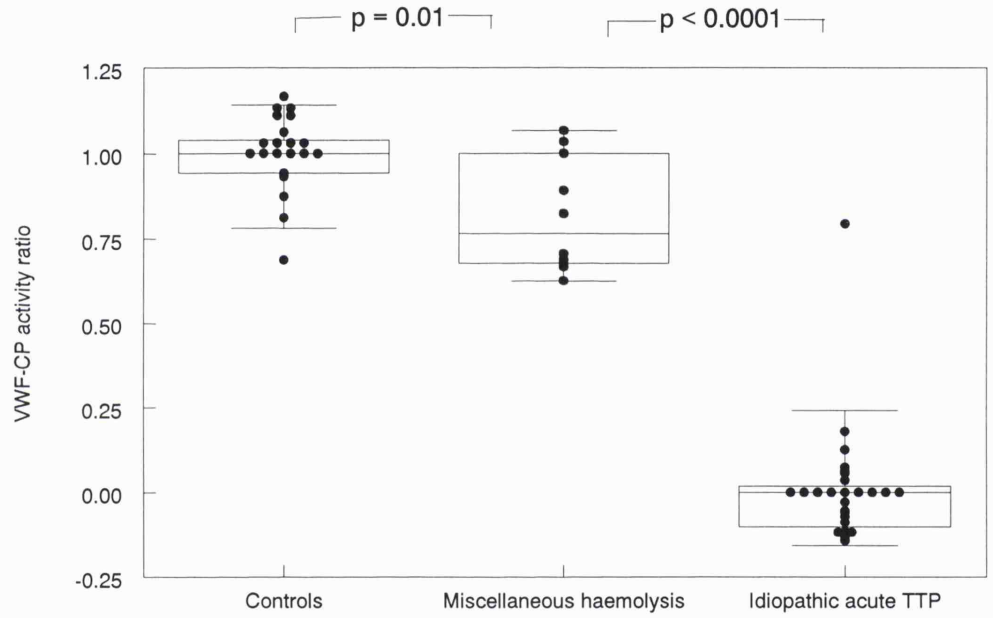


Figure 4.7 *Plasma levels of VWF-CP activity in controls (n=25), miscellaneous haemolytic conditions (n=10) and idiopathic acute TTP (n= 32).*
Horizontal line indicates median, box the 25th to 75th percentile and bars the 5th and 95th percentile.

A moderate reduction in VWF-CP activity was seen in HUS, pregnancy associated microangiopathy (non TTP), post BMT associated TTP and miscellaneous microangiopathic haemolytic conditions as compared to normal controls. Neither abnormal renal function nor increased VWF:Ag levels appeared to account for this difference since there was no significant correlation between VWF-CP activity and plasma creatinine or VWF:Ag concentration (see *Figure 4.8*).

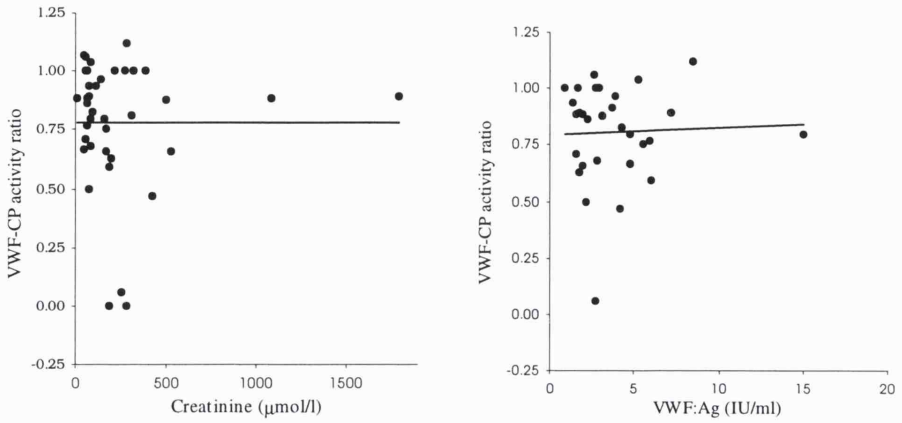


Figure 4.8 *Correlation between VWF-CP activity and creatinine and VWF:Ag concentration*

4.3.6 Correlation between VWF-cleaving protease activity and recovery from acute idiopathic TTP

A total of 405 samples from 16 patients (patient numbers 1-3, 5-14, 16,17 and 19, Table 4.1) were studed serially to assess the relationship between disease and protease activity. Platelet count was used as a surrogate marker of disease activity. In seven cases VWF-CP activity returned to the normal range within seven days of attaining clinical remission (defined as normal neurological status, platelet count and LDH with a rising haemoglobin) whilst in the remaining six patients who were followed to remission, VWF-CP remained persistently depressed. Two contrasting examples (patients 7 and 14) are shown in *Figures 4.9* and *4.10* respectively. Once clinical remission was attained, VWF-CP activity was subject to fluctuation with time: both recovery and loss of activity were seen in the absence of any clinical symptoms or significant therapeutic intervention (see *Figures 4.11* and *4.12*).

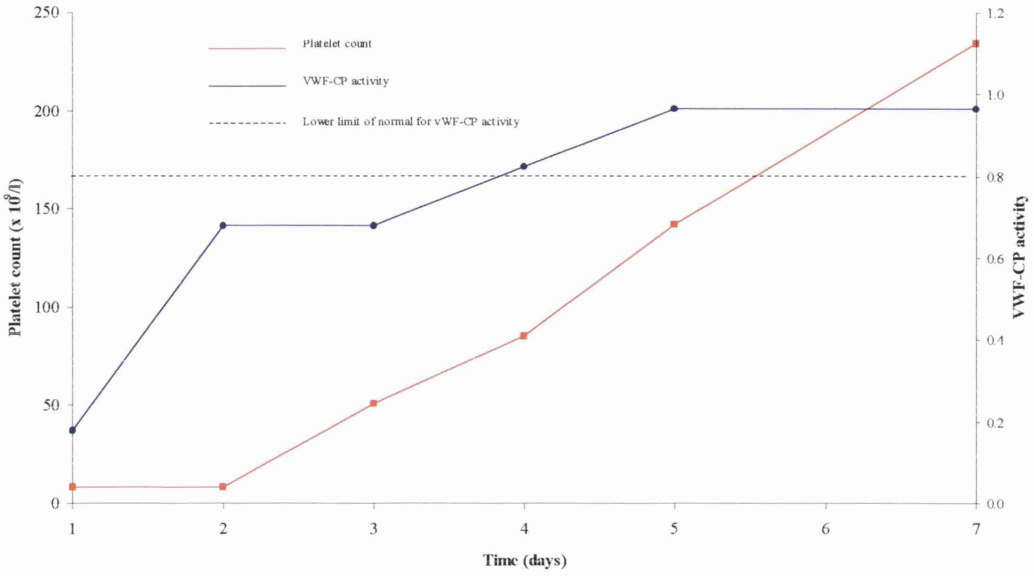


Figure 4.9 *Patient 7 presented at day 1 and received daily plasma exchange (PEX). Clinical remission (CR) was attained at day 6. There is a clear correlation between VWF-CP and progressive normalisation of platelet count. The patient remains in remission at 31 months follow-up.*

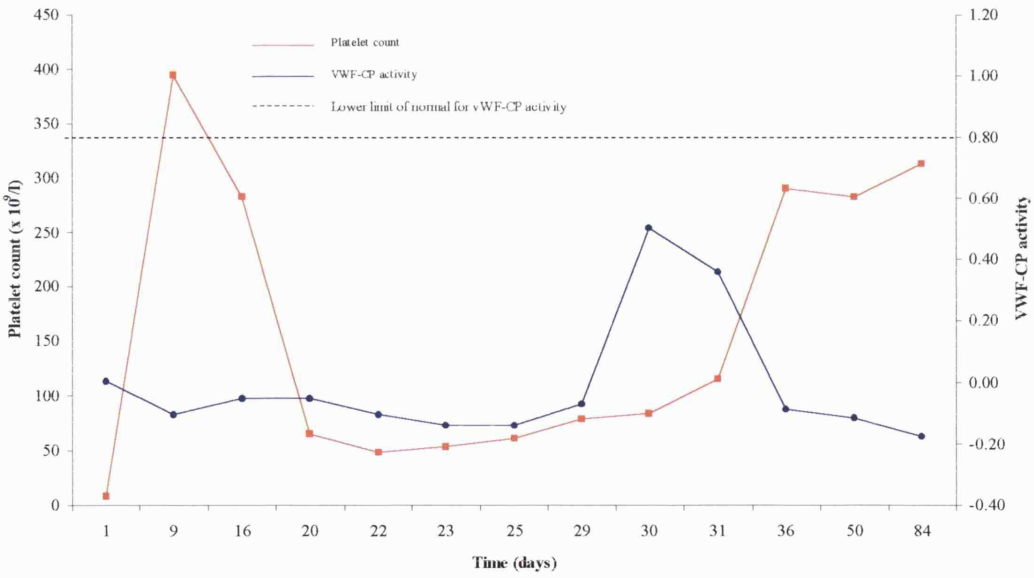


Figure 4.10 *Patient 14 presented at day 1 and received daily PEX. CR was attained on day 6 despite persistently reduced protease activity. Exacerbation quickly followed and PEX was recommenced. Durable remission was attained on day 36 although there was only transient and partial recovery of protease activity. A further relapse occurred at 12 months.*

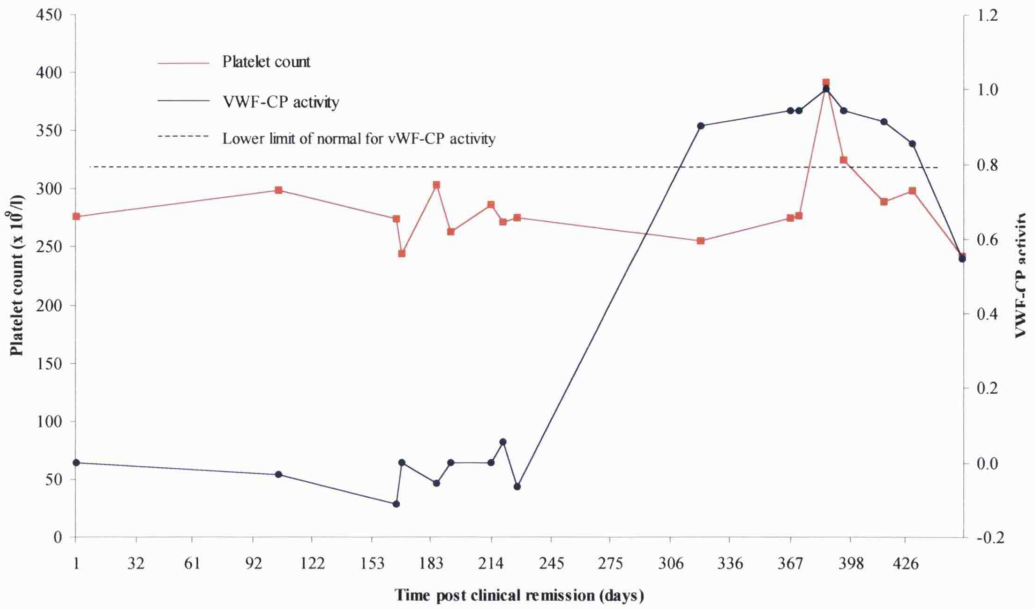


Figure 4.11 *Patient 6 entered clinical remission on day 1 (day 38 post index presentation) despite persistent severe protease deficiency. Apparent spontaneous recovery of protease activity occurred between days 265 and 359. The patient was on maintenance prophylaxis due to concomitant pregnancy and received a single plasma exchange and four plasma infusions during this time interval. A total of 30 exchange procedures had been required to attain remission.*

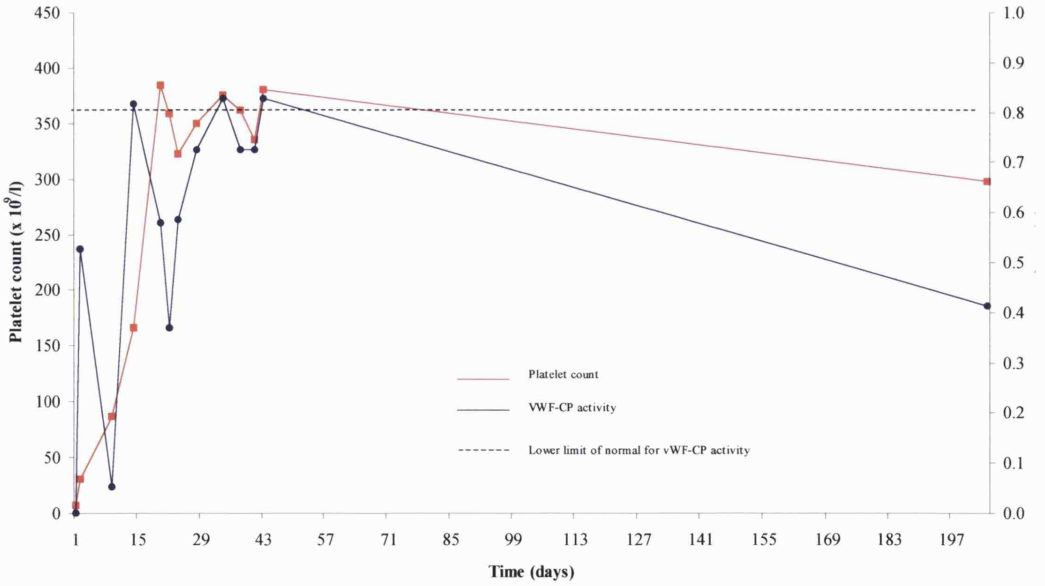


Figure 4.12 *Patient 3 entered CR on day 23 after an early exacerbation. There was a parallel recovery in VWF-CP activity and platelet count. However protease activity was found to be significantly depressed at routine follow-up. Relapse occurred 13 months later.*

Linear regression suggested that overall there was a statistically significant positive weak correlation between platelet count and VWF-CP activity ($p < 0.0001$, $rs = 0.2949$) (see *Figure 4.13*). However it is also possible that this data represents two subgroups: one of which is characterised by platelet count recovery in association with resolution of protease deficiency and the other, typified by persistent protease deficiency despite apparent clinical remission.

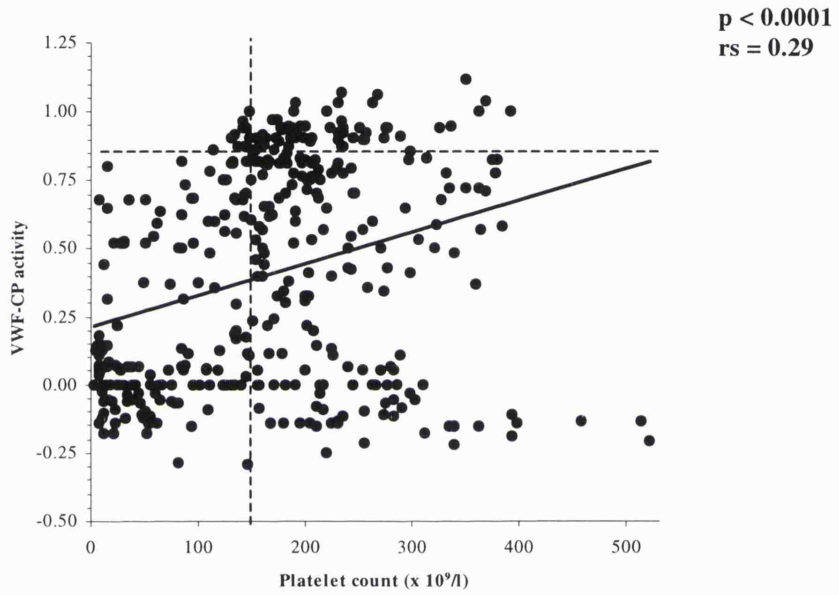


Figure 4.13 *Correlation between VWF-CP activity and platelet count in acute idiopathic TTP.*
The dotted lines represent lower limit of normal for platelet count and VWF-CP activity

There was a significant association between a reduced protease activity (defined as a VWF-CP activity ratio of less than 0.4, equivalent to a protease activity 5% normal) and the presence of ULVWF multimers ($p = 0.0011$, Chi squared test).

	VWF-CP activity ≤ 0.4	VWF-CP activity > 0.4	Total
ULVWF multimers	156	43	199
Normal multimeric profile	44	157	201
Total	200	200	400

Table 4.6 *Association between VWF-CP activity and the presence of ULVWF multimers*

Since the serial study had indicated that clinical remission may be acheived and/or maintained despite abnormal levels of protease activity, VWF-CP activity was assessed in patients with a previous history of TTP who remained under routine haematological follow-up. A total of 142 samples from 26 patients were analysed. Patient details are given in *Table 4.7*. Samples were taken after a median remission period of four months (range one week to 228 months). Remission was defined as before (normal neurological status, platelet count and LDH with a rising haemoglobin).

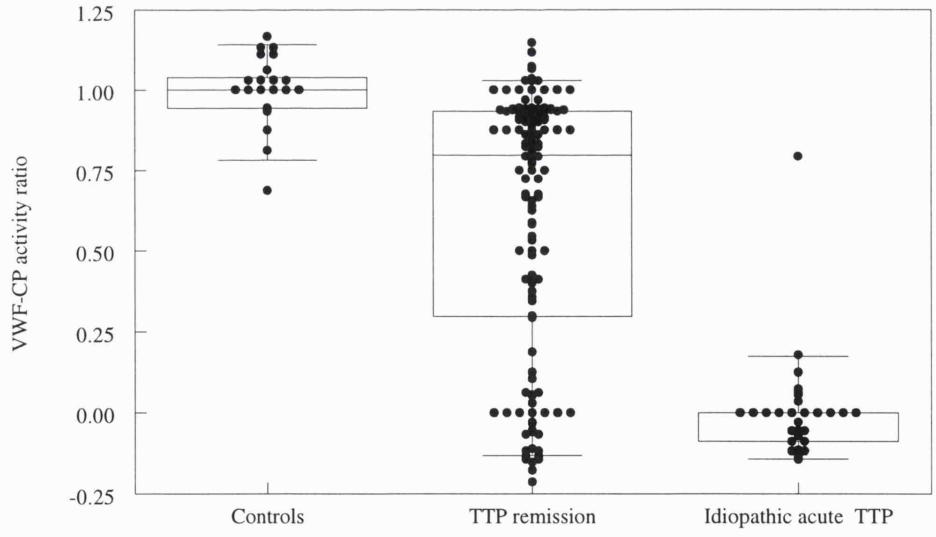


Figure 4.14 *Plasma levels of VWF-CP activity in controls (n=25), patients with previous history of TTP in remission (n=142) and acute idiopathic TTP (n=32)*
Horizontal line indicates median, box the 25th to 75th percentile and bars the 5th and 95th percentile.

Patient	Sex	Age (at initial presentation)	History of intermittent TTP	Duration of clinical remission (months)	Maintenance treatment		
					ASA	CyA	Other
1	F	38	-	0.25	+	-	-
2	M	50	+	38	+	+	-
3	M	47	+	8	+	-	-
6	F	26	+	24.5	+	-	-
8	M	35	+	25	+	+	-
9	M	37	+	9	+	+	-
10	F	24	-	12	+	-	-
11	F	54	+	0.25	+	-	-
12	F	54	-	6	+	+	-
13	F	24	-	7	+	+	-
14	F	45	+	11	+	+	-
21	F	32	-	0.25	+	-	-
25	M	49	+	36	+	-	-
26	M	32	-	3	+	-	-
27	F	28	-	32	+	-	-
28	F	51	+	6	+	-	-
29	F	24	+	27	+	-	-
30	F	26	-	96	+	-	-
31	F	59	+	0.75	+	-	AZA
32	M	44	+	32	+	-	Cyclo
33	F	44	-	228	-	-	-
34	F	30	-	12	+	-	-
35	F	27	+	2	+	-	-
36	F	31	-	4	-	-	-
37	F	31	+	13	-	-	-

Table 4.7 *Clinical features of 26 patients with a previous history of TTP tested for VWF-CP activity whilst in remission.In 13 patients serial samples were available. In such cases the duration of clinical remission refers to the longest time interval between resolution of symptomatic disease and sampling for protease activity during clinical remission.*

F = female; M = male; ASA = aspirin; CyA = cyclosporin; AZA = azaathioprine; Cyclo = cyclophosphamide

During clinical remission from TTP, VWF-CP activity was significantly increased as compared to patients at acute presentation ($p < 0.0001$) whilst remaining significantly reduced as compared to normal controls ($p < 0.0001$). However there was a wide range in activity with overlap into both groups. Since VWF-CP activity had been demonstrated to vary with time, VWF-CP activity of 16 of these patients (patients 2, 3, 6, 8 - 10, 12 - 14, 25, 26, 28, 29, 33, 34 and 36) was studied six to nine months after attainment of remission from 17 episodes of TTP.

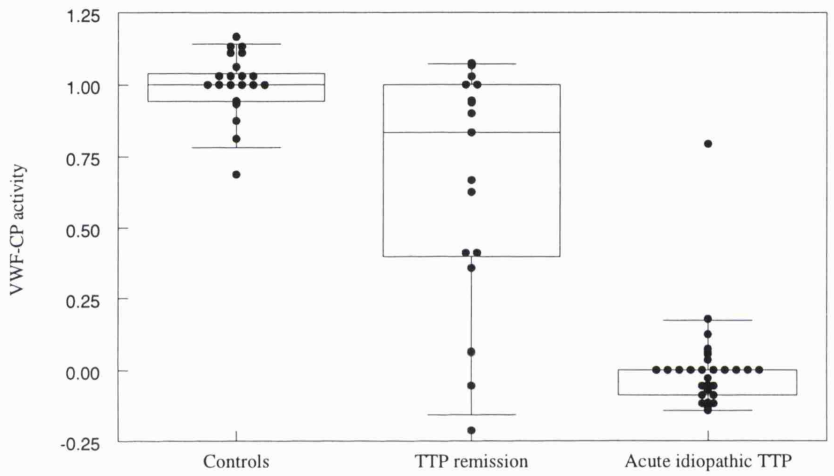


Figure 4.15 *Plasma levels of VWF-CP activity in controls (n=25), patients with previous history of TTP after 6-9 months remission (n=17) and acute idiopathic TTP (n=32)*
Horizontal line indicates median, box the 25th to 75th percentile and bars the 5th and 95th percentile.

Even after a durable clinical remission of at least six months, there was a wide range in protease activity amongst those patients with a history of TTP that differed significantly from both normal controls ($p = 0.001$) and cases of acute TTP at presentation ($p < 0.0001$). Four patients had a protease activity ratio less than 0.4 and ULVWF multimers were detected in three of these four patients. The heterogeneous nature of TTP was further demonstrated below. Paired samples from presentation and durable remission (six to nine months after attaining CR) were available in ten of these

patients and were assayed for VWF-CP activity (*Figure 4.16*). VWF-CP activity returned to the normal range in only four of these ten patients.

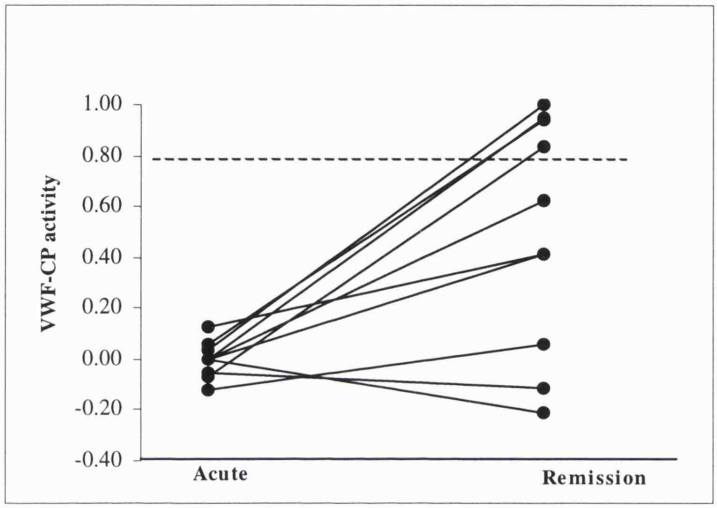


Figure 4.16 *VWF-CP activity in paired samples from presentation and durable remission of acute TTP.*
Dashed line represents the lower limit of normal for VWF-CP activity.

Although there was an overall statistically significant correlation between VWF-CP activity and platelet count, there was clearly a subpopulation of samples in which protease activity remained severely depressed despite normalisation of platelet count. The possibility that the apparent correlation could be the consequence of two distinct subgroups representing intermittent and single episode TTP was assessed in these seventeen episodes of TTP. There was no significant difference in protease activity at six to nine months after attainment of clinical remission between these two groups of patients (see *Figure 4.17*).

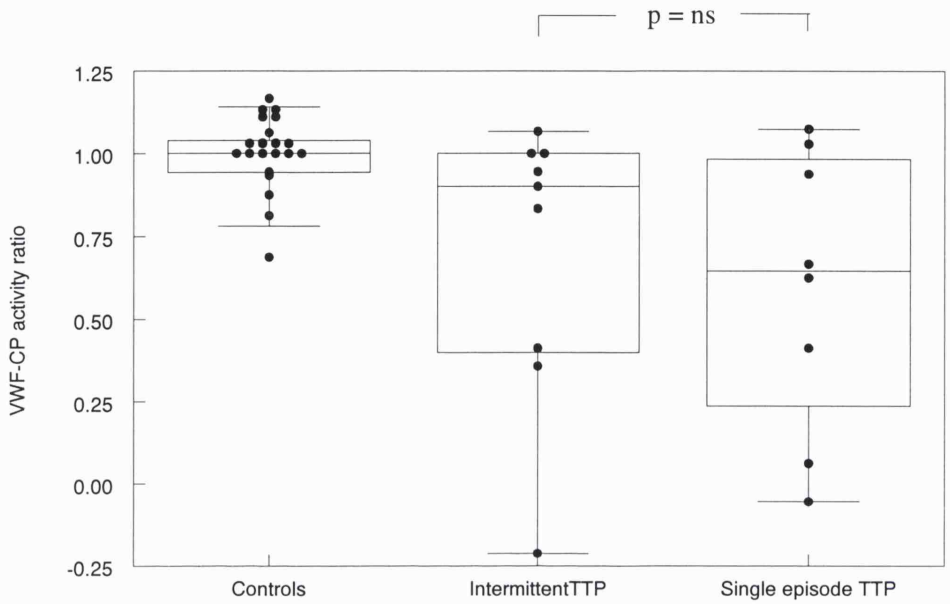


Figure 4.17 *Plasma levels of VWF-CP activity in controls (n=25) and patients with a history of intermittent TTP (n=9) or single episode TTP (n=8) after 6-9 months remission.*
Horizontal line indicates median, box the 25th to 75th percentile and bars the 5th and 95th percentile.

Similarly, after an additional median follow-up of 14 months, neither persistent depression of VWF-CP activity (defined as VWF-CP activity less than 0.4) or the presence of ULVWF multimers predicted subsequent relapse (*Tables 4.8* and *4.9*). Patients with apparently normal protease activity also relapsed. In fact, four of the five relapses occurred in patients with a protease activity ratio greater than 0.4. In only one case was protease activity moderately reduced at 0.41 whilst the three remaining cases had an activity ratio within the normal range (activity ratio 1 – 1.1 in all three cases). The former relapse may be explained by the presence of ULVWF multimers that were detected. In contrast, multimeric profile was normal in the other cases.

	VWF-CP activity ratio less than 0.4	VWF-CP activity ratio greater than 0.4	Total
Relapse	1	4	5
Continued remission	3	9	12
Total	4	13	17

Table 4.8 *Association between VWF-CP activity in remission and subsequent relapse*

	ULVWF multimers	Normal multimeric profile	Total
Relapse	2	3	5
Continued remission	2	10	12
Total	4	13	17

Table 4.9 *Association between VWF multimer distribution in remission and subsequent relapse*

4.3.7 Influence of cyclosporin on VWF-cleaving protease activity

Although cyclosporin is associated with an increased risk of post bone marrow transplant microangiopathy there are reports of its successful application to the treatment of refractory (Hand *et al*, 1998), severe intermittent (Pasquale *et al*, 1998) and post autologous BMT TTP (Van Ojik *et al*, 1997). This is consistent with the current auto-immune model of TTP and cyclosporin's immunosuppressive action. The optimal therapeutic range for cyclosporin is unknown although trough serum levels of 200 – 300μg/l have been used successfully in the above studies. Six of the patients previously described, who were followed to durable remission, were treated with cyclosporin. Indications for treatment were recurrent relapse (n = 2), fulminant

progressive disease despite plasma exchange and standard adjuvant therapy (n = 1) and inability to withdraw plasmapheresis (n = 3). Patients 8 and 14 had suffered a total of ten episodes of TTP and had had persistently reduced protease activity documented during the nine and eighteen months preceding commencement of cyclosporin. Plasma exchange could not be withdrawn from patients 2, 12 or 13 without precipitating disease exacerbation despite adjuvant therapy with steroids, vincristine and defibrotide. Fulminant progressive disease with development of seizures and profound reduction of level of consciousness (Glasgow coma scale = 8) prompted early initiation of cyclosporin in patient 9 who had had a single previous episode requiring in excess of 30 plasma exchange procedures before remission was attained. Low intensity immunosuppression with cyclosporin was commenced in all cases aiming for a trough serum concentration of 100 – 200μg/l.

VWF-CP activity

Cyclosporin therapy was initiated in patients 8 and 14 for recurrent disease. Both had persistently reduced VWF-CP activity during periods of clinical remission despite normalisation of platelet count. After three months of therapy, VWF-CP activity had returned to the normal range in patient 8 and there have been no further episodes over the past 22 months (see *Figure 4.18*). Follow-up in patient 14 is much shorter: only 4 months have elapsed since cyclosporin was commenced. However *in vitro* results show for the first time protease levels above 0.4 in the absence of intensive plasmapheresis (see *Figure 4.19*).

Three patients were commenced on cyclosporin due to an inability to withdraw plasmapheresis without precipitating disease exacerbation. Within five weeks of iniatiating cyclosporin plasma exchange was being successfully tapered in all three patients with attainment of remission after a median of 8 days therapy (range 5 - 27). Patients 12 and 13 both remain in remission six months later with protease levels within the normal range. Patient 2 remains in remission after 37 months follow-up and is now receiving aspirin alone. Cyclosporin was gradually tapered after 28 months of maintenance therapy and was completely stopped five months later. Whilst protease

levels had been stable during maintenance there has been a progressive fall in activity since the initiation of cyclosporin withdrawal. VWF-CP activity and cyclosporin levels throughout this time period are shown in *Figure 4.20*.

Cyclosporin was commenced acutely in one patient due to progressive fulminant neurological disease. Following initiation of cyclosporin, a durable remission was achieved after 30 plasma exchange procedures. Maintenance therapy was continued for 18 months before a gradual reduction in dosage was started. One month after complete cessation of therapy there was a further relapse which responded rapidly to plasma exchange (total of five procedures) and reintroduction of cyclosporin.

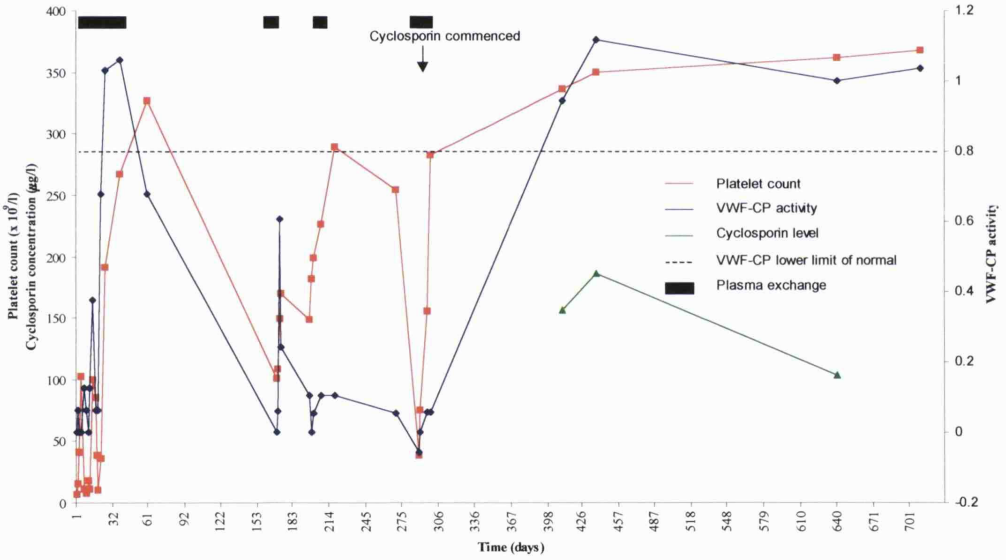


Figure 4.18 *Patient 8 was commenced on cyclosporin in September 1999 due to recurrent relapse. Prior to the initiation of therapy VWF-CP activity was not maintained in the absence of exchange procedures. Three months later protease activity was within the normal range. This was sustained at fourteen months follow-up and there have been no further relapses.*

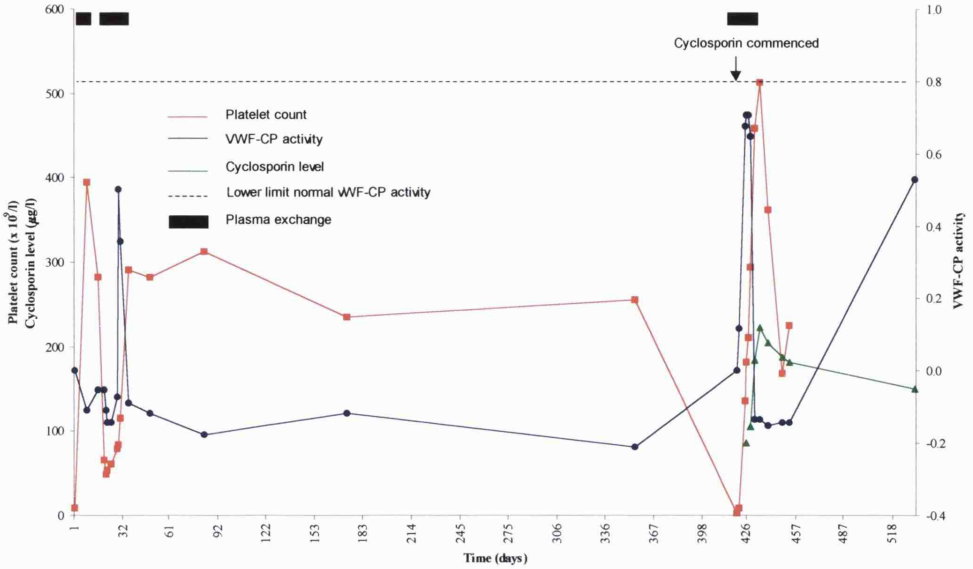


Figure 4.19 *Patient 14 was commenced on cyclosporin during a seventh episode of TTP. Persistently reduced protease activity had been documented during a preceding prolonged period of remission. After three months of therapy, VWF-CP activity had risen above 0.4 for the first time in the absence of plasmapheresis.*

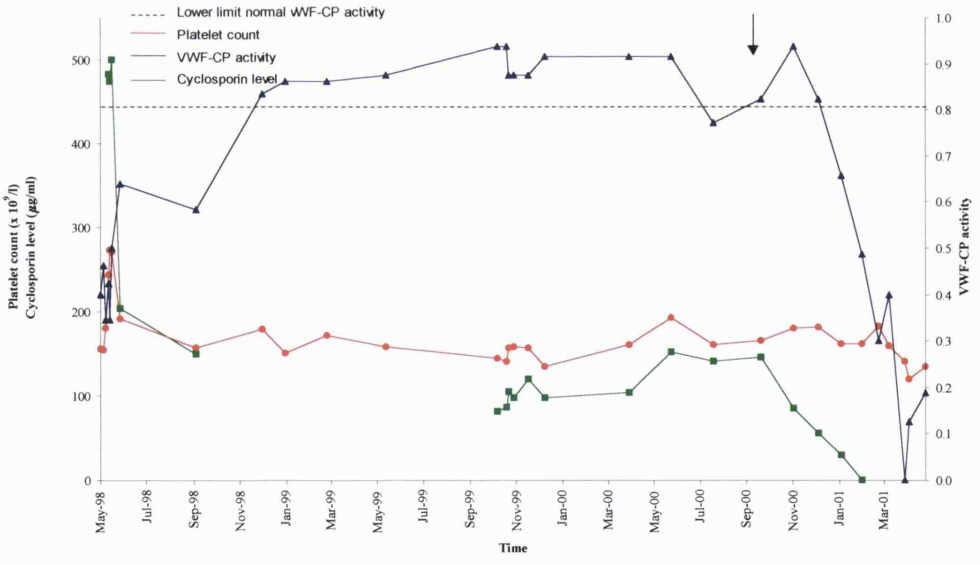


Figure 4.20 *Patient 2 was commenced on cyclosporin due to recurrent exacerbations in May 1998. Plasma exchange was successfully discontinued x days later. Thereafter VWF-CP remained withing the normal range until cyclosporin was gradually reduced (arrow). There was a subsequent progressive fall in protease activity although the platelet count has remained stable.*

4.4 Discussion

It has recently been proposed that deficiency of a novel VWF-CP activity is critical to the pathogenesis of TTP (Furlan *et al*, 1998b). In this investigation, 32 episodes are described in 24 patients diagnosed with idiopathic TTP. Thirty one were characterised by a severe deficiency of protease activity (median 0.0, range –0.29 – 0.17). The remaining case had a protease activity just below the lower limit of normal at 0.79 (normal range 0.8 – 1.25). There was no significant correlation between the severity of protease deficiency and degree of thrombocytopenia in this study. This is perhaps surprising if protease deficiency is central to the pathological process but might reflect assay limitations. The assay is only semi-quantitative since it measures multimeric degradation which is used as a surrogate marker for cleavage of the Tyr 842-Met 843 bond. Multiple bonds must be cleaved before loss of firstly high and subsequently intermediate molecular weight multimers can be identified by gel electrophoresis. This is reflected in the relatively high CV of the assay (intra-assay CV 9.2%, inter-assay CV 9.8%). The levels of protease activity associated with acute TTP are also at the lower limit of sensitivity of the assay. In contrast, using an immunoradiometric technique, VWF-CP deficiency was associated with the severity of the thrombocytopenia ($p < 0.01$) (Veyradier *et al*, 2001a). Increased VWF:Ag concentrations have also been reported as a confounding factor (Mannucci *et al*, 2001) but this was not confirmed here.

Two groups independently reported severely reduced VWF-CP activity in association with acute TTP in 1998. In one series of 39 cases, 38 had no detectable VWF-CP activity whilst one patient had a level 3% that of the control value (Tsai & Lian, 1998). Likewise, Furlan reported that in a series of 24 cases, 20 had severe deficiency of VWF-CP activity whilst the remaining four had moderate deficiency (defined as less than 5% and 5 – 25% of the activity of pooled normal plasma respectively) (Furlan *et al*, 1998b). TTP is presently a clinical diagnosis and the possibility of misdiagnosis must therefore be considered for patient number 23. Although four components of the classical TTP pentad were present in this case, all of these features

may be seen in other conditions such as DIC. An underlying malignancy was suspected clinically but could not be confirmed as a post mortem was not performed.

Malignancy has itself been associated with reduced VWF-CP activity. Reduced protease levels were associated with the presence of ULVWF multimers and metastatic disease in 20 patients. However none of these patients developed clinical features suggestive of TTP (Oleksowicz *et al*, 1999). One possible explanation for this observation is that although VWF-CP activity was reduced as compared to normal controls, it remained significantly higher than those values associated with acute TTP. Moderate decreases in protease activity have now also been reported in the context of acute inflammatory states such as DIC, decompensated liver disease, chronic renal insufficiency, SLE and leukaemia (Moore *et al*, 2001;Loof *et al*, 2001;Moore *et al*, 2001;Mannucci *et al*, 2001) but the majority of these conditions are not associated with TTP. A similar phenomenon was demonstrated here in ten miscellaneous microangiopathic/thrombocytopenic states (see below). Release of VWF is one component of the acute inflammatory response. It is conceivable that this results in secondary consumption of VWF-CP, accounting for the mild reductions in protease activity seen in sepsis and malignancy. When an excess of VWF was present *in vitro*, protease was completely bound to VWF as demonstrated by coelution of protease from a Sephacryl-500 column (Fujikawa *et al*, 2001). Alternatively the protease may bind to some other acute inflammatory plasma protein(s). Complex formation would explain a number of observations: the long biologic plasma half-life (Furlan *et al*, 1999), unusually high molecular mass (200 – 300kDa) of protease in earlier reports on partial VWF-CP purification (Furlan *et al*, 1996;Tsai, 1996) and almost simultaneous elution of protease with several protein molecules of different sizes on gel filtration (Gerritsen *et al*, 2001). However the identification of a carrier protein remains elusive: both α_2-macroglobulin and clusterin have been excluded (Gerritsen *et al*, 2001).

Protease activity has also been demonstrated to fall slightly with age (Mannucci *et al*, 2001). In the ten patients with miscellaneous microangiopathic states studied here, five were aged over 65 years. The median protease activity in this older group of patients

was 0.69 (range 0.625 – 1). In those aged less than 65 years it was 0.89 (range 0.68 – 1.07). The moderate reduction in protease activity seen in malignancy or sepsis may therefore be the composite effect of an acute inflammatory reaction and advancing age. Any one of these factors could have contributed to the marginal reduction in VWF-CP activity seen in patient 23. This would suggest that the patient was erroneously diagnosed with TTP on the basis of clinical features. The current definition of TTP, a microangiopathic haemolytic anaemic and thrombocytopenia in the absence of any other underlying cause, is likely to result in over-diagnosis and represent a number of heterogeneous conditions. This might explain the failure of some cases to respond to plasmapheresis as illustrated by patient 23. Alternatively however, reduced VWF-CP activity might not be a prerequisite for TTP. In support of this, one group has recently reported that of 66 patients presenting with TTP, seven (10%) had normal VWF-CP activity. It should, however, be noted that this phenomenon was only seen in secondary TTP and never in idiopathic TTP (Veyradier *et al*, 2001a). Both of these concepts will be discussed below.

Adult-onset TTP may be defined as secondary or idiopathic depending on the presence or absence of recognised associated factors. These include drugs, auto-immune disease, pregnancy and bone marrow transplantation. There are few reports of VWF-CP activity in cases of secondary TTP. Of the 61 patients first described, precipitating factors were recognised in only 7 (11%) (Furlan *et al*, 1998b;Tsai & Lian, 1998). Two of these were HIV positive, one had SLE, one had undergone allogeneic transplantation for acute leukamia and three had their first episode during pregnancy. All had severe protease deficiency. Forty one patients with secondary TTP have recently been included in a prospective national study. Of these, thirty four had protease activity below the lower limit of normal but only twenty two were reduced to values associated with idiopathic TTP (Veyradier *et al*, 2001a). Fourteen patients with secondary TTP and six cases of gestational thrombotic microangiopathy are presented here. Three patients had HIV infection and all had CD4 counts below 250 x 10^9/l, a recognised risk factor (de Man *et al*, 1997). Of the HIV positive cases, one had severely reduced protease activity. Moderate reduction of VWF-CP activity was seen

in the two remaining patients. This may reflect prior steroid treatment in one case. However in the second, protease activity was reduced in the absence of any antecedent therapy. It is possible that several diverse pathophysiological conditions might give rise to a microangiopathic haemolytic anaemia in the context of active HIV infection, only one of which is classical TTP. This is similar to the concept that HIV associated thrombocytopenia has multiple aetiologies.

Nine post BMT TTP cases were studied. The median age at presentation was 32 years (range 16 – 55 years), comparable to that recently reported by a single centre (median age 29 years, range 4 – 49 years) (Fuge *et al*, 2001). However in contrast to this patient series there was no female preponderance (male: female ratio 2:1). Female sex has previously been reported as a risk factor for the development of BMT-associated TTP (Iacopino *et al*, 1999). Other clinical risk factors found in some (but not all) studies include acute graft versus host disease (GvHD) (Holler *et al*, 1989) and cyclosporin prophylaxis for GvHD (Iacopino *et al*, 1999). Here eight of the nine patients were receiving either cyclosporin or tacrolimus which has also been associated with TTP (Gharpure *et al*, 1995;Holman *et al*, 1993). Although a trend to higher cyclosporin levels has been associated with severe microangiopathy (Holler *et al*, 1989), toxic plasma concentrations were only seen in three cases. VWF-CP activity was only moderately reduced in BMT-associated TTP as compared to normal controls (median 0.91, range 0.59 – 1 versus median 1.0, range 0.69 – 1.17, $p = 0.003$) but significantly greater than acute idiopathic TTP ($p < 0.0001$). These results are in accordance with published literature: a single previous series of eight patients with BMT-associated TTP demonstrated increased mean breakdown of VWF (0.91 ± 0.092) as compared to the classic TTP group (0.086 ± 0.094, $n = 5$) ($p = 0.0016$) (van der Plas *et al*, 1999). This observation is consistent with the hypothesis that these two conditions may have distinct underlying pathologies and is supported by the lack of efficacy of plasmapheresis in the treatment of BMT-associated as opposed to classical TTP (Sarode *et al*, 1995). In fact BMT-associated TTP has a dismal prognosis with reported mortality rates of up to 86% (Fuge *et al*, 2001). However, like HIV, microangiopathic states post BMT may be heterogeneous: one patient reported by

Furlan developed TTP following an allogeneic transplant but was found to have severely depressed VWF-CP activity (Furlan *et al*, 1998b).

Thus, if not all, at least the majority of BMT-associated TTP cases appear to have a distinct underlying pathophysiology as compared to idiopathic TTP. Deficiency of VWF-CP activity does not appear to be of major importance. Since cyclosporin and GvHD are apparent risk factors, it has been suggested that endothelial activation or damage may play a role. Cyclosporin toxicity is associated with elevated VWF:Ag concentrations *in vivo* (Brown *et al*, 1986) whilst *in vitro* suppression of endothelial prostacyclin generation (Voss *et al*, 1988) and induction of tissue factor expression is seen (Carlsen *et al*, 1988). Elevated levels of VWF:Ag and thrombomodulin, markers of endothelial cell damage, are prevalent in BMT-associated TTP (Zeigler *et al*, 1996). Moreover, cessation of cyclosporin therapy can result in disease amelioration. In contrast, in autografted patients, as illustrated here by patient 8, TTP develops in the absence of cyclosporin therapy and paradoxically cyclosporin may be an effective treatment for TTP in such cases (Van Ojik *et al*, 1997;van der Plas *et al*, 1999). A possible mechanism of action is the stimulation by cyclosporin of endothelial cell nitric oxide production (Stroes *et al*, 1997). As a consequence, inhibition of platelet activation and aggregation ensues. Immune modulation is an alternative proposal.

TTP may be seen in association with autoimmune processes such as scleroderma, rheumatoid arthritis, polyarteritis nodosa, Sjögren's syndrome and systemic lupus erythematosus (SLE). The association between TTP and SLE has been well recognised but the diagnosis may be challenging since both share similar clinical and histological features. One autopsy study described a clinical syndrome consistent with TTP among 14 of 50 cases of SLE with systemic small vessel thrombi noted in 50% patients (Devinsky *et al*, 1988). An antemortem diagnosis of TTP was made in only one of these 14 patients. Conversely an earlier study described histological changes consistent with SLE among 34 of 151 (23%) autopsy specimens of patients with TTP (Levine & Shearn, 1964). This frequency has not been confirmed clinically (Bray *et al*, 1994) and only one such patient was available for study here. The patient, a 44 year

old woman, had been diagnosed with SLE 13 years earlier and presented with a florid vasculitic rash, thrombocytopenia and microangiopathic haemolysis. Antiphospholipid antibodies were present but were only minimally elevated (ACL IgG 5.7 GPL units, normal range < 5 GPL units, $a\beta_2$ GPI IgG 3.6%, normal range < 3.5%). Neurological features were absent. Plasmapheresis was instituted with normalisation of clinical and laboratory parameters after eight procedures. However retrospective analysis of VWF-CP activity at presentation showed this to be minimally reduced at 0.77. This was in the presence of a grossly elevated VWF:Ag concentration of 5.9 IU/ml. Whether this is a consistent observation in microangiopathic states associated with SLE will require prospective study. Nevertheless this does suggest that apparent cases of TTP secondary to SLE may be distinct from idiopathic disease. Proposed alternative pathological mechnisms for the formation of microvascular thrombi in SLE include direct endothelial damage by autoantibodies, a hypofibrinolytic state and defective prostacyclin generation.

TTP may also arise secondary to drug therapy. Several drugs have been implicated including cyclosporin, oral contraceptive, quinine, ticlopidine (Bennet *et al*, 1998), clopidogrel (Bennet *et al*, 2000) and mitomycin C. Protease deficiency was not seen in the single mitomycin associated TTP case studied here. This is perhaps unsurprising since plasma exchange is often inefficacious in the management of such cases. Likewise cyclosporin, possibly a risk factor for the development of TTP post transplantation, appears to act via a protease independent mechanism. Again plasmapheresis is often ineffectual. Ticlopidine and clopidogrel are both thienopyridine derivatives which block the platelet ADP receptor. However they may precipitate TTP via different mechanisms. Whilst 95% ticlopidine associated cases have an onset within 2 – 12 weeks of therapy (Bennet *et al*, 1998), ten of the 11 clopidogrel associated cases reported in one recent series received clopidogrel for 14 days or less (Bennet *et al*, 2000). Ticlopidine-associated disease has been associated with protease deficiency secondary to inhibitory IgG antibodies (Tsai *et al*, 2000). Protease deficiency secondary to inhibitory IgG was identified in the two clopidogrel treated patients tested, but TTP occurred after only three to five days of treatment in

some patients (Bennet *et al*, 2000). Thus an antibody mediated mechanism seems unlikely. Furthermore, unlike ticlopidine, clopidogrel is not associated with apoptosis of microvascular cell lines in culture (Mauro *et al*, 1999). It has also been argued that clopidogrel may not in fact increase the risk of TTP since the estimated incidence, 11 cases per 3 million pateients treated, is similar to that of idiopathic TTP. In contrast the estimated incidence of ticlopidine-associated TTP is much higher at one per 1600 to 5000 patients treated.

Only small numbers of patients with a diagnosis of secondary TTP could be included here due to the scarcity of the condition. Nevertheless the findings do suggest that those patients currently diagnosed with secondary TTP are a heterogeneous population. Whether this concept also applies to idiopathic TTP is discussed further in chapter 6. However clinical presentation of those primary and secondary TTP cases, currently defined as a microangiopathic haemolytic anaemia in the absence of any other underlying cause, and characterised by severe deficiency of VWF-CP activity, appears to be indistinguishable from those in whom a near normal protease activity is maintained. Variations in protease activity may nevertheless have a clinical impact. Histological changes may be distinct in those microangiopathic states associated with reduced as opposed to near normal protease levels. In depth studies have yet to be performed. Similarly protease activity might predict response to plasmapheresis. The post allogeneic BMT and mitomycin associated TTP cases studied here had normal/near normal protease activities. Failure to respond to plasmapheresis is often seen in such cases. Thus distinct pathophysiological mechanisms may give rise to similar clinical features such that TTP defined according to current accepted diagnostic criteria represents a heterogeneous group of conditions. Further prospective studies of idiopathic and secondary TTP will be required to investigate the possible diagnostic and predictive value of protease activity.

VWF-CP activity has been suggested as a sensitive and specific discriminating factor between TTP and HUS: in one series of 23 patients with a diagnosis of HUS (13 with sporadic and ten with familial disease) all had normal or only mildly reduced levels of

VWF-CP activity (Furlan *et al*, 1998b). This is potentially of great clinical importance, since HUS is characterised by a microangiopathic haemolytic anaemia, thrombocytopenia and renal failure (Gasser *et al*, 1955) and diagnostic uncertainty may arise particularly when neurological problems coexist. In this investigation, deficiency of VWF-CP had a sensitivity of 97% and a specificity of 80% for discrimination between acute idiopathic TTP and HUS diagnosed according to conventional clinical criteria. However the clinical features of TTP and HUS overlap: in one series, HUS (defined by the presence of acute renal failure) could not be distinguished from TTP by other clinical features (coma, seizure, focal neurological signs or mental status changes) or routine laboratory data (Vesely *et al*, 2001). Should microangiopathic haemolytic anaemias therefore be diagnosed according to protease activity rather than clinical features? This can be acceptable only if it can be demonstrated that the biological course of the disease is determined by protease activity in preference to clinical symptoms and signs. Of the ten patients presenting with acute HUS studied here, two had severe reductions in protease activity with values similar to those associated with acute idiopathic TTP. Neither had neurological dysfunction and in fact one had documented *E coli* 0157 infection. Both responded to supportive care and neither were plasma exchanged. That reduced protease activity was temporally related to the microangiopathic process was supported by recovery of protease activity to the normal range in both patients with remission. These two cases are not unique. A case of HUS associated with VWF-CP deficiency secondary to an inhibitory antibody was recently reported (Hunt *et al*, 2001). Similarly in a prospective study of 33 children with HUS, five had total deficiency of protease activity during the acute phase. One child had a D+ HUS related to *E. Coli* 0157 and four had recurrent D- HUS. None had a detectable inhibitor to VWF-CP (Veyradier *et al*, 2001b). Neither study provided evidence to suggest that those cases of HUS characterised by reduced protease activity failed to respond to standard supportive care. Protease determined diagnosis of microangiopathic haemolytic anaemias does not therefore appear to be superior to diagnosis according to clinical features.

Despite the observation that severe deficiency of VWF-CP may occur during an episode of HUS, it is thought that TTP and HUS are distinct pathophysiological entities. Firstly TTP and HUS have contrasting histological appearances. Arteriolar and capillary thrombosis is prominent in TTP and thrombi are largely composed of platelets that stain strongly for VWF:Ag but only weakly for fibrin and fibrinogen (Asada *et al*, 1985). In contrast, the primary histological changes in HUS are glomerular and arteriolar fibrin thrombi with subendothelial widening of the glomerular capillary wall apparent on electron microscopy (Remuzzi & Ruggenenti, 1995). Secondly whilst plasmapheresis is the treatment of choice for TTP and has had a dramatic impact on survival rates (Rock *et al*, 1991), it has no demonstrable efficacy in HUS (Gianviti *et al*, 1993). If TTP and HUS are distinct, HUS occurring in the context of severe protease deficiency must be explained.

Assessment of VWF-CP activity is at present a functional assay. Whether VWF proteolyis *in vivo* is achieved by the sole activity of VWF-CP is unknown. VWF proteolysis may be the consequence of an enzymatic cascade or require one or more cofactors for optimal activity. Whilst a defect in any of one of these may result in loss of protease activity *in vitro*, the resulting clinical phenotype may vary widely depending on the specific abnormality. Reduced protease activity could therefore be an *in vitro* phenomenon and a coincidental finding in HUS. This would suggest that VWF-CP deficiency is of high prevalence. However, in the limited studies published to date, only moderate reductions of protease activity have been detected, most commonly in the context of another underlying pathology such as malignancy (Oleksowicz *et al*, 1999), DIC (Loof *et al*, 2001) or decompensated liver cirrhosis (Mannucci *et al*, 2001). Decreased protease activity has been reported in uncomplicated gestations in the last two trimesters and healthy neonates but such reductions are only of moderate magnitude (Mannucci *et al*, 2001). Severe deficiency has therefore yet to be implicated in conditions other than TTP or HUS. The recent purification and partial sequencing of VWF-CP reported simultaneously by two independent groups (Gerritsen *et al*, 2001;Fujikawa *et al*, 2001) will allow this aspect

to be addressed in detail through the development of quantitative functional and antigenic assays.

The pathogenesis of HUS is multifactorial. Although more than 90% diarrhoea associated HUS cases are secondary to verotoxin secreting enterococci, only a minority of infected individuals progress to develop HUS. Susceptibility may be increased by both exogenous and endogenous factors. In a prospective cohort study of children younger than ten years of age with diarrhoea caused by *E coli* 0157:H7, the frequency of the development of HUS was related to the severity of illness (shown by initial white cell count and day of initial stool culture) and antibiotic administration. Of those children given antibiotics, 56% developed HUS as compared to 8% who were not (Wong *et al*, 2000). Several antimicrobial agents increase the release of toxin from *E coli* 0157:H7 *in vitro* (Grif *et al*, 1998), furthermore some are potent inducers of the Shiga toxin 2 gene (Zimmerhacki, 2000). The use of antimotility drugs promotes the probability of developing HUS (Cimolai *et al*, 1992) whilst in the recent Scottish outbreak in Lanarkshire adults with low gastric acid (previous gastrectomy or proton pump inhibitor medication) were at significantly increased risk of HUS and death (Dundas *et al*, 2001). The susceptibility of any particular individual may be further influenced by complement. Factor H deficiency results in excessive C3 activation, membrane deposition and endothelial cell injury. Reduced levels of C3 have been reported in 73% patients with familial HUS compared to 16% controls ($p = 0.001$) (Noris *et al*, 1998) although published literature regarding sporadic HUS is lacking. Thus reduced VWF-CP activity might promote the generation of glomerular and arteriolar thrombi seen in HUS whilst not being an absolute requirement for disease. Certainly multimeric analysis may demonstrate ultra large multimers during an episode of HUS (Rose *et al*, 1984).

Alternatively, VWF-CP deficiency may be a consequence of HUS rather than a contributory factor. Although VWF-CP activity is found in both plasma and serum, cell surface binding may be of functional importance. The endothelium is heterogeneous and endothelial phenotype varies from one vascular bed to the next. If a

large fraction of the VWF-CP pool were bound to the renal endothelium, a secondary deficiency could arise from renal endothelial damage. However, since protease activity appears unaffected by renal impairment and pathological conditions characterised by widespread endothelial damage have not, to date been associated with a severe protease deficiency, this appears unlikely. Further studies are required to ascertain the role of protease deficiency in acute HUS.

Assessment of protease activity may nevertheless assist in the differential diagnosis of thrombotic microangiopathic states. As stated above, VWF-CP activity had a sensitivity of 97% and a specificity of 80% for discrimination between acute idiopathic TTP and HUS diagnosed according to conventional clinical criteria. Pregnancy is complicated by several microangiopathic conditions including pre-eclampsia, HELLP and sepsis in addition to HUS and TTP. Accurate diagnosis is complicated by the virtue that each feature of the clinical pentad of TTP can occur in pre-eclampsia and HELLP. This is however a prerequisite for appropriate management: urgent delivery is required for fulminant pre-eclampsia whereas termination of pregnancy does not alter the clinical course of TTP that instead warrants immediate plasma exchange. This investigation included six cases of pregnancy associated microangiopathic haemolysis. Protease activity was extremely varied suggesting that it might be a useful diagnostic discriminator. Two of the patients were clinically diagnosed with TTP and responded to plasmapheresis. VWF-CP activity was severely reduced in both cases. In contrast, the remaining patients with clinical diagnoses of HELLP and DIC had protease activities within or just below the normal range. This may reflect the observation that VWF-CP activity falls during the second and third trimester of normal gestation (Mannucci *et al*, 2001). However a more substantial reduction in protease activity than that seen here has been reported in the setting of HELLP (mean ± SD, 36 ± 6% as compared to normal pregnancy 65 ± 16%) (Lattuada *et al*, 2001). If levels of protease activity in TTP and HELLP overlap this will preclude the use of protease activity as a diagnostic discriminator of thrombotic microangiopathy during pregnancy. Much larger studies will be required to address this issue.

Why pregnancy should be associated with TTP is obscure. Although VWF-CP activity is reduced from the second trimester, the levels remain substantially higher than those associated with acute TTP at presentation. Values for the second and third trimester, expressed in percentage activity of normal plasma, were recently reported as 64 ± 34% and 64 ± 15% (mean ± SD) respectively (Mannucci *et al*, 2001). A decrease of this magnitude may be sufficient to precipitate TTP in a predisposed individual since the majority of pregnancy associated TTP cases present at or before 24 weeks gestation (Weiner, 1987). However additional factors may also be at play since similar reductions have been reported in liver cirrhosis (46 ± 33%) and post-operatively (58 ± 27%), neither of which are associated with an increased risk of TTP (Mannucci *et al*, 2001). Pregnancy is characterised by a maternal systemic inflammatory response (Redman *et al*, 1999). Maternal endothelial activation is one component of this and may contribute to the gestational predisposition to TTP. The progressive rise in VWF:Ag concentration during pregnancy could also play a role. A statistically signficant negative correlation between VWF:Ag concentration and protease activity has been described (Mannucci *et al*, 2001) and VWF-CP has now been shown to bind to VWF *in vitro* albeit with relatively low affinity (Fujikawa *et al*, 2001). The relative importance of all these factors has yet to be addressed.

Further specificity of VWF-CP deficiency for acute TTP was demonstrated by the observation that there were only minor reductions in protease activity in those patients presenting with miscellaneous haemolytic/thrombocytopenic conditions as compared to normal controls. Decreased activity could not be ascribed to impaired renal function or increased VWF:Ag concentrations. However as discussed above, this finding may have been secondary to the advanced years of this subgroup. The median age of this group of patients was 64.5 years as compared to 35 years for the normal controls.

The bulk of current scientific evidence seems to suggest that deficiency of VWF-CP activity is a prerequisite for the development of idiopathic TTP and central to the underlying pathophysiology. Protease deficiency does explain the presence of

circulating ultra large multimers associated with the condition which were found either at presentation or during 26 of the 32 (81%) acute episodes studied here. In previous studies abnormalities of VWF multimers were identified at the onset of or during 86% cases of single episode TTP (Moake & McPherson, 1989). Although variable multimeric patterns have been observed and no correlation made with clinical course (Rock *et al*, 1998), it is thought that ULVWF multimers play a central role in the pathophysiology of TTP (Moake *et al*, 1994;Moake *et al*, 1985;Moake *et al*, 1986). The failure to detect ULVWF multimers in the remaining six cases reported here may reflect preferential platelet binding of large multimeric forms (Tsai *et al*, 1991b) rather than suggesting an alternative pathological mechanism. Slightly higher protease levels were found in this subgroup of patients and this could be interpreted as representing a subgroup of less severely affected individuals. In fact this is likely to simply reflect underlying methodology since it is the presence of ULVWF multimers in the test plasma which results in a negative value. Evaluation of protease activity using an alternative technique such as IRMA could address this issue.

A pivotal role of VWF processing in the aetiology of TTP would be supported by a significant positive correlation between recovery of protease activity and clinical remission. Published literature is extremely limited. Protease activity in 23 acute TTP samples taken after plasma exchange ranged from 0 – 70% (mean ± SD, 16 ± 20%) (Tsai & Lian, 1998). Although a significant positive correlation was demonstrated in this study of recovery from acute idiopathic TTP, there were clearly samples in which, despite an extremely depressed protease activity (such as are typically seen at presentation of acute TTP), the platelet count remained within the normal range. Furthermore when these samples were taken there were no other clinical or laboratory features of TTP. A number of explanations are possible. Firstly differences in protease activity which lie below the sensitivity of the assay may be of physiological importance. This would suggest that protease levels equivalent to less than 3% activity of pooled normal plasma (PNP) may prevent an episode of TTP. However a protease activity of less than 0.4, equivalent to 5% activity of PNP, was consistently associated with circulating ULVWF multimers which are implicated in the formation of platelet

microthrombi. *In vitro* studies have shown such multimers mediate increased platelet retention (as measured by the modified Adeplat S test) which is typical of TTP (Karpman *et al*, 1996) and are capable of supporting primary platelet aggregation under the shear conditions which exist within the microcirculation (Moake *et al*, 1988). It therefore appears that simple protease deficiency resulting in circulating ULVWF multimers is insufficient to precipitate TTP. This is corroborated by the observation that although ULVWF multimers are found circulating in the healthy neonate (Weinstein *et al*, 1989) and those patients with the rare Vicenza variant of von Willebrand disease (Mannucci *et al*, 1988) there is no associated increase in TTP. Furthermore if circulating ULVWF multimers secondary to protease deficiency was the sole factor involved in the pathogenesis of TTP, thrombi might be expected to form throughout the vasculature. Although this may be seen at post mortem, there is a predilection for thrombi to form within the renal and cerebral circulation.

The study of VWF-CP activity during remission further supports a "multi-hit" hypothesis for the aetiology of TTP. Of the 142 samples from 26 patients studied, 39 (27%) had a protease activity below 0.4. In a previous paper, 15 patients were studied during the acute event and remission. In only five of fifteen patients was there complete recovery of VWF-CP activity with return of the platelet count to normal. However in five additional patients protease activity became evident after extended periods of clinical remission (Furlan *et al*, 1998b). Normal or near normal levels have been reported in seven cases of TTP studied in remission (Tsai & Lian, 1998). This suggests that the observed reductions in protease activity during remission could be a time-lag phenomenon. Variations in the speed of protease recovery were seen but so too were apparently spontaneous fluctuations. In an attempt to minimise this confounding factor, protease activity was studied six to nine months after attainment of remission following seventeen episodes of TTP. Severe protease deficiency as defined above was still seen in 24%. There was no skewing attributable to the single patient who was analysed following two episodes of TTP because protease activity was greater than 0.4 on both occasions. No clinical consequences could be attributed to these disparate clinical features. Persistent protease deficiency was not associated

with a past history of intermittent disease nor did it predict future relapse. In fact of the five relapses, only one had a protease activity below 0.4. The failure to demonstrate an association between protease activity and relapse may be because follow-up is relatively short. It has been shown that the first relapse may occur up to eight years after the index event (Shumak *et al*, 1995). Although ULVWF multimers have been associated with future relapse this was not a feature of this study. Thus the factors responsible for the development of intermittent disease remain elusive.

These discrepancies suggest that whilst VWF-CP activity is important in the pathogenesis of TTP, hitherto unidentified factors are also involved. Possible candidates include endothelial modulation, additional VWF processing pathways, complement activation and immune regulation. The endothelium is a heterogeneous organ and is subject to regulation by multiple factors including cytokines (Drake *et al*, 1993), microenvironment (Aird *et al*, 1997) and shear stress (White & Fujiwara, 1986). Alterations in any one of these parameters could influence either VWF-cleaving protease activity *per se* or the susceptibility of VWF to proteolysis. Even in the absence of shear stress and under uniform culture conditions, the susceptibility of endothelial cells to apoptosis induced by TTP plasma appears to be determined by cell lineage. Increased apoptosis was detected in microvascular cell lines of renal, cerebral and dermal origin as compared to those derived from hepatic or pulmonary microvasculature (Mitra *et al*, 1997) or macrovascular endothelium (human umbilical vein endothelial cells) (Laurence *et al*, 1996). This is of particular interest because it reflects the anatomical predilection for thrombi to form in the microcirculation of the brain and kidneys during an acute episode of TTP. Whilst endothelial phenotype appears to confer susceptibility, the mechanism responsible remains elusive.

Cleavage of the Tyr 842-Met 843 bond secondary to VWF-CP activity may not be the only mechanism involved in post secretory VWF processing. Evidence for additional mechanisms is provided by the presence of ULVWF multimers in a patient with recurrent TTP in the context of normal VWF processing activity (Daghistani *et al*, 1996). In addition, VWF secreted by cultured endothelial cells into the medium in the

presence of plasma cryosupernatant is of smaller average size than the VWF deposited into the subendothelial matrix (Frangos *et al*, 1989). It has been proposed that a substance in plasma cryosupernatant is responsible for reducing the size of large VWF multimeric forms secreted into the vascular lumen to the smaller multimers ordinarily found in the plasma. The VWF depolymerising activity is thought to be a type of limited disulphide bond reductase since it is inactivated by thiol blocking agents and no evidence for proteolysis has been found (Phillips *et al*, 1993). One group has recently proposed that the reductase is the trimeric glycoprotein, thrombospondin-1 (TSP-1) (Xie *et al*, 2001a). This is of particular interest since TTP has been associated with anti CD36 antibodies (Tandon *et al*, 1994) and CD36 is a membrane receptor for thrombospondin. Since the A3 domain of VWF is thought to mediate TSP-1 binding, it is also conceivable that mutations within this domain might result in reduced VWF depolymerisation. To date, TSP-1 activity has only been reported in a single TTP case and three first degree relatives. TSP-1 activity was reduced in all four although only the index case was symptomatic (Xie *et al*, 2001b). This suggests that isolated TSP-1 deficiency is unlikely to explain TTP occurring in the context of normal VWF-CP activity (such as in patient 23). However, whether reductions in both VWF-CP and TSP-1 activity increase the risk or severity of clinical disease is unknown.

In direct contrast it has been suggested that the acute phase of TTP/HUS is characterised by enhanced rather than reduced VWF proteolytic cleavage. Reduced VWF-CP activity might therefore be a coincidental *in vitro* phenomenon rather than being of pathophysiological importance. It has been demonstrated that an increase of low molecular weight multimers rather than the presence of ULVWF multimers is the most consistent abnormality seen in TTP (Furlan *et al*, 1998a). There have been previous reports of a relative decrease in the native 225kDa VWF subunit accompanied by a relative increase of fragments that can only derive from cleavage of the native subunit (Mannucci *et al*, 1989). Enhanced fragmentation might arise from excessive activity of alternative plasma proteases from that initially described by Furlan and Tsai or from an intrinsic modification of the VWF molecule. Certainly excessive VWF fragmentation reflected by an abnormal low: high molecular weight

VWF multimer ratio has been seen in the presence of absent VWF-CP activity (Galbusera *et al*, 1999) but preferential binding of higher molecular weight VWF forms to platelets (Tsai *et al*, 1991b) could be the cause rather than increased fragmentation *per se*. This cannot however explain the presence of novel VWF fragments other than those consistently found in normal plasma of 189, 176 and 140kDa, generated by cleavage of the Tyr 842-Met 843 bond (Galbusera *et al*, 1999). The responsible enzyme(s) have yet to be identified. Elastase and other enzymes released from activated polymorphonuclear cells, calpain and plasmin proteolyse VWF *in vitro* (Berkowitz *et al*, 1988;Hamilton *et al*, 1985;Thompson & Howard, 1986) and are possible candidates. Although such enzymes may not play a physiological role in VWF processing, they may nevertheless be involved in pathological states. This hypothesis is however contentious as it is generally accepted that the larger VWF multimeric forms are most haemostatically active (Moake *et al*, 1986). A single group has suggested that VWF proteolysed by calpain *in vitro* acquires increased capacity to bind to both ADP and calpain activated platelets (Moore *et al*, 1990). Until this is confirmed however, the pathological relevance of an apparent increase in VWF proteolysis must remain in doubt.

Immune regulation may also be important in the pathogenesis of TTP. An immune mechanism has been suggested as there are reports of increased platelet associated IgG (Morrison & McMillan, 1977), immune complex formation (Zimmerman *et al*, 1982) and anti-CD36 antibodies (Tandon *et al*, 1994). More recently, deficiency of VWF-CP activity has been associated with the presence of a circulating autoantibody of IgG subtype (Tsai & Lian, 1998;Furlan *et al*, 1998b). Cases of TTP lacking central nervous system abnormalities other than headache, have been shown to respond to steroid treatment alone, although of 54 such patients 44% required plasma exchange due to deterioration or failure to improve (Bell *et al*, 1991). Reports of successful outcome following the use of azathioprine, cyclophosphamide and cyclosporin have also been published. Cyclosporin is a potent immunosuppressive drug with effects primarily on T cells. As demonstrated by patients 2 and 8, long-term therapy with cyclosporin (trough 100-200µg/l) was associated with recovery and maintenance of normal

protease activity. Clinical efficacy was suggested by the following observations: protease levels fell to values associated with acute TTP at presentation when cyclosporin was discontinued in patient 2 and has now been followed by relapse, relapse occurred shortly after treatment was stopped in patient 9 and plasma exchange was sucessfully tapered in patients 13 and 14. Although indirect evidence this does suggest that aberrent immunity may play a role in the pathogenesis of TTP. Whether restoration of normal or near normal levels of VWF-CP activity should be a therapeutic goal requires further study. Subsequent relapse following cessation of cyclosporin as demonstrated by patient numbers 2 and 9 and reported elsewhere in the literature (Hand *et al*, 1998;Pasquale *et al*, 1998), coupled with the observation of a temporal association between recurrent protease deficiency and tailing of cyclosporin therapy suggest investigation is warranted.

In conclusion this study suggests that whilst not specific, deficiency of VWF-CP activity has a high sensitivity for the diagnosis of acute idiopathic TTP. Isolated protease deficiency may not however necessarily result in clinical disease nor apparently increase the risk of subsequent relapse. Thus hitherto unidentified factors appear involved in the pathogenesis of acute idiopathic TTP and determination of future relapse. The recent purification and partial sequencing of VWF-CP will aid not only the differential diagnosis and pathophysiology of the various microangiopathic states but also our understanding of VWF homeostasis.

5. An investigation of VWF-cleaving protease activity and congenital TTP

5.1 Introduction

Congenital TTP is extremely rare with fewer than 60 cases reported in the literature since 1953 when, in retrospect, a case was included in a series of atypical congenital haemolytic anaemias (Dacie *et al*, 1953). Characteristically the condition presents during infancy or early childhood with recurrent episodes of haemolysis and thrombocytopenia at predictable intervals (usually every 21-28 days) (Chintagumpala *et al*, 1992;Moake *et al*, 1994). Regular replacement infusions, typically with fresh-frozen plasma (Chintagumpala *et al*, 1992) are required to avert such episodes. In contrast, milder variants have been described in which patients may present at a later age with more intermittent episodes of TTP (Karpman *et al*, 1996;Barbot *et al*, 2000). Episodes are still characterised by the classic clinical pentad of microangiopathic haemolytic anaemia, thrombocytopenia, fluctuating neurological signs, renal dysfunction and fever and often appear to be precipitated by, for example, febrile illnesses (Upshaw, 1978;Karpman *et al*, 1996). Both presentations are typified by the presence of circulating ultra-large von Willebrand factor (ULVWF) multimers during remission periods consistent with defective post-secretory VWF processing (Moake & McPherson, 1989;Chintagumpala *et al*, 1992). Like acute idiopathic adult onset TTP, congenital TTP has recently been associated with deficiency of VWF-CP activity. In a series of six patients with familial TTP, all lacked VWF-CP activity although no circulating inhibitor was detected (Furlan *et al*, 1998b).

If reduced VWF-cleaving protease activity alone was of integral importance to the pathophysiology of TTP, it is possible that disease severity might be proportional to residual protease activity. Moreover, it would be expected that the clinical efficacy of FFP infusions, currently the mainstay of treatment for congenital TTP, is secondary to its intrinsic VWF-cleaving protease activity. Thus alternative replacement products

containing substantial protease activity could be equally efficacious. There are limited reports suggesting that in some cases a factor VIII/VWF concentrate may have therapeutic effect (Karpman *et al*, 1996;Karpman *et al*, 1997;Miura *et al*, 1984). *In vitro* experiments support these clinical findings: administration of both *Haemate*® (Aventis Behring, Germany) and FFP to two patients with congenital TTP during remission reduced excessive platelet retention as measured by the modified Adeplat S glass bead test (Karpman *et al*, 1997).

To address these hypotheses, VWF-cleaving protease activity was determined in four cases of congenital TTP, two of whom had severe disease requiring prophylaxis every 10 - 14 days whilst the others, two brothers, had moderate disease requiring only symptomatic treatment. Successful prophylaxis had been achieved in one of the severely affected patients with an intermediate purity factor VIII concentrate (*Kryobulin*), cryoprecipitate and FFP. An *in vitro* assessment of VWF-cleaving protease activity of both plasma and plasma derived products was therefore also undertaken.

5.2 Methods

5.2.1 Patients

Patient 1, a Middle Eastern boy from Kuwait, was born in 1982 to consanguinous parents (first cousins). He has five younger siblings all of whom are alive and well. There is no significant family history. He presented at the age of seven months with easy bruising and was found to be thrombocytopenic with an excess of high molecular weight VWF multimeric material. A diagnosis of congenital TTP was made and thereafter he was satisfactorily maintained at home on fortnightly infusions of *Kryobulin* (Hyland Immuno, Baxter Healthcare Ltd, Newbury, UK), an intermediate purity factor VIII concentrate which successfully averted episodes of haemolysis and sustained his platelet count above 50 x 10^9/l. Since its withdrawal in 1998, he has received fortnightly infusions of cryoprecipitate (8 units), the most readily available

product in Kuwait. Eight samples were taken over a period of six weeks when he attended University College London Hospitals for assessment.

Patient 2, an Indian girl was born in 1987 to consanguinous parents (first cousins). She was delivered at 37 weeks gestation by caesarian section for foetal distress and became jaundiced within 12 hours of birth. At this juncture her haemoglobin (Hb) was 12.9g/dl and platelets 39 $x10^9$/L. She received a whole blood exchange transfusion primarily for jaundice with subsequent clinical improvement and normalization of the platelet count. She represented at seven weeks of age with pallor and poor feeding. Microangiopathic haemolytic anaemia, thrombocytopenia and ULVWF multimers were found on investigation and a diagnosis of congential TTP was made. Since then fortnightly prophylactic FFP infusions have successfully averted symptomatic haemolytic episodes. Twenty four samples were taken over a period of 81 days when she underwent a therapeutic trial with *BPL 8Y* (Bio-Products Laboratory, Elstree, UK), an intermediate purity factor VIII concentrate.

Patient 3, a Caucasian boy, was born in 1995. His parents are healthy and unrelated. There is no significant family history. He became jaundiced within 12 hours of delivery and received phototherapy and exchange transfusion. No definitive diagnosis was reached. He re-presented at the age of nine months with jaundice and bruising and was found to be thrombocytopenic with marked red cell fragmentation. Extensive investigations revealed an excess of ULVWF multimeric material and a diagnosis of congenital TTP was made. To date he has had a total of 22 haemolytic episodes which appear to be triggered by viral infections or routine childhood vaccinations. Although prophylactic infusions are not required and some of the episodes have been self-limiting, more severe episodes have been successfully treated with 15ml/kg FFP or solvent-detergent (S/D) treated plasma (Octaplas, Octapharma Ltd, Coventry, UK).

Patient 4 born in 1997, the brother of patient 3, has a similar clinical picture. He became jaundiced and developed severe thrombocytopenia (platelets 11 x 10^9/l) within 48 hours of birth. Since then he has had episodes of haemolysis and thrombocytopenia

at infrequent intervals. There are no other siblings. As above, an excess of ULVWF material has been found during periods of remission. He has now had a total of 16 haemolytic episodes, the majority of which have required treatment with S/D plasma. Samples from patients 1 and 2 were taken over a period of six weeks encompassing three haemolytic episodes whilst those from patients 3 and 4 were taken during routine clinical attendance when both were systemically well and in haematological remission (*Table 5.2*). Parents gave verbal consent to have extra samples of blood collected. Control subjects for VWF-CP activity were 25 healthy adult volunteers (14 male, 11 female, median age 35 years).

5.2.2 Blood collection

A vacutainer system® was used to obtain blood samples from the older children (patients 1 and 2). In the two younger children blood was collected into 0.106M citrate (1 in 10) using a butterfly and syringe technique. Plasma was prepared for specific assays as outlined in section 2.1.

5.2.3 Transfusion products/FVIII concentrates

Intrinsic VWF-CP activity of the following transfusion products: FFP, cryosupernatant, cryoprecipitate (all routine donations supplied by National Blood Service (NBS), England) and S/D plasma (Octaplas, Octapharma Ltd, UK) was assessed *in vitro*. In addition, a variety of commercially available factor VIII concentrates were also tested. Product characteristics are given in *Table 5.1*.

Product	Purity	Purification process	Viral inactivation
Kryobulin, Baxter Healthcare Limited	Intermediate	Isoelectric precipitation/ ammonium sulphate	Steam treatment (1200mbar in an inert atmosphere, 10 hours)
Haemate P, Aventis Behring Limited	Intermediate	$Al(OH)_3$/glycine	Pasteurisation (10 hours at 60°C)
BPL 8Y, Bio Products Laboratory	Intermediate	Glycine precipitation/gel filtration	Dry heat treatment (80°C for 72 hours)
Fahndi, Grifols UK Limited	High	PEG precipitation/affinity chromatography	Solvent/detergent and heat treatment (72-74 hours at 81± 1°C)
Liberate, Scottish National Blood Transfusion Service	High	Ion exchange chromatography	Solvent/detergent (TNBP/polysorbate 80)
Beriate® HS, Centeon Limited	High	PEG precipitation/affinity chromatography	Heat treatment in aqueous solution (10 hours at 60°C)
Koate®-HP, Bayer plc	High	PEG and glycine precipitation/gel chromatography	Solvent/detergent (TNBP/polysorbate 80)
Koate®-DVI, Bayer plc	High	PEG and glycine precipitation/ gel chromatography	Solvent/detergent (TNBP/polysorbate 80) and heat treatment (72 hours at 80°C)
Hemofil® M, Baxter Healthcare Limited	High	Monoclonal immunoaffinity /ion exchange chromatography	Solvent/detergent (TNBP/Triton X-100)
Kogenate®, Bayer Limited	Recombinant (bhk)	Recombinant	

Table 5.1 *Product characteristics of factor VIII concentrates assayed for endogenous VWF-cleaving protease activity*
PEG = polyethylene glycol; TNBP = tri-n-butylphosphate

5.2.4 VWF-cleaving protease activity

VWF-CP activity of patient plasma was assessed according to the final method described in section 2.5. Intrinsic VWF-cleaving protease activity of conventional and possible alternative treatment modalities was determined by substitution of citrated plasma with the relevant transfusion product. Factor VIII concentrates were reconstituted according to manufacturer's instructions and in all cases other than *Kryobulin*, 100µl of each transfusion product or commercial factor preparation was substituted for 100µl patient plasma. Only a single aliquot of *Kryobulin* was available for testing since this product is not now commercially available. Protease activity of this product was assayed according to the initial method described in section 2.5 using VWF purified by gel filtration from a cryoprecipitate of normal human plasma.

5.2.5 Multimers

Electrophoretic VWF multimer analysis using 0.8% agarose gels was performed using the PhastSystem as described in section 2.4.

5.3 Results

5.3.1 VWF-cleaving protease activity in congenital TTP

Patients

The four patients with congenital TTP were studied on a total of 40 occasions. Eight samples were taken immediately prior to prophylactic or symptomatic treatment and 32 during asymptomatic periods. In the case of patients 1 and 2 asymptomatic periods occurred during the time interval between prophylactic infusions and were therefore a maximum of ten to fourteen days following treatment. However three of the remaining five samples from patients 3 and 4 were taken two to twenty weeks post therapy. Representative results of simultaneously performed routine laboratory investigations are shown in *Table 5.2*.

	Date	Platelets (x 10^9/l)	Hb (g/dl)	LDH (U/l)	Creatinine (μmol/l)	Bilirubin (μmol/l)
Normal range		150-450	11.5-14.5	211-423	0 - 133	0-18
Patient 1	18.1.99	50	16.6	526	101	21
	22.1.99	13	14.3	1057	101	49
	26.1.99	103	15.2	628	95	13
	2.2.99	29	15.6	565	99	22
	5.2.99	23	15.2	782	102	26
	8.2.99	14	14.2	820	96	29
	22.2.99	9	14.5	-	88	47
Patient 2	20.11.00	49	12.7	-	-	24
	20.11.00	57	12.6	-	-	-
	21.11.00	113	12.8	-	-	-
	22.11.00	156	12.1	-	-	-
	23.11.00	218	12.0	-	-	-
	24.11.00	278	12.0	-	-	-
	27.11.00	437	12.5	-	-	-
	29.11.00	448	12.0	-	-	-
	1.12.00	264	11.4	-	-	-
	4.12.00	45	12.1	-	-	-
Patient 3	5.10.98	411	12.7	-	-	-
	18.10.99	482	11.9	-	37	22
Pre-	8.2.01	126	11.6	-	-	-
1 hour post	8.2.01	141	11.5	-	-	-
Patient 4	18.10.99	222	11.7	-	31	6
Pre-	7.2.01	120	11.4	-	30	6
1 hour post	7.2.01	126	11.1	-	-	-

Table 5.2 *Laboratory findings in patients 1, 2, 3 and 4 at time of sampling for VWF-cleaving protease activity*

VWF-CP activity

All congenital TTP samples assayed had severely reduced VWF-CP activity as compared to healthy controls. Protease activity was significantly decreased not only in those samples taken immediately prior to treatment but also in those taken during asymptomatic episodes (p values < 0.0001 in both instances). However there was no significant difference in activity between these two groups (see *Figure 5.1*). In addition protease activity was comparable between patients 1 and 2 who had clinically severe disease and patients 3 and 4 who were only moderately affected. (see *Figure 5.2*).

VWF multimeric analysis

Ultra large VWF multimers were a consistent finding in all four patients.

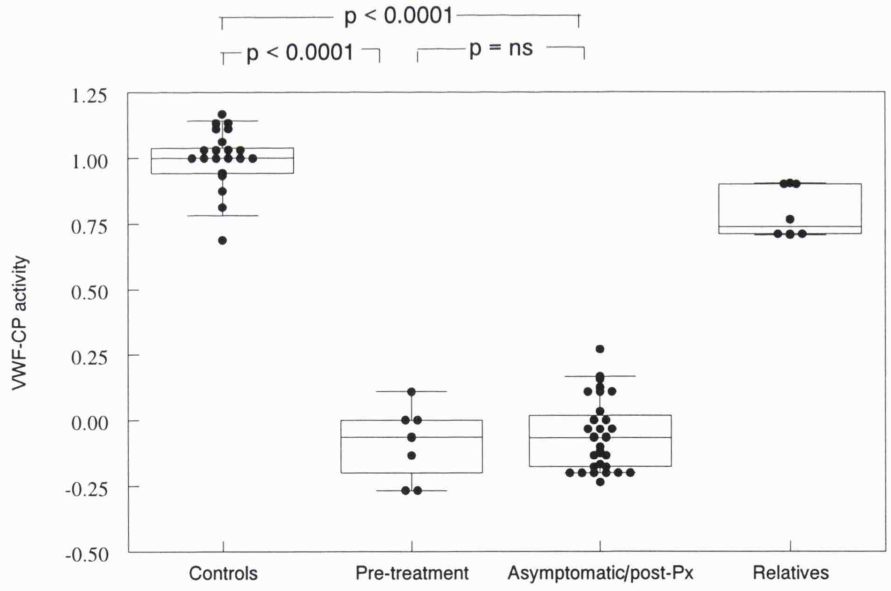


Figure 5.1 *Plasma levels of VWF-CP activity in controls (n=25), congenital TTP pre-treatment (n = 8) and post-treatment/remission (n=32) and first degree relatives (n = 8)*

Horizontal line indicates median, box the 25th to 75th percentile and bars the 5th and 95th percentile.

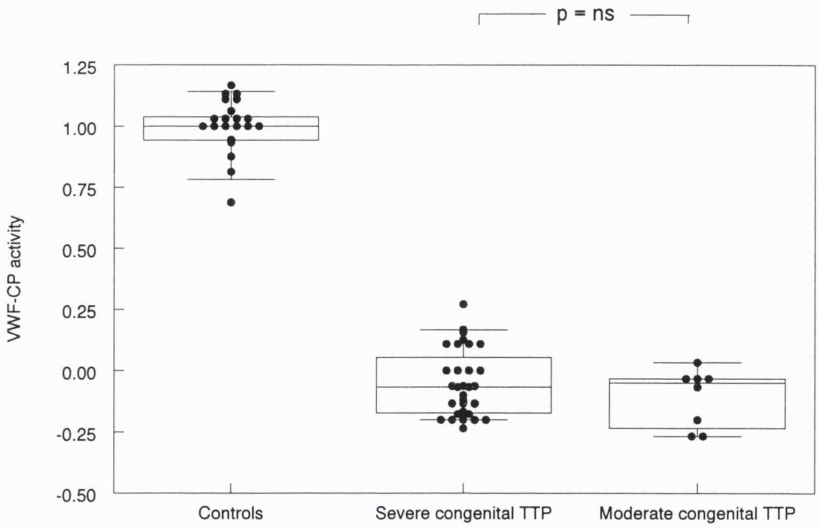


Figure 5.2 *Plasma levels of VWF-CP activity in controls (n=25), congenital TTP with clinically severe disease requiring prophylaxis (n=32) and congenital TTP with clinically moderate disease requiring symptomatic treatment only (n=8)* Horizontal line indicates median, box the 25th to 75th percentile and bars the 5th and 95th percentile.

First degree relatives

VWF-cleaving protease activity and VWF multimeric analysis was assessed in all available first-degree relatives. Samples were obtained from both parents of families 1, 2 and 3 in addition to the two younger female siblings of patient 1. All had normal VWF multimeric analysis. Protease activity was normal (> 0.80) in the unaffected members of family 3 but this was not the case in families 1 and 2. Here there was evidence of a slight reduction in protease activity (range 0.71 – 0.76 which equates to an activity of 25 – 50% of pooled normal plasma, PNP) in both sets of parents and one sibling (*Figure 5.1, Figure 5.3 and Figure 5.4*). None of these individuals has to date had any symptoms suggestive of TTP.

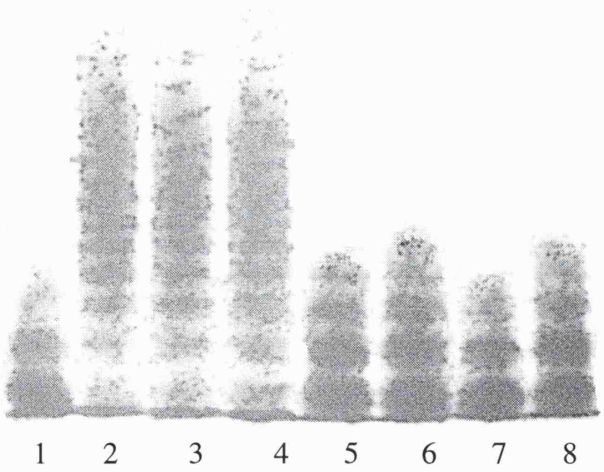


Figure 5.3 *VWF-CP activity in family 1. A positive and negative control (activated PNP incubated with VWF substrate, the latter in the presence of 0.2M EDTA) are shown in lanes 1 and 2 respectively. Samples from patient 1 tested on two separate occasions run in lanes 3 and 4. Persistent intermediate and high molecular weight VWF multimers indicate severe protease deficiency. Samples from parents and two younger sisters are shown in lanes 5 to 8. There is a slight increase in intermediate size VWF multimers as compared to the positive control in both parents (lanes 5 and 6) and one sister (lane 8), which corresponds to a moderate reduction in protease activity equivalent to 25 – 50% activity of PNP.*

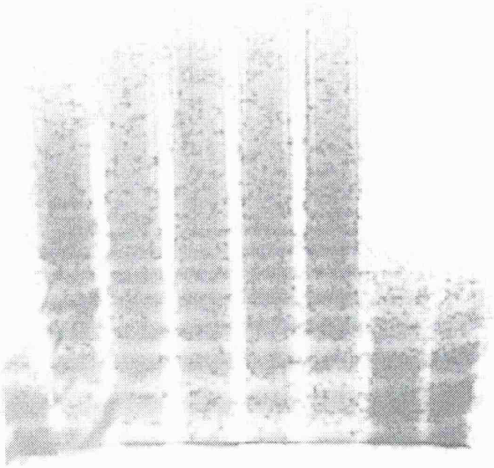


Figure 5.4 *VWF-CP activity in family 2. A positive and negative control run in lanes 1 and 2 as above. Samples from patient 2 taken on four separate occasions are shown in lanes 3 to 6. The persistence of intermediate and high molecular weight multimers indicates severe protease deficiency. Samples from her parents run in lanes 7 and 8. Both demonstrate a slight increase in intermediate size vWF multimers as compared to the positive control corresponding to a moderate reduction in protease activity equivalent to 25-50% activity of PNP.*

5.3.2 VWF-cleaving protease activity and plasma/plasma derived products

VWF-cleaving protease activity was determined in FFP, cryosupernatant, cryoprecipitate, S/D plasma, ten commercial factor VIII and two factor IX preparations. Although infusions of FFP (Moake *et al*, 1985), cryosupernatant (Moake *et al*, 1985) and solvent-detergent treated plasma (Moake *et al*, 1994) are accepted treatments for congenital TTP, cryoprecipitate is relatively contra-indicated in this condition due to its high VWF concentration. Nevertheless, all were shown to possess protease activity equivalent to that of PNP (*Figure 5.5*). This is consistent with the successful treatment of patient 1 for 18 months using prophylactic cryoprecipitate infusions.

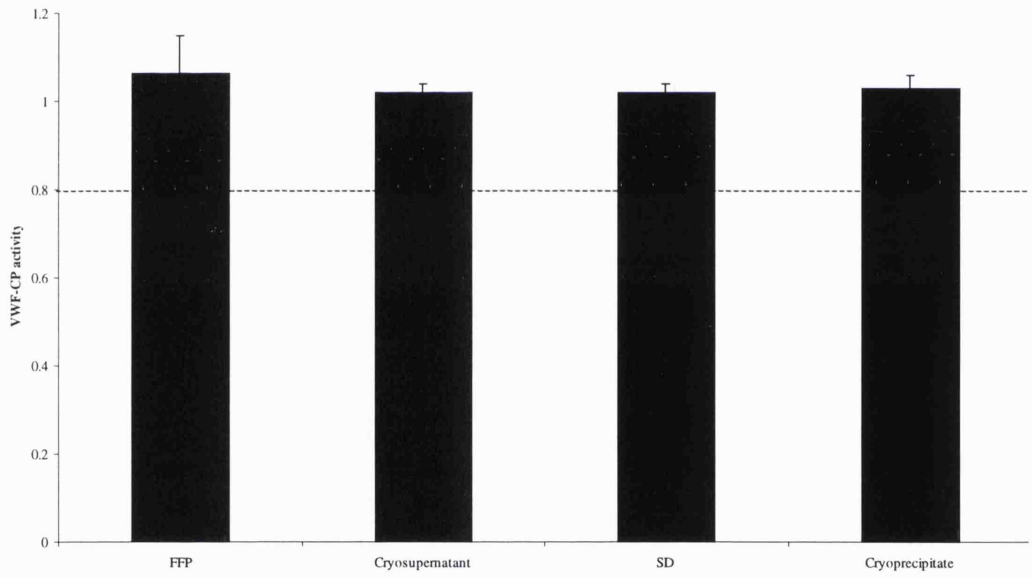


Figure 5.5 *Intrinsic VWF-cleaving protease activity of FFP, cryosupernatant, solvent-detergent treated plasma (SD) and cryoprecipitate. Each plasma product was assessed on three separate occasions. The solid bars represent the mean activity and error bars the standard deviation. The dotted line represents the lower limit of normal for VWF-cleaving protease activity.*

Results of VWF-cleaving protease activity determined in a variety of factor VIII concentrates including recombinant, high purity and intermediate purity products and two factor IX concentrates are shown in *Figures 5.7* and *5.8*. Of the intermediate

purity concentrates, *Kryobulin* and *BPL 8Y* possessed protease activity at least equal to that of PNP. Quantitation of protease activity greater than that of PNP is hampered by the fact that assay conditions have been optimised to allow virtual complete degradation of VWF substrate by PNP. Estimation of protease activities above 100% may be achieved by dilultion studies. There was insufficient material to perform a dilution study with *Kryobulin*. Dilution studies performed using *BPL 8Y* did not appear to demonstrate significantly higher protease activity as compared to PNP (see *Figure 5.6*). Although *Kryobulin* and *BPL 8Y* are both intermediate purity concentrates, intrinsic VWF-cleaving protease activity was not simply a function of purity. *Haemate P* is also an intermediate purity concentrate but had a mean protease activity of only 0.52 (equivalent to approximately 12.5% activity of PNP). In addition, although minimal (< 0.1) protease activity was found in those monoclonally purified and recombinant products tested, two high purity products, *Koate®-HP* and *Koate®-DVI* possessed protease activity equal to that of PNP (*Figure 5.7*). Neither factor IX concentrate tested possessed significant VWF-CP activity.

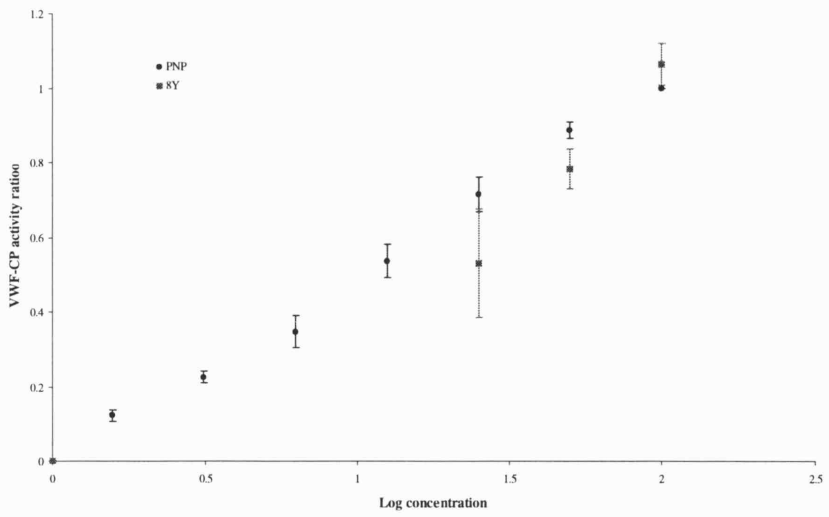
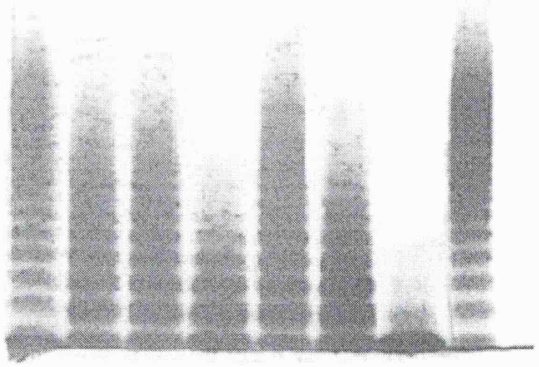


Figure 5.6 *Dilution studies comparing intrinsic VWF-CP activity ratio of PNP and BPL 8Y.*
Mean ± SD (n = 3) are shown in each case.



1 2 3 4 5 6 7 8

Figure 5.7 *VWF-cleaving protease activity of plasma products and factor VIII concentrates assayed according to the initial method described in section 2.5. A negative and positive control (normal healthy adult control plasma in the absence or presence of Ba^{2+} respectively) run in lanes 1 and 2. The positive control has comparable protease activity to FFP (lane 3). Enhanced loss of high and intermediate molecular weight VWF multimers is seen in lanes 4 and 6 and most strikingly in lane 7. Cryoprecipitate runs in lane 4, Fahndi in lane 6 and Kryobulin in lane 7. Persistence of high molecular weight multimers is seen in lanes 5 and 8 corresponding to Haemate P and plasma from patient number 1 respectively.*

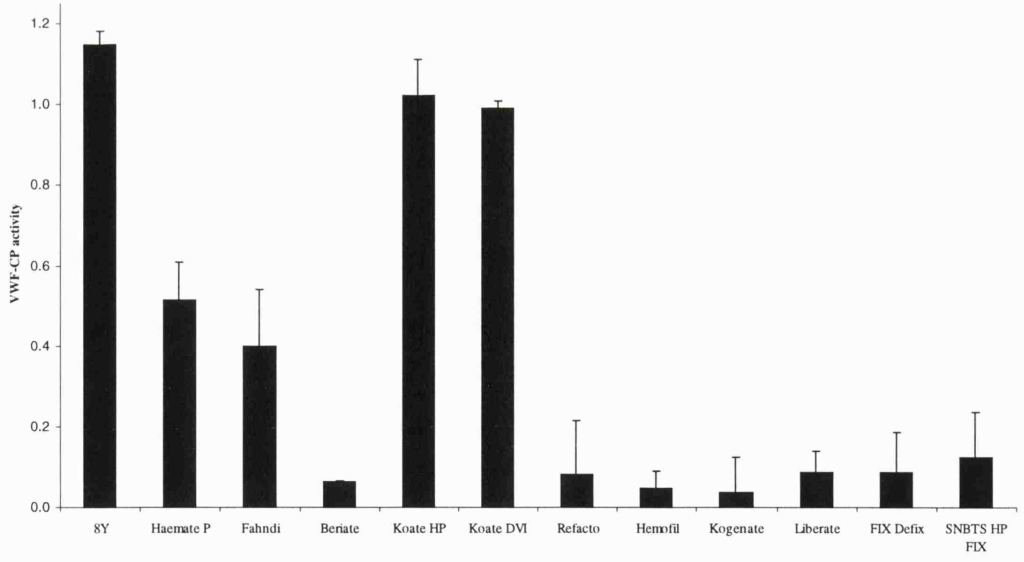


Figure 5.8 *VWF-cleaving protease activity in ten factor VIII and two factor IX concentrates.*

Each concentrate was assessed on three separate occasions. The solid bars represent the mean activity and error bars the standard deviation.

5.3.3 Therapeutic trial of factor VIII concentrates in congenital TTP

Trial of Fahndi and Haemate P

As described above, patient 1 had for many years been satisfactorily maintained on fortnightly infusions of *Kryobulin* (10 units/kg) (Hyland Immuno, Baxter Healthcare Ltd, UK) before its withdrawal from the market. During a six week period when attending University College London Hospitals for assessment, patient 1 underwent a trial of two alternative factor VIII products: *Fahndi* (Grifols UK Ltd, Thetford, UK) and *Haemate P* (Aventis Behring, Haywards Heath, UK). These two concentrates were selected before the results of intrinsic VWF-CP activity were known.

Clinical response

Patient 1 failed to respond to either of the factor VIII concentrates as demonstrated by standard markers of disease activity (platelet count and lactate dehydrogenase, LDH). However both platelet count and LDH values subsequently normalised following his usual maintenance cryoprecipitate infusion (see *Figure 5.9*).

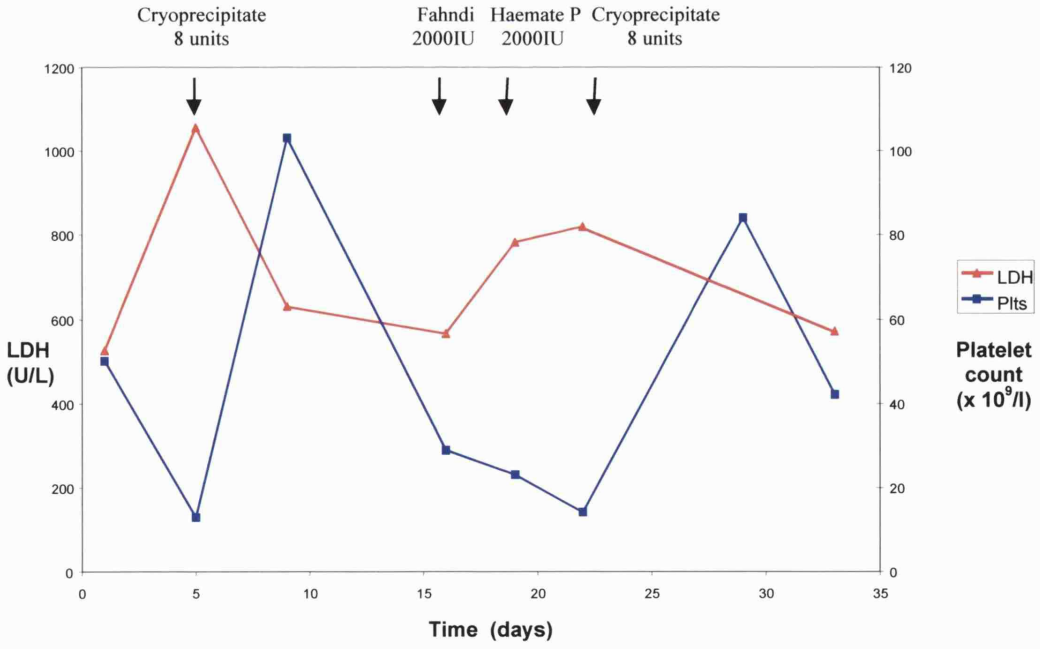


Figure 5.9 *Patient 1: response of platelet count and lactate dehydrogenase (LDH) to cryoprecipitate, Fahndi and Haemate P over 33 days.*

Trial of 8Y

Of the commercially available concentrates, the intermediate factor VIII product *BPL 8Y* was identified as that containing the greatest intrinsic VWF-CP activity in the comparative studies reported above in section 5.3.2. This was therefore selected for a therapeutic trial in patient 2. Platelet count and VWF-CP activity were measured serially over the following fourteen days to assess response.

Clinical response

A bolus of 1500 units (30U/kg) *BPL 8Y* was initially administered to patient 2 at a nadir platelet count of 49 x 10^9/l. Although there was a progressive rise in platelet count over the next seven to nine days only a minimal increase in VWF-CP activity was detected at six hours. By fourteen days the platelet count had fallen back to baseline (see *Figure 5.10*). This response was reproducible: for the past eight months the patient has been satisfactorily maintained on 500 units (10U/kg) *BPL 8Y* administered weekly.

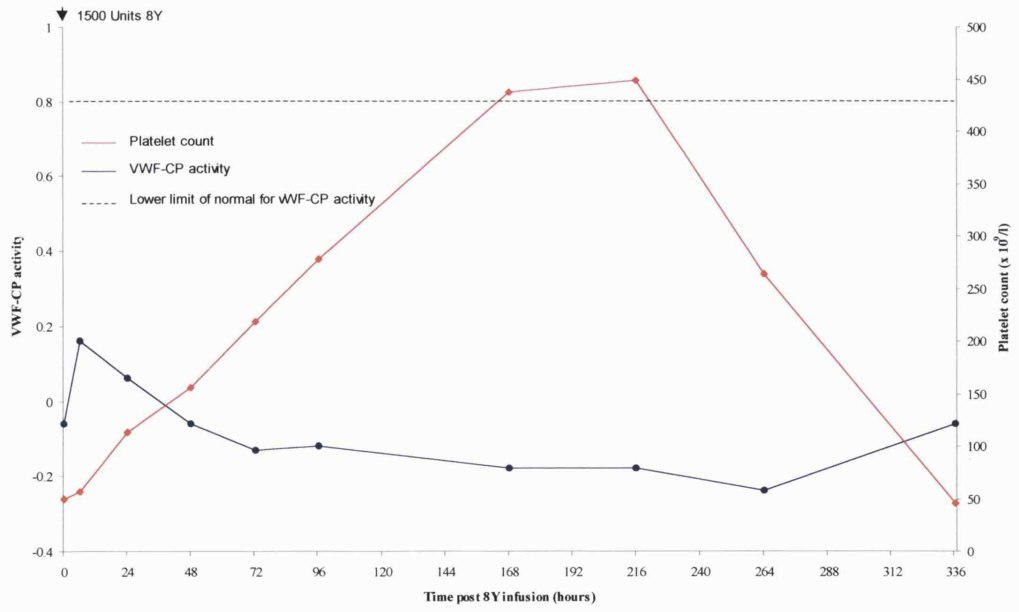


Figure 5.10 *Platelet count and VWF-CP activity in patient 2 following trial of 1500 units (30U/kg) BPL 8Y.*

5.4 Discussion

Congenital TTP was first associated with an absolute deficiency of VWF-cleaving protease activity in 1997 (Furlan *et al*, 1997). Severe protease deficiency in the absence of a circulating inhibitor has been a consistent finding in subsequent reports (Furlan *et al*, 1998b;Barbot *et al*, 2001). Using a genome wide linkage scan in four pedigrees with seven affected inidividuals, DNA analysis identified mutations in all four pedigrees within the *ADAMTS13* gene located on chromosome 9q34 (Levy *et al*, 2001). Simultaneous purification and sequence analysis of the amino-terminal amino acids of VWF-CP by two independent groups has confirmed the identity of ADAMTS13 as the VWF-CP. These findings support the hypothesis that reduced VWF-cleaving protease activity is pivotal to the pathogenesis of congenital TTP.

The classical presentation of congenital TTP is one of symptomatic anaemia and thrombocytopenia which is usually present at birth. However it is increasingly recognised that some individuals may present much later in life. In one recent series, only ten of 23 subjects with constitutional VWF-CP deficiency had suffered a first TTP event in the first five years of life (median age 2 years). Eleven of the remainder had not become symptomatic until the third or fourth decade of life (median age 24 years) and two remained asymptomatic aged 37 and 44 years respectively (Lämmle *et al*, 2001). If VWF-cleaving protease activity alone was the sole determinant of congenital TTP it is conceivable that such variations in disease severity are secondary to endogenous VWF-cleaving protease activity. To address this issue four cases of congenital TTP, two of whom had clinically severe disease requiring prophylaxis whilst the remaining two, who were brothers, had relatively mild disease manifestations needing only symptomatic treatment, were studied. Patient 1 was tested on seven occasions over a period of 36 days and patient 2 on twenty four occasions over a period of 81 days encompassing a total of eight mild haemolytic episodes. In contrast, patients 3 and 4 were assessed during clinical and haemolytic remission two to twenty weeks after the last symptomatic episode as well as at relapse. Despite the wide variation in clinical severity, VWF-cleaving protease activity was significantly

reduced in all cases as compared to healthy adult controls. Not only was protease activity significantly reduced in pre-treatment samples but also in those obtained during apparent remission or post treatment (p value < 0.0001 in both instances). Furthermore there was no significant difference in protease activity between the two severely affected individuals as compared to the two brothers with milder disease. This suggests that variations in clinical phenotype are not solely determined by endogenous VWF-CP activity.

However as previously stated this assay does not allow accurate quantitation below a level corresponding to 3% activity of PNP. Differences in activity below this may nevertheless have an impact on clinical presentation. It is conceivable that less than 1% normal protease activity results in clinical symptoms even under steady state conditions whilst slightly higher levels (1–5% normal protease activity), although sufficient for normal basal post-secretory VWF processing, are inadequate following external precipitants. Certainly it is recognised clinically that precipitants such as bacterial and viral infections often precede haemolytic episodes in less severely affected cases of congenital TTP (Karpman *et al*, 1997). Increased VWF is released from the endothelium as part of the acute phase response and could overwhelm VWF-processing activity if this were already impaired. Although theoretical, several parallels for this model exist: for example, severe haemophilia A is characterised by spontaneous haemorrhage requiring prophylactic treatment whilst this is rare in moderate disease with slightly higher factor VIII levels.

VWF-CP deficiency does explain the presence of circulating ULVWF multimers in the plasma of patients with congenital TTP during periods of remission (Moake & McPherson, 1989;Chintagumpala *et al*, 1992). Several *in vitro* studies have implicated ULVWF as the platelet aggregating factor responsible for formation of platelet microthrombi. Circulating ULVWF multimers were a consistent feature irrespective of clinical symptoms in all four patients. This suggests either that there may be a threshold concentration or molecular size above which ULVWF must rise before spontaneous platelet aggregation is initiated or that simple protease deficiency

resulting in circulating ULVWF multimers is insufficient to precipitate TTP. Certainly a sibling from a family with a history of congenital TTP was found to have severe VWF-cleaving protease deficiency activity but has not, to date, had any reported symptoms suggestive of TTP (Furlan *et al*, 1998b). A possible explanation for the apparent failure of absolute protease activity to determine disease severity is that other genetic/environmental factors influence clinical phenotype. This is supported by the observation that, even within the same family the age of onset may be highly variable. Indeed in one family neonatal onset was seen in an individual with a protease activity of 0.06U/ml (6% normal) whilst a younger sibling with a protease activity of 0.02U/ml did not present until eight years of age (Levy *et al*, 2001). The possible role of endothelial heterogeneity, endothelial modulation, additional VWF processing pathways and complement activation has been considered in the previous chapter.

In this study the parents of patients 1 and 2, who both had severe disease, had moderately reduced levels of VWF-CP activity (median value 0.71 corresponding to 25 – 50% activity of PNP) as did one of the siblings of patient 1. Since these individuals were entirely asymptomatic, this suggests that a carrier state may exist and be identified in some kindreds. This has recently been confirmed in a study of four pedigrees in which plasma protease levels in the parents of the affected individuals were 0.51 – 0.68U/ml (equivalent to 51 – 68% of normal). A clear bimodal distribution for protease activity was seen between carriers and normal individuals (Levy *et al*, 2001). In contrast the parents of the two less severely affected individuals studied here, patients 3 and 4, had protease activity within the normal range. However this apparent anomaly may reflect assay variability. That employed here is semi-quantitative and is by design less sensitive at higher protease activities. A protease activity equivalent to 50% that of PNP gives an activity ratio of approximately 0.85. The parents of patients 3 and 4 both had a protease activity ratio of 0.9 which may therefore be comparable to those levels reported in parents of affected individuals by Levy *et al*. Comparative studies of protease assays and identification of *ADAMTS13* mutations in the parents would address this issue. All of the identified carriers in this series were entirely asymptomatic and to date no clinical phenotype has been reported

in *ADAMTS13*-mutation heterozygotes. Whether such heterozygote carrier individuals themselves have an increased susceptibility to thrombosis or idiopathic or secondary TTP is currently a matter of conjecture.

Although it appears that VWF-CP activity may be modulated by as yet unidentified factors the *in vitro* studies performed support the hypothesis that VWF-cleaving protease activity is critical to the pathogenesis of congenital TTP. Plasma products known to be clinically efficacious in the treatment of congenital TTP (FFP, cryosupernatant and S/D treated plasma) were associated with VWF-cleaving protease activity comparable to that of PNP. Somewhat unexpectedly, so too was cryoprecipitate. However, this is consistent with previous reports of effective treatment of congenital TTP in response to a factor VIII/VWF product (Karpman *et al*, 1996;Karpman *et al*, 1997;Miura *et al*, 1984;Hara *et al*, 1986) and the sustained response for over ten years to both *Kryobulin* and cryoprecipitate seen here in patient 1. Moreover loss of high and intermediate sized VWF multimers was greater following incubation of VWF with *Kryobulin* than with PNP. In contrast, patient 1 showed no clinical response to either *Haemate P* or *Fahndi*. This therapeutic failure, however, can be explained by the fact that both these products had a mean protease activity below 0.55 (equivalent to 12.5% activity of PNP). After screening of several factor VIII concentrates, *BPL 8Y* was identified as a possible alternative since *in vitro* it possessed protease actitiviy at least equivalent to that of FFP. Dilution studies performed using *BPL 8Y* appeared to demonstrate an intrinsic protease activity comparable to that PNP. However the methodology used is likely to underestimate the intrinsic protease activity of the factor VIII concentrate due to its much higher relative VWF:Ag concentration as compared to PNP. *BPL 8Y* was therefore selected for use in patient 2. As demonstrated in *Figure 5.10* there was a progressive rise in platelet count following an infusion of 1500 units before falling back towards baseline values 14 days later. This is a consistent response: *BPL 8Y* infusions (10 units/kg) administered weekly have now been used as maintenance treatment for six months. Symptomatic haemolytic episodes have been prevented and the nadir platelet count maintained above 150 x 10^9/l. Thus the efficacy of plasma and plasma derived products in the

treatment of congenital TTP appears to parallel their intrinsic VWF-cleaving protease activity, supporting the theory that absence of this novel protease activity is of prime importance in this condition.

The explanation for the disparate intrinsic VWF-cleaving protease activities of the factor VIII concentrates is unclear. Although there was a trend to decreasing protease activity with increasing purity: no monoclonal antibody purified or recombinant factor VIII product tested had a protease activity greater than 0.25 (equivalent to 3% activity of PNP), of those concentrates showing protease activity comparable to PNP, two are classified as high purity whilst the remaining two are intermediate purity concentrates. Moreover, not all intermediate purity factor VIII products possessed significant protease activity (defined as VWF-CP activity > 0.8). Although the reasons for these discrepancies remain unclear, the heterogeneous nature of factor VIII concentrates has long been recognised (Lawrie *et al*, 1989) and it is accepted that this has a profound effect on the efficacy of factor VIII concentrates in the treatment of von Willebrand's disease (Pasi *et al*, 1990;Mannucci *et al*, 1992). Presumably a similar phenomenon is at play here and reflects methodological differences in manufacturing resulting in either loss or relative preservation of protease activity.

Despite the observation that clinical efficacy of plasma and plasma derived products correlated with *in vitro* assessment of VWF-CP activity there was no dramatic rise in protease activity following infusion of *BPL 8Y* in patient 2. One hour after infusion of 1500 units protease activity rose to 0.16. However this is still less than 3% activity of PNP. Furthermore by 48 hours levels had fallen to pre-infusion values. Despite this there was a progressive rise in platelet count for seven days following the infusion before it plateaued and then fell back to baseline. The biologic half-life of VWF-CP has been determined in two patients with constitutional deficiency and calculated to be 3.3 and 2.1 days respectively (Furlan *et al*, 1999). Although this is uniquely long for a proteolytic enzyme it is still appears insufficient to account for the dosage frequency in view of the minimal increment in protease activity. Uptake of protease onto endothelial cells or circulation of VWF-CP as an inactive precursor that is slowly but

continuously activated in the presence of calcium ions might account for this discrepancy. However as previously discussed this protease assay is only semi-quantitative since it is based on multimeric degradation which is used as a surrogate marker for cleavage of the Tyr 842-Met 843 bond. Complete multimeric degradation is not a physiological phenomenon. Instead proteolysis to multimers of a range of molecular weights from 500 to 20,000kDa is seen. This may require a much lower protease activity than that necessary for complete loss of high and intermediate molecular weight multimers *in vitro*. The evolution of more sensitive protease assays will allow this question to be addressed.

In conclusion, severe VWF-CP deficiency does appear central to the pathogenesis of congenital TTP but clinical phenotype appears to be modulated by as yet unidentified factors. Carrier individuals may be identified by moderate decreases in VWF-CP activity but this does not seem to confer a disease association. Like idiopathic adult onset TTP, low levels of VWF-CP activity do not invariably result in clinical symptoms suggesting additional external precipitants or as yet unidentified pathological factors may be required for disease manifestation. Nevertheless *in vitro* assessment of VWF-cleaving protease activity of plasma derived products and factor VIII concentrates correlates with clinical efficacy. Further research is warranted to address issues such as batch to batch variation in protease activity. If factor VIII concentrates are to be used clinically the potential hazards of increased plasma concentrations of factor VIII and VWF must also be considered. Platelet transfusions in TTP have been temporally associated with clinical deterioration (Gordon *et al*, 1987) (Harkness *et al*, 1981) and gross elevations in circulating VWF concentrations, particularly if a concentrate contained high molecular weight multimers, could have a similar deleterious effect. However, such an approach would allow home treatment with consequent improvements in quality of life. The recent identification of *ADAMTS13* as the VWF-CP should allow those factor VIII concentrates with high *ADAMTS13* content to be identified although it is likely that future therapy will focus on the production of recombinant *ADAMTS13*.

6. Investigation into the nature of VWF-cleaving protease deficiency in TTP

6.1 Introduction

In the two preceding chapters both adult-onset idiopathic and congenital TTP were associated with VWF-CP deficiency in accordance with published literature (Furlan *et al*, 1998b;Tsai & Lian, 1998). Congenital TTP responds promptly to simple plasma infusion (Chintagumpala *et al*, 1992) whilst adult onset idiopathic TTP requires multiple plasma exchange procedures, often over a prolonged time period, before clinical remission is attained (Rock *et al*, 1991). An explanation for such clinical disparity is that congenital TTP has been linked to a constitutional deficiency of VWF-CP activity (Furlan *et al*, 1997;Furlan *et al*, 1998b) secondary to mutations within the *ADAMTS13* gene (Levy *et al*, 2001). In contrast, protease deficiency in adult-onset TTP appears to be secondary to the acquisition of a circulating inhibitory antibody of IgG subtype (Furlan *et al*, 1998b;Tsai & Lian, 1998). Adult-onset TTP does have some clinical features typical of an auto-immune disease: female preponderance and familial clustering. It is also associated with other auto-immune disorders such as SLE, rheumatoid arthritis and Sjögren's syndrome (Stricker *et al*, 1992;Musio *et al*, 1998;Porta *et al*, 1999;Krupsky *et al*, 1991). Immune complexes and several autoantibodies such as anti-CD36 antibodies and AECA have been reported as pathogenic factors in the aetiology of TTP (Burns & Zucker-Franklin, 1982;Koenig *et al*, 1993;Tandon *et al*, 1994;Schultz *et al*, 1998). Although the presence of autoantibodies does not necessarily infer causality, which could equally be coincident to or the consequence of clinical events, a recent case report describing deteriorating clinical course with increasing inhibitory activity supports the former hypothesis (Tsai, 2000). The disparate response to plasmapheresis which is seen in clinical practice has not been consistently explained by presenting platelet count (Pereira *et al*, 1995;Rock *et al*, 1998) or degree of neurological impairment (Pereira *et al*, 1995). Whether this

may be attributable to presenting inhibitory titre is unknown and is further addressed in this chapter.

It was demonstrated in chapter 4 that prolonged clinical remission may occur in the context of persisting deficiency of VWF-CP activity. This suggests that hitherto unidentified factors are also involved in the pathogenesis of TTP or may modulate the clinical effects of protease deficiency. TTP has been proposed to be primarily a disease of the endothelium. Markers of endothelial damage are elevated in TTP (Anthony *et al*, 1998;Wada *et al*, 1998) and increased levels have been associated with increased mortality (Wada *et al*, 1998). Initial endothelial damage may lead to the activation and local exposure of negatively charged phospholipid on the surface of endothelial cells and platelets. Phospholipid binding proteins eg. β_2-glycoprotein I (β_2GPI) and prothrombin may then bind to such potentially reactive phospholipids. If present, antiphospholipid antibodies (APA) may concentrate on the cell surface, bind to cellular FcγRII receptors and induce prothrombotic changes. APA have been shown to promote platelet activation (Wiener *et al*, 1991;Martinuzzo *et al*, 1993;Forastiero *et al*, 1998) and tissue factor expression on endothelial cells (Branch & Rodgers, 1993). They might, therefore, contribute to the development of TTP which is characterised by widespread microvascular thrombosis. Certainly APA are common in sytemic lupus erythematosus (SLE) which has been associated with TTP (Musio *et al*, 1998). In a review examining the results of 21 studies of 1428 patients with a diagnosis of SLE, 554 (39%) possessed APA and there were 346 patients with a history of thrombosis (24%). Thrombosis was much more frequent in those patients with APA (42%) than those without (13%) suggesting a direct pathogenic role for APA in the promotion of thrombosis (McNeil *et al*, 1991).

To investigate the influence of protease inhibitory activity on clinical course, correction studies (50:50 mix of patient plasma with PNP) were performed in a total of 13 patients presenting with adult-onset idiopathic TTP. Three adult patients with recurrent episodes of TTP maintained on long-term prophylactic FFP infusion regimens were also studied. In order to verify the association and specificity of

inhibitory VWF-CP activity for adult-onset TTP, four patients with congenital disease were also included in the investigation. The *in vitro* effects of affinity purified patient IgG on protease activity of PNP was subsequently assessed both at presentation and during remission and correlated with clinical outcome. To investigate the possible facilitatory role of APA in the development of TTP, anticardiolipin (ACL), anti-β_2GPI and anti-prothrombin (PT) antibodies were measured at presentation of adult-onset idiopathic TTP and secondary TTP cases. VWF-CP activity was also measured in a group of previously well characterised patients with a diagnosis of primary antiphospholipid syndrome (PAPS) or SLE.

6.2 Methods

6.2.1 Patients

Thirteen patients (nine female, four male) with adult onset idiopathic TTP presenting to University College Hospital were studied at diagnosis and serially throughout an episode of TTP. Four patients with congenital TTP and three adults on prophylactic infusion programmes for recurrent disease were also studied. All of these patients and nine with a diagnosis of secondary TTP were assessed for the presence of ACL, anti-β_2GPI and anti-PT antibodies whilst VWF-CP activity was measured in 23 well documented cases of PAPS and seven cases of SLE, four of whom were APA positive.

6.2.2 Blood collection

A vacutainer system® was used to obtain blood samples in adults and children over the age of 12. In children under the age of 12 blood was collected into 0.106M citrate (1 in 10) using a butterfly and syringe technique. Plasma and serum was prepared for specific assays as outlined in section 2.1.

6.2.3 Von Willebrand factor-cleaving protease activity

VWF-CP activity was assessed according to the final method described in section 2.5.

6.2.4 Von Willebrand factor-cleaving protease activity correction studies

VWF-CP activity correction studies were performed as described in section 2.5.1.

6.2.5 *In vitro* effect of patient IgG on VWF-cleaving protease activity of PNP

Patient IgG was isolated by affinity purification on a protein G column and concentrated to 2mg/ml as outlined in section 2.6. Patient or control IgG (VIGAM, Bio Products Laboratory, UK), which had undergone identical elution and concentration procedures, was added to 100µl PNP and 10mM Tris (hydroxymethyl)methylamine 0.015M NaCl pH 8.0 to give the following IgG concentrations: 0.1, 0.2, 0.4, 0.8 and 1.6mg/ml in a final volume of 500µl. This was then incubated at 37°C for one hour. 10µl $BaCl_2$ 100 mM was subsequently added to a 90µl aliquot of the incubation mix. Further dilution in sample buffer was not required since the PNP had already undergone a 1 in 5 dilution. The remainder of the assay was performed in accordance with the final method described in section 2.5.

6.2.6 Enzyme Linked Immunosorbent Assays (ELISA)

All TTP, SLE and PAPS patients had the following parameters measured using an ELISA technique as described in section 2.3: ACL, anti-β_2GPI and anti-PT antibodies.

6.3 Results

6.3.1 Idiopathic adult onset acute TTP

Patient characteristics

The characteristics of the 13 patients presenting with idiopathic adult onset TTP to University College Hospital, London are shown in *Table 6.1* and were also presented in Tab*le 4.1*. Three of the patients subsequently relapsed, one on two occasions allowing a total of 17 episodes to be studied.

Correction studies at presentation

All of the patients presenting with acute idiopathic TTP had a protease activity ratio of less than 0.2 (median value 0.0, range –0.14 – 0.18). This is equivalent to an activity below 3% that of PNP and is the lower limit of sensitivity of the assay. Correction of protease deficiency was defined as a protease activity ratio in a 50:50 mix of patient and PNP that was within the normal range (greater than 0.8). This was seen in only two of the 17 cases although seven in total overlapped with values of protease activity obtained in normal controls (protease activity ratio greater than 0.7). The remaining ten samples (59%) had protease activity ratios of less than 0.55, equivalent to an activity of 12.5% that of PNP (*Figure 6.1*). This was suggestive of circulating inhibitory activity.

Patient	Sex	Age (at initial presentation)	Platelet count (x 10^9/l)	CNS dysfunction	Fever	Past history of TTP	Number of plasmaphereses to CR	Protease correction
1	F	38	19	+	-	-	28	0.70
2	M	50	8	+	+	-	32	0.73
			82	-	-		48	0.74
3	M	47	7	+	+	+	14	0.74
			12	-	-	-	6	0.18
4	F	37	19	+	-	-	†	0.28
6	F	26	16	+	+	-	26	0.12
7	F	40	9	+	-	+	6	0.82
8	M	35	14	+	+	-	33	0.19
9	M	37	7	+	+	+	48	0.53
10	F	24	7	+	+	-	17	0.82
11	F	54	14	+	-	-	15	0.22
12	F	54	4	+	+	-	76	0.13
13	F	24	9	+	+	-	29	0.70
14	F	45	17	-	+	+	Not treated at UCH	0.52
			8	-	?		19	-0.03
			3	-	+		7	-0.06

Table 6.1 *Clinical and laboratory features of 13 patients presenting with idiopathic TTP.*

F =female; M = male. † = arrested and died during first plasmapheresis. Protease correction represents protease activity ratio of 50:50 mix of patient plasma and PNP at acute presentation before initiation of plasmapheresis. All patients had had severe protease deficiency (protease activity ratio < 0.2) documented at presentation.

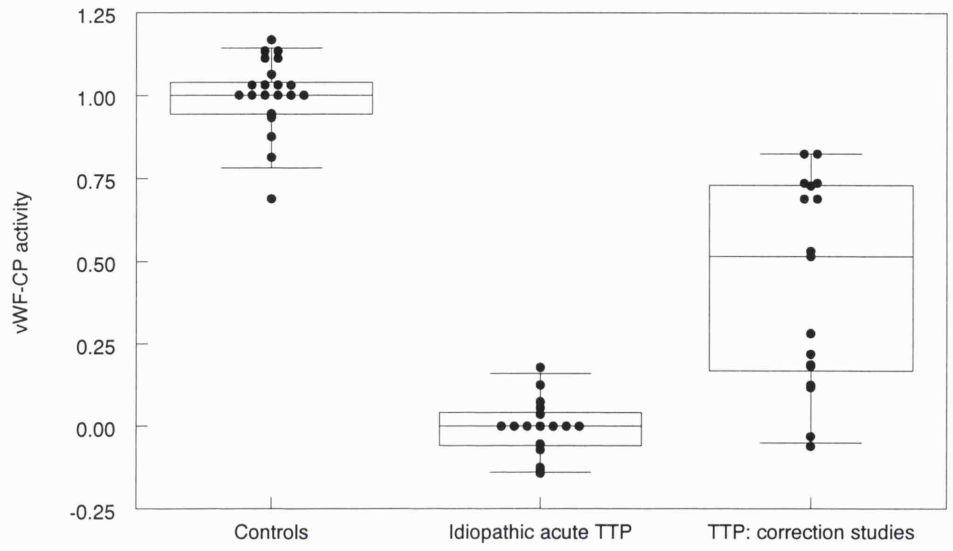


Figure 6.1 *Plasma levels of VWF-CP activity in controls (n=25) and acute TTP either at presentation or in a 50:50 mix of patient plasma and PNP (n=17).* Horizontal line indicates median, box the 25th to 75th percentile and bars the 5th and 95th percentile.

In the majority of patients VWF-CP activity showed some, if not complete, recovery with treatment. However three of the patients (patient numbers 6, 8 and 14) achieved clinical remission despite persistent protease deficiency (protease activity ratio less than 0.2). As described in chapter 4, there was apparent spontaneous recovery in protease activity in one of these patients, patient 6, between days 265 and 359 post index presentation. Pregnancy had been confirmed on day 198 and a prophylactic regimen of weekly plasmapheresis and infusion was temporarily introduced before reducing the intensity of treatment. From days 265 to 359 only a single plasma exchange and four plasma infusions were administered. As illustrated in *Figure 6.2*, protease activity of a 50:50 mix normalised approximately three months before the intrinsic protease activity of patient plasma. There has been no relapse after a further 18 months follow-up in the absence of maintenance therapy other than low dose aspirin.

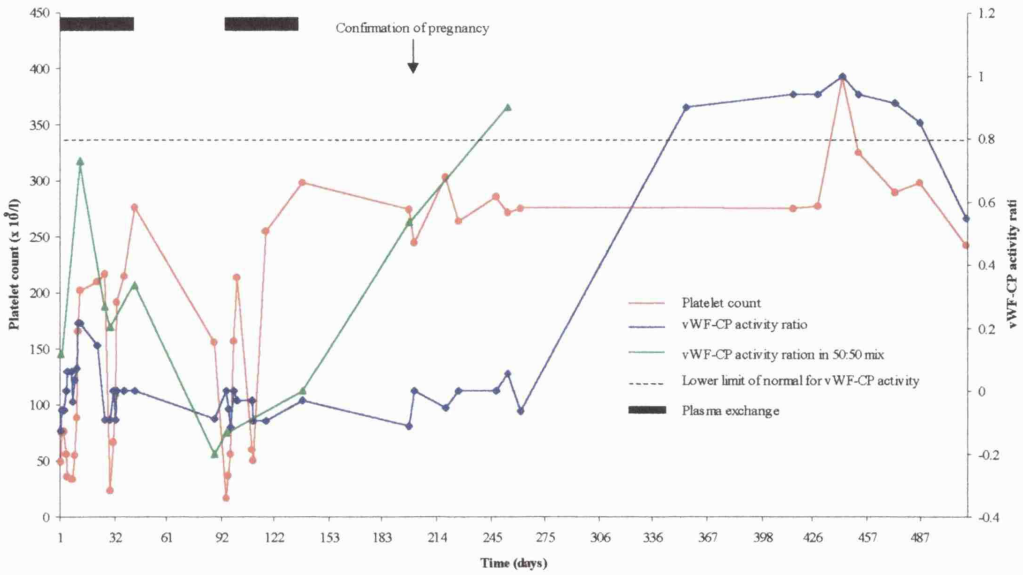


Figure 6.2 *Patient 6 presented on day 1 and attained clincial remission on day 38. There was an early relapse necessitating reinstitution of plasmapheresis on day 84. A durable remission ensued despite persistent severe protease deficiency. Protease activity of a 50:50 mix of patient and PNP normalised approximately three months before the intrinsic protease activity of patient plasma.*

It has already been shown in chapter 4 that protease activity ratio at presentation of acute TTP does not discriminate between those patients with an uneventful versus a protracted course. However the above observation suggested that the protease activity ratio of a 50:50 mix of patient and PNP at presentation might predict clinical response. Patients were therefore placed into two subgroups according to the protease activity ratio of the 50:50 mix at presentation. The normal range of protease activity in normal controls is 0.8 – 1.2. Some normal individuals however have an activity ratio of only 0.7. This was chosen as the discriminating value as it represented the lower limit of the range of activity seen in healthy controls. The number of plasmaphereses required to attain durable remission was used as a surrogate marker for clinical response. To minimise the influence of prolonged, and possibly unnecessary, tapering of plasma exchange procedures, a durable remission was defined as normalisation of platelet count and LDH which was maintained for four weeks after cessation of plasmapheresis. If exacerbation occurred within this time period and reinstitution of plasma exchange was required, the total number of procedures undertaken was used in

the analysis. The median platelet count at presentation of those patients with demonstrable inhibitory protease activity was 10 x 10^9/l (range range 3 – 16 x 10^9/l). In comparison, it was 9 x 10^9/l (range 7 – 82 x 10^9/l) in those presenting with a protease activity ratio greater than 0.7 on a 50:50 mix with PNP. A median number of 27 plasmaphereses were required to attain CR in the former group as compared to 17 procedures in the latter.

In vitro effect of patient IgG on VWF-CP activity of PNP

The correction studies suggested that some, but not all patients presenting with idiopathic TTP had potent circulating inhibitors of protease activity. To determine whether this was attributable to auto-antibodies of IgG subtype, the *in vitro* effect of patient IgG on VWF-CP activity of PNP was determined in eight patients presenting with idiopathic adult onset TTP. Protease activity of a 50:50 mix of patient plasma with PNP was less than 0.2 in four cases (patient numbers 6, 8, 12 and 14), consistent with a circulating inhibitor. The *in vitro* effect of IgG purified from these four was compared with IgG prepared from four patients in whom the protease activity ratio was greater than 0.7 in a 50:50 mix of patient plasma with PNP (patient numbers 1, 2, 3 and 7). A commercial IgG preparation (VIGAM, BPL, UK) eluted and concentrated in an identical manner was used as a negative control. In all cases, IgG was added over a range of final concentrations from 0.1 – 1.6mg/ml.

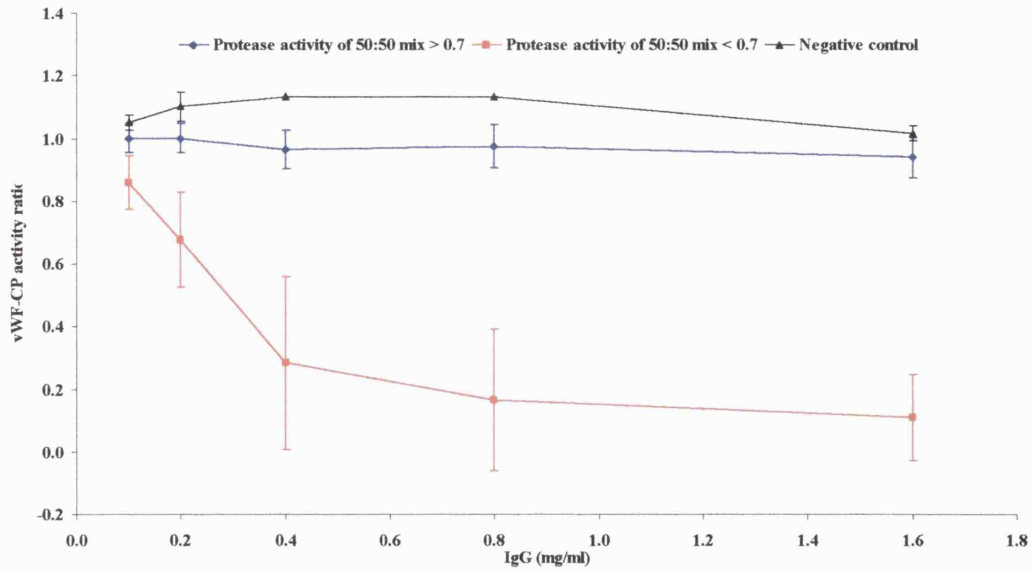


Figure 6.3 *The in vitro effect of IgG purified from patients in whom protease activity ratio was less than 0.7 in a 50:50 mix of patient plasma with PNP (n=4) as compared with IgG prepared from those in whom protease activity ratio was greater than 0.7 in a 50:50 mix of patient plasma (n=4). A commercial IgG preparation serves as a negative control.*

Mean ± SD are indicated.

Over the concentration range tested, as compared to the negative control (VIGAM), IgG eluted from patient sera in whom presenting protease deficiency was largely corrected by a 50:50 mix with PNP showed no demonstrable inhibition of intrinsic protease activity of PNP. In contrast there was a concentration dependent inhibition of protease activity with IgG isolated from patient sera in whom protease deficiency was not corrected by the addition of PNP. There was however no difference in the median number of exchange procedures required to attain a platelet count above 150 x 10^9/l between these two groups (11.5 as compared to 12.25). In contrast, a durable remission, defined as above, required a median of 18.5 procedures in the former case as compared to 45.5 in the latter.

Remission

If such inhibitory IgG activity were of importance in the pathogenesis of TTP, inhibitory titre should fall with disease resolution. In chapter 4, VWF-CP activity was assayed serially in 24 patients presenting with idiopathic TTP. This allowed

identification of three patients (patient numbers 6, 8 and 14) who had persistent severe protease deficiency which failed to correct on a 50:50 mix with PNP despite attainment of durable clinical remission (*Table 6.2*). The *in vitro* effects on intrinsic protease activity of PNP of IgG derived from patient sera at remission was compared to that of IgG isolated at acute presentation and shown in *Figure 6.4.*

Patient	Hb (g/dl)	Platelet (x 10^9/l)	Retics (%)	Creat (µmol/l)	Bili (µmol/l)	LDH (IU/l)	VWF-CP activity ratio	VWF-CP in 50:50 mix
6	11.3	271	1.4	55	5	337	0.05	0.9
8	14.9	254	0.6	79	4	365	0.05	0.70
14	11.2	235	1.4	-	-	377	-0.1	0.07

Table 6.2 *Laboratory findings in patient numbers 6, 8 and 14 when in apparent haematological remission from TTP at time of investigation of in vitro effect of patient IgG on intrinsic VWF-CP activity of PNP.*

The *in vitro* inhibitory activity of IgG isolated from remission samples of these three patients was less potent in comparison to IgG derived from serum at presentation. Nevertheless there was still a trend a concentration dependent inhibition of VWF-CP activity. Two of these three patients have subsequently relapsed and protease activity of the third (patient number 6) spontaneously recovered (as discussed above).

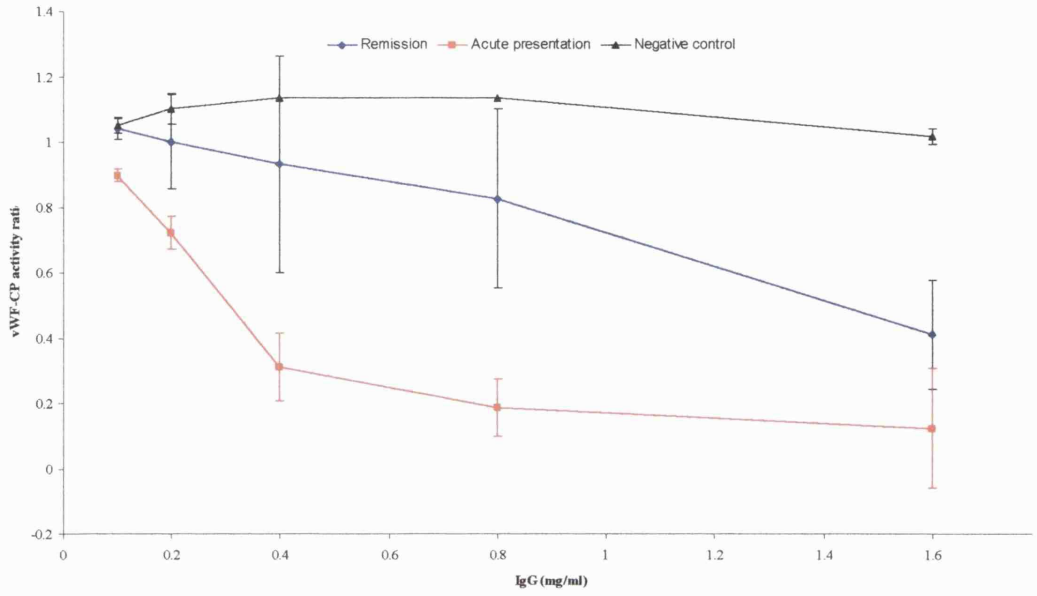


Figure 6.4 *The in vitro effect of IgG on intrinsic VWF-CP activity of PNP. IgG was prepared from both acute presentation and remission samples of patient numbers 6, 8 and 14 who had persistent VWF-CP deficiency when in apparent haematological remission from TTP. A commercial IgG preparation serves as a negative control.*

Mean ± SD are indicated.

In chapter 4, VWF-CP activity was measured in a total of 26 patients with a history of TTP who remained under routine clinical haematological follow-up. This allowed identification of an additional eight patients with a protease activity ratio of less than 0.2 in apparent clinical remission (patient numbers 2, 11, 22, 27, 33, 35, 36 and 37). Three (patient numbers 11, 22 and 37) were recovering from a recent acute episode and subsequently relapsed within four weeks. Thus they were not in durable remission and will not be considered further here. Of the remaining five patients, two (patient numbers 2 and 35) had a history of severe intermittent disease and both relapsed within four months of analysis. In contrast the remaining three (patient numbers 27, 33 and 36 were studied in durable remission, remote from active therapy (other than aspirin) and have remained asymptomatic for at least six months following protease analysis. Haematological indices were within the normal range and there were no clinical symptoms of note. Results of VWF-CP and correction studies are given in *Table 6.3.*

Patient	VWF:Ag	VWF multimer analysis	VWF-CP activity ratio	VWF-CP in 50:50 mix
27	0.86	ULVWF	0	0.82
33	2.1	ULVWF	-0.05	-0.13
36	0.79	Normal	0.06	0.82

Table 6.3 *Results of VWF-CP activity and correction studies of patients 27, 33 and 36 studied in durable clinical remission in the absence of treatment other than aspirin.*

Protease deficiency was corrected by a 50:50 mix with PNP in two of the three cases but was persistent in the third. IgG was purified from two of the three patients, patient number 27 in whom protease deficiency was corrected by a 50:50 mix with PNP and patient number 33 in whom it was not. This allowed the *in vitro* effect of patient IgG on the intrinsic protease activity of PNP to be studied. In keeping with the correction studies, IgG purified from patient number 27 had no demonstrable inhibitory protease activity, its effects being comparable to the negative control. In contrast that purified from patient number 33 resulted in profound inhibition of the intrinsic protease activity of PNP even at a final concentration of 0.1mg/ml. This was more extreme than that seen with IgG prepared from presentation samples of the four patients studied in whom a 50:50 mix with PNP failed to correct protease deficiency (*Figure 6.5*).

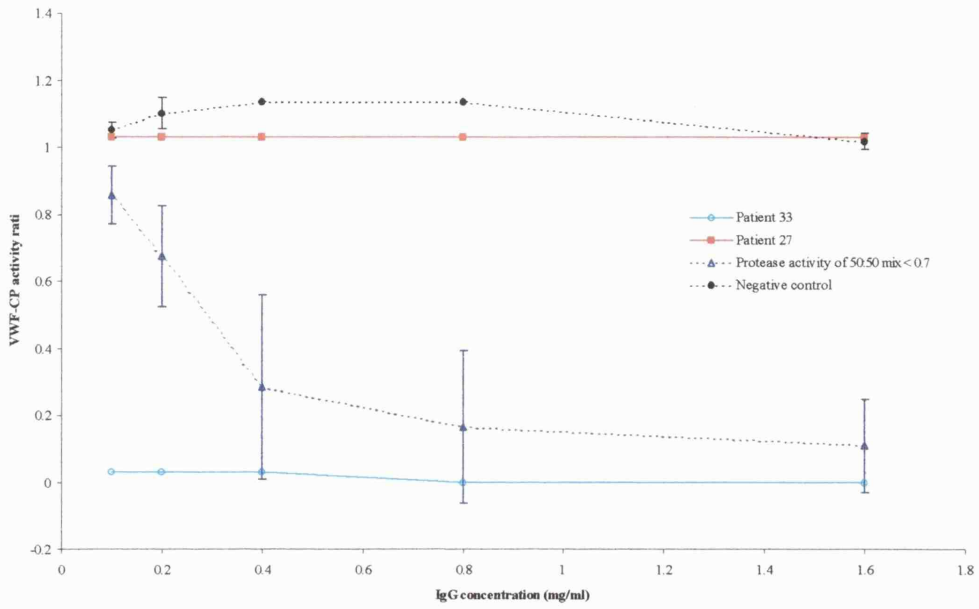


Figure 6.5 *The in vitro effect of IgG on intrinsic VWF-CP activity of PNP. IgG was prepared from two patients (patient 27 and 33) who were in haematological remission at the time of study despite persistent VWF-CP deficiency. The effects of IgG prepared from presentation samples of the four patients studied in whom protease activity ratio was less than than 0.7 on a 50:50 mix of patient plasma with PNP is included for comparison. A commercial IgG preparation serves as a negative control.*

Mean ± SD are indicated for control samples.

6.3.2 Recurrent TTP (ADULT)

TTP is a clinically heterogeneous group. Idiopathic, secondary, single episode and intermittent TTP are all recognised as clinical variants. However some adult patients may not easily fit with this classification. A small number of adult patients with a prior history of recurrent disease are maintained on prophylactic intermittent plasma infusions in an attempt to prolong remission. Whether such cases represent late presentation of constitutional deficiency of protease activity (cf congenital TTP) or the presence of persistent inhibitory protease activity is unclear. The protease activity and *in vitro* inhibitory IgG activity of three such patients was therefore investigated and compared to that of congenital and adult onset idiopathic disease.

Patient characteristics

Patient A is a 39 year old African woman. She first presented aged 30 with sudden onset of aphasia and weakness of the right hand and face. A diagnosis of TTP was made in the presence of thrombocytopenia and MAHA. A left parietotemporal infarct was confirmed on CT scanning. Since this index presentation she has suffered six relapses. Five of these have occurred despite weekly maintenance FFP infusions in combination with antiplatelet medication, immunosuppressive therapy (azathioprine, cyclophosphamide) and prostacyclin infusions. Protease activity and correction studies were performed whilst in clinical and haematological remission ten months after the last relapse. During this period she was receiving weekly prophylactic S/D plasma (600ml) and prostacyclin infusions in addition to aspirin 75mg od and dipyridamole 100mg tds. Plasma had been administered six days prior to venepuncture.

Patient B is a 51 year old male. He presented aged 47 with confusion, thrombocytopenia and MAHA and was diagnosed with TTP. Remission was attained after a course of plasmapheresis. Within 12 months he relapsed and suffered an occipital infarct. A maintenance programme of FFP infusions was initiated at this juncture. Despite this he has had two further relapses with additional permanent neurological deficits. He has now been in remission for two years. Prophylaxis includes aspirin 75mg od and fortnightly infusions of FFP (600ml). Samples were taken 24 hours preceding maintenance FFP infusion on three separate occasions.

Patient C is a 46 year old female who initially presented with TTP at six months gestation in her one and only pregnancy. Since then she has required fortnightly infusions of FFP to prevent relapses. If these are delayed, abdominal pain and occasionally neurological symptoms develop which are rapidly ameliorated by plasma infusion. Of interest she appears to have had intermittent anaemia and thrombocytopenia from 18 months of age. She has three siblings who are unaffected. Protease activity and mixing studies were performed immediately prior to FFP infusion.

Patient	Hb (g/dl)	Platelet (x 10^9/l)	Reticulocyte count (%)	Creatinine (μmol/l)	Bilirubin (μmol/l)	LDH (IU/l)
A	11.9	215	1.04	82	20	353
B	12.9	159	0.89	120	7	393
C	13.3	261	-	128	6	-

Table 6.4 *Representative laboratory features of three patients with recurrent TTP.*

Correction studies

When investigated, all three patients were in apparent clinical remission as shown by standard markers of disease activity shown in *Table 6.4*. Nevertheless, VWF-CP activity was severely depressed in all samples assayed (median –0.06, range –0.1 - – 0.03). Protease activity of a 50:50 mix of patient plasma with PNP showed complete correction only in the case of patient C whilst this was incomplete in patients A and B (*Figure 6.6*). Of note, over the duration of prophylaxis, patient C has suffered no overt haemolytic episodes whilst both patients A and B have had severe relapses necessitating reinstitution of plasmapheresis.

In vitro effect of patient IgG on VWF-CP activity of PNP

There was no uniform effect of patient IgG on VWF-CP activity of PNP. Instead the results mirrored those of the correction studies. Thus there was no evidence of inhibition with IgG derived from patient C. This was similar to those results obtained with congenital TTP (see section 6.3.3 below). In contrast, IgG derived from the two remaining patients in whom protease activity was not corrected by a 50:50 mix with PNP showed concentration dependent inhibition consistent with the presence of an inhibitory IgG autoantibody. These effects were comparable to those obtained with IgG isolated from sera of patients with TTP in remission (*Figure 6.7*).

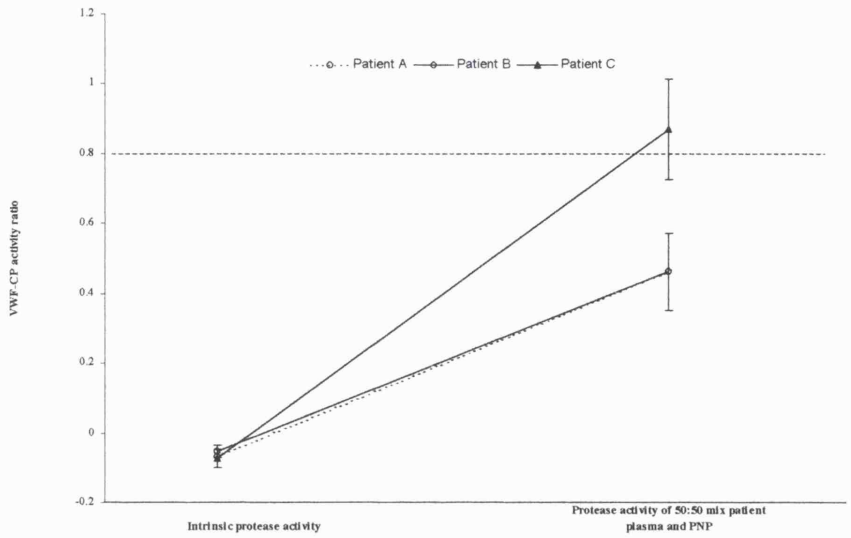


Figure 6.6 *VWF-CP activity during remission of three adult patients with recurrent TTP on prophylactic intermittent FFP infusions. Paired samples of intrinsic VWF-CP activity and that of a 50:50 mix of patient plasma and PNP are shown. Mean ± SD are shown in the cases of patients B and C.*

The dotted line represents the lower limit of normal of VWF-CP activity.

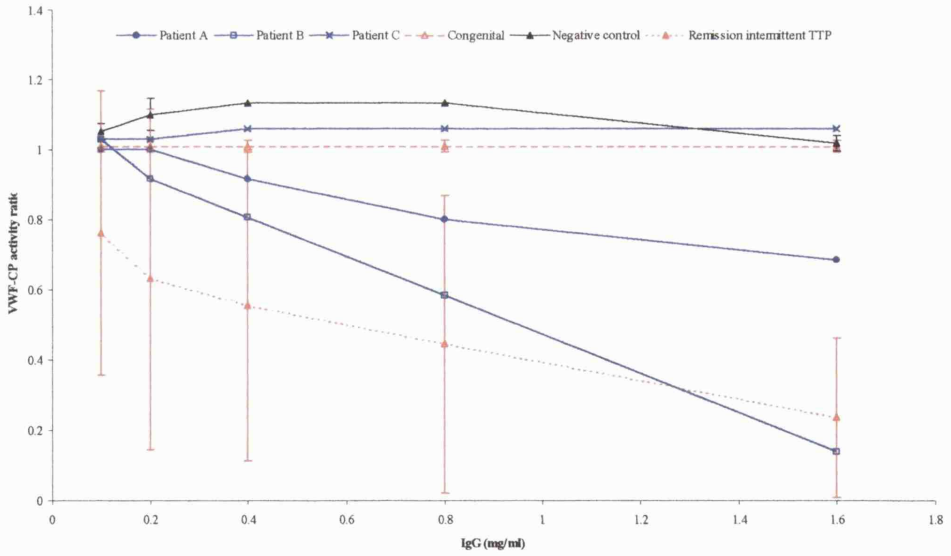


Figure 6.7 *The in vitro effect of IgG on intrinsic VWF-CP activity of PNP. IgG was prepared from three adult patients with recurrent TTP maintained on prophylactic intermittent FFP infusions. The effects of IgG prepared from patients with a previous history of TTP in haematological remission despite persistent depression of VWF-CP activity (n=6) and congenital TTP (n=4) are included for comparison. A commercial IgG preparation serves as a negative control.*

6.3.3 Correction studies in congenital TTP

Patient characteristics

The characteristics of the four patients diagnosed with congenital TTP have been described previously in chapter 5 and laboratory findings presented in *Table 5.2*.

Correction studies

A total of 25 samples were tested. Severe protease deficiency was demonstrated in each sample as shown in chapter 5. In contrast to those adults presenting with idiopathic TTP, protease deficiency was corrected in a 50: 50 mix (protease activity ration greater than 0.8) in all instances.

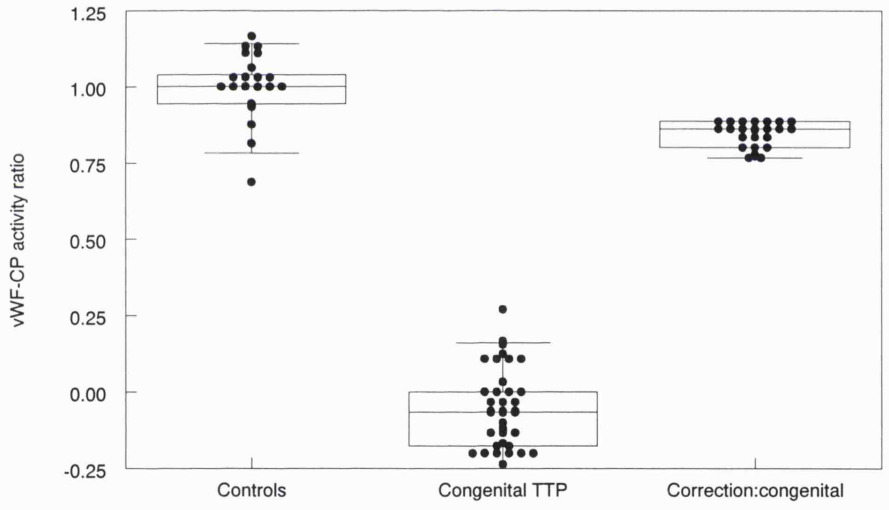


Figure 6.8 *Plasma levels of VWF-CP activity in controls (n=25), patients with congenital TTP (n = 25) and a 50:50 mix of congenital TTP plasma and PNP (n=25)*

Horizontal line indicates median, box the 25th to 75th percentile and bars the 5th and 95th percentile.

In vitro effect of patient IgG on VWF-CP activity of PNP

IgG purified from patients with congenital TTP had no effect on intrinsic VWF-CP activity of PNP over the range of IgG concentrations studied.

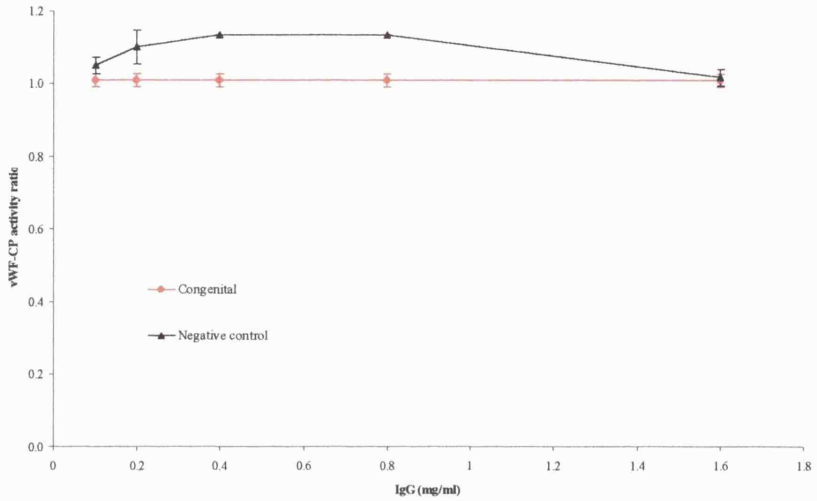


Figure 6.9 *The in vitro effect of IgG on intrinsic VWF-CP activity of PNP. IgG was prepared from four patients with congenital TTP. A commercial IgG preparation serves as a negative control.*

6.3.4 Antiphospholipid antibodies and TTP

The results above illustrate that even when there is *in vitro* evidence of inhibitory VWF-CP activity and secondary protease deficiency, symptoms of TTP do not automatically ensue although such patients do apppear to have a higher incidence of subsequent episodes. Presumably a "second-hit" is required to initiate microvascular platelet aggregation. Antiphospholipid antibodies (APA) may arise secondary to chronic endothelial perturbation which has been proposed to be involved in the pathogenesis of TTP. APA are heterogeneous and have a range of *in vitro* phenomenon including endothelial cell activation (Simantov *et al*, 1995), induction of endothelial cell apoptosis (Nakamura *et al*, 1994) and upregulation of TF on human umbilical vein endothelial cells (HUVEC) and monocytes (Branch & Rodgers, 1993;Amengual *et al*, 1998). Such phenomena are also reported in TTP. It was therefore proposed that APA may be involved in the pathophysiology of TTP. ACL,

anti β_2-GPI and anti-PT antibodies were measured at presentation of 16 patients with idiopathic adult onset TTP and nine patients with secondary TTP. The four congenital TTP patients and three adult patients with recurrent TTP on prophylactic regimens were also studied since such patients are hypothetically at greater risk of endothelial damage.

Results are shown in *Tables 6.5, 6.6* and *6.7.* Only four episodes of idiopathic TTP were characterised by elevated levels of APA. However only minor abnormalities were seen and these were not consistent between individuals. There was no association between the presence of APA and the failure of PNP to correct protease deficiency. Although the nine cases of secondary TTP had a higher incidence of APA, with an overall incidence of 55%, all but one individual had a concomitant diagnosis of HIV or SLE with which APA are independently associated. Similarly there were no striking APA abnormalites in patients with congenital TTP or those adults with recurrent disease on maintenance prophylactic regimens even though they are theoretically at higher risk of endothelial damage.

Patient Upper limit of normal range	ACL IgG 5 GPL units	ACL IgM 5 MPL units	$a\beta_2$ GPI IgG 3.5%	$a\beta_2$ GPI IgM 3.9%	aPT IgG 3.9%	aPT IgM 20.1%
1	2.6	1.0	0.8	**5.1**	**5.1**	7.9
2	1.7	2.6	1.5	1.6	3.4	8.2
3	1.3	17.7	1.4	1.4	3.4	5.5
4	1.7	2.6	1.2	2.3	2.1	11.4
5	0.5	2.6	1.1	2.5	2.0	4.4
6	3.1	2.6	1.0	2.6	2.2	11.0
7	1.1	2.6	1.7	3.6	2.5	7.6
8	3.6	1.2	0.9	**4.9**	2.2	2.3
9	0.4	0.8	2.3	1.6	3.3	6.3
10	0.6	2.6	1.7	1.4	3.5	5.8
11	1.7	0.2	3.3	1.8	3.2	6.4
12	**14.7**	2.6	**4.7**	2.1	3.4	6.4
13	**5.5**	1.0	**3.7**	1.8	3.7	8.5
14	3.8	0.4	3.3	1.6	3.8	9.4
19	4.8	0.4	0.9	3.0	3.2	9.3
23	3.1	2.6	1.1	2.6	3.4	5.1

Table 6.5 *Anticardiolipin, anti β2-GPI and anti-PT antibodies in 16 patients with idiopathic adult-onset TTP*

Patient Upper limit of normal range	Clinical details	ACL IgG 5 GPL units	ACL IgM 5 MPL units	$a\beta_2$ GPI IgG 3.5%	$a\beta_2$ GPI IgM 3.9%	APT IgG 3.9%	APT IgM 20.1%
1	Post BMT	1.6	2.6	1.4	3.01	3.5	6.5
2	Post BMT	0.1	0.1	**5.4**	2.0	3.3	3.8
3	Post BMT	0.3	2.6	1.1	2.1	3.3	3.7
4	Post BMT	0.3	1.2	0.8	1.7	3.3	4.4
5	Post BMT	1.5	2.6	0.5	3.1	3.3	6.2
10	HIV	20.3	2.6	2.0	**4.6**	2.4	**61.6**
11	HIV	4.7	0.4	**3.9**	1.9	**4.7**	15.9
12	HIV	**7.0**	0.4	**3.9**	**4.8**	**5.5**	18.3
13	SLE	**5.7**	1.4	**3.6**	2.9	2.7	18.5

Table 6.6 *Anticardiolipin, anti β2-GPI and anti-PT antibodies in nine patients with secondary adult-onset TTP*

Patient Upper limit of normal range	Clinical	ACL IgG 5 GPL units	ACL IgM 5 MPL units	$a\beta_2$ GPI IgG 3.5%	$a\beta_2$ GPI IgM 3.9%	aPT IgG 3.9%	aPT IgM 20.1%
A	Recurrent: FFP	0.7	3.3	1.6	3.3	3.9	11.1
B	Recurrent: FFP	0.5	0.0	1.3	2.7	2.1	2.3
C	?congenital	1.8	0.7	1.1	**4.3**	2.1	2.5
1	Congenital	**6.0**	2.6	2.2	2.7	2.5	2.2
2	Congenital	1.4	0.3	1.4	**4.5**	2.2	12.1
3	Congenital	4.1	0.3	1.0	3.3	2.1	6.7
4	Congenital	1.0	1.7	**16.8**	3.1	1.8	3.4

Table 6.7 *Anticardiolipin, anti β_2-GPI and anti-PT antibodies in three adult patients with recurrent TTP on prophylactic FFP infusion regimens and four patients with congenital TTP.*

6.3.5 VWF-cleaving protease activity in PAPS

Although there was no supportive evidence for the involvement of APA in the pathophysiology of TTP, one of the patients with a history of TTP studied in remission was also diagnosed with the primary antiphospholipid syndrome (PAPS) (patient number 27). She has had no further haemolytic episodes since the index episode despite a persistently depressed protease activity ratio of 0.2. This corrects on a 50:50 mix with PNP (protease activity ratio of 50:50 mix with PNP was 0.8) and as demonstrated previously there is no evidence of inhibitory IgG activity (*Figure 6.5*). However there is no history suggestive of congenital disease. In view of the concomitant diagnosis of PAPS, VWF-CP activity was measured in 23 patients with well defined PAPS and seven patients with SLE to determine the prevalence of reduced protease activity in such connective disorders which are associated with an increased incidence of TTP.

Patients

The antiphospholipid syndrome comprises both clinical and laboratory features which were first proposed as criteria by Harris (Harris, 1987) and may be diagnosed when arterial or venous thrombosis or recurrent miscarriage occurs in a subject in whom laboratory tests for antiphospholipid antibody (ACL, LA or both) are positive. Persistence of the positive tests must be demonstrated. An international workshop recently reported on a consensus statement on classification criteria (Wilson *et al*, 1999). Twenty three patients (M:F = 8: 15) with well defined PAPS were included in this study. Clinical and laboratory features are shown in *Table 6.8.*

Patient	Sex	Mis-carriage	Thrombosis	Therapy	LA	ACL IgG GPL units	ACL IgM MPL units	$a\beta_2$ GPI IgG %	$a\beta_2$ GPI IgM %	aPT IgG %	aPT IgM %	VWF-CP activity
1	M	-	+	Warfarin	+	48.5	64.2	74.8	19.0	1.2	30.6	0.89
2	M		Arterial	Warfarin	-	1.2	2.2	7.4	1.6	1.9	4.8	0.89
3	F	-	+	Warfarin	+	60.0	13.3	210.0	4.9	7.4	53.5	0.97
4	F	-	Venous and arterial	Aspirin and warfarin	-	14.6	16.9	2.1	1.8	1.2	36.2	0.86
5	M	-	Arterial	Aspirin	-	8.7	0.3	0.9	3.7	2.4	4.7	0.91
6	F	-	Venous and arterial	Warfarin	+	18.0	73.0	2.8	27.0	2.1	45.7	0.97
7	F	-	Venous	Aspirin	+	18.1	0.1	15.1	1.5	2.5	5.8	1.09
8	M	-	Venous and arterial	-	+	89.7	52.0	45.4	30.8	6.4	27.9	0.89
9	F	-	Arterial	Aspirin	-	0.1	28.5	2.7	3.4	3.1	53.6	0.81
10	F	-	-	Aspirin	Equivocal	30.2	57.1	2.0	46.4	7.9	41.3	1.13
11	M	-	Arterial	Aspirin and warfarin	+	18.2	2.9	16.8	4.2	8.4	8.9	0.91
12	F	+	Arterial and venous	-	+	1.3	23.6	1.6	5.4	1.8	20.3	1.00
13	M	-	+	Warfarin	+	20.5	4.7	2.5	2.6	5.7	134.7	0.97
14	F	+	Venous	Aspirin and warfarin	+	67.6	2.9	45.7	1.6	1.9	6.8	**0.76**
15	M	-	Venous	Aspirin	+	50.1	1.2	11.0	2.5	1.2	29.0	1.03
16	F	-	Arterial	Warfarin	+	-	-	-	-	-	-	1.03
17	F	+	Arterial	Aspirin and warfarin	-	16.4	121	13.8	120.0	2.3	9.5	0.91
18	F	+	-	-	+	82.2	39.9	22.5	42.8	2.8	27.2	**0.03**
19	F	+	Venous and arterial	Aspirin and warfarin	+	0.8	2	0.3	2.0	2.9	23.3	1.06
20	F	+	-	-	Equivocal	15.6	0.5	24.0	3.9	12.9	57.9	1.06
21	F	+	Venous	Aspirin	+	53.3	3.3	17.8	1.9	3.5	3.3	**0.77**
22	M	-	Arterial	Aspirin and warfarin	+	100.0	5.5	71.3	2.4	2.2	8.8	**0.61**
27	F	-	Arterial	Aspirin	-	2.4	17.0	2.4	1.3	2.2	16.0	**-0.07**

Table 6.8 *Clinical and laboratory features of 27 patients with primary antiphospholipid syndrome*

Patient	Sex	Mis-carriage	Thrombosis	Therapy	LA	ACL IgG GPL units	ACL IgM MPL units	$a\beta_2$GPI IgG %	$a\beta_2$GPI IgM %	aPT IgG %	aPT IgM %	VWF-CP activity
1	M	-	?	Aspirin	Equivocal	41.8	3.5	2.6	2.8	5.8	27.9	0.87
2	F	-	-	Aspirin	+	3.3	0.0	1.2	2.3	2.3	5.5	0.97
3	F	-	Arterial	Warfarin	+	19	0.6	2.8	2.4	3.7	48.3	1.03
4	F	+	Arterial	Warfarin	+	34	20	32	50	4.4	22.7	1.03
5	F	-	Venous and arterial	Warfarin	+	125	8	250	2	2	2	1.21
6	F	-	-	Aspirin	-	0.8	6.9	1.4	0.9	2.1	3.5	1.13
7	F	+	Venous	Warfarin	+	0	0	1	2	2.7	25.9	1.09

Table 6.9 Clinical and laboratory features of seven patients with SLE
M = male; F = female.

VWF-CP activity in PAPS and SLE

Twenty three patients with well documented PAPS were studied. There was a median protease activity ratio of 0.91 (range –0.07 – 1.1) (*Figure 6.10*). Two patients (patient numbers 18 and 27) had severely reduced protease activity, only one of whom had a history of TTP 32 months earlier. Although both had elevated titres of ACL IgM antibodies, this was seen in ten other PAPS patients without any observed untoward effect on protease activity.

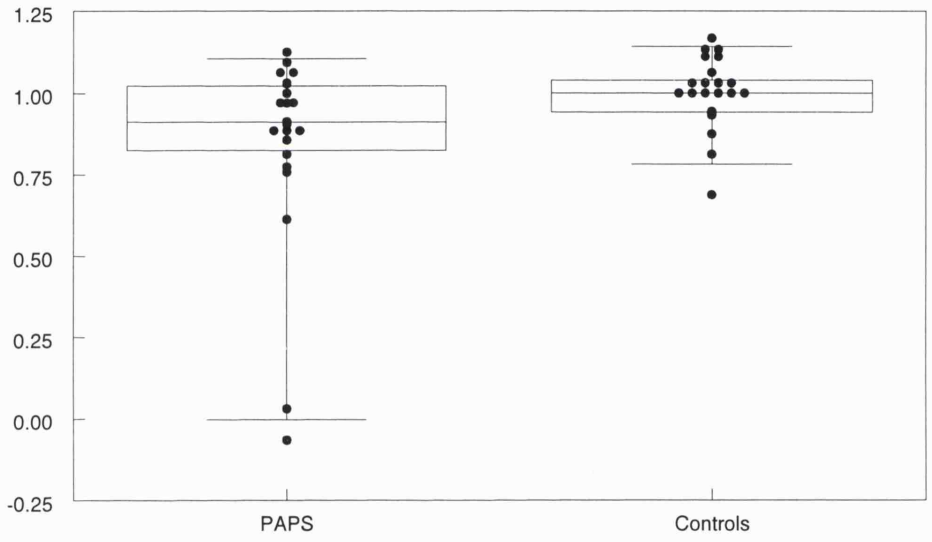


Figure 6.10 *Plasma levels of VWF-CP activity in patients with PAPS (n = 23) and controls (n=25).*
Horizontal line indicates median, box the 25th to 75th percentile and bars the 5th and 95th percentile.

As already discussed, in patient 27 protease deficiency corrected on a 50:50 mix of patient plasma with PNP to an activity ratio of 0.8. However that of patient 18 remained depressed at 0.2. In keeping with these results some evidence of inhibitory IgG activity was seen in patient 18 but not patient 27 (*Figure 6.11*). Such inhibitory activity cannot be attributable to the documented abnormalities of antiphospholipid antibodies. In this case, there was a positive lupus anticoagulant and elevated titres of

the following antibodies: ACL IgG, ACL IgM, $a\beta_2$-GPI IgG, $a\beta_2$-GPI IgM and anti-PT IgM. This picture was seen in three other patients diagnosed with PAPS (patient numbers 1, 3 and 8). However all had a protease activity within the normal range.

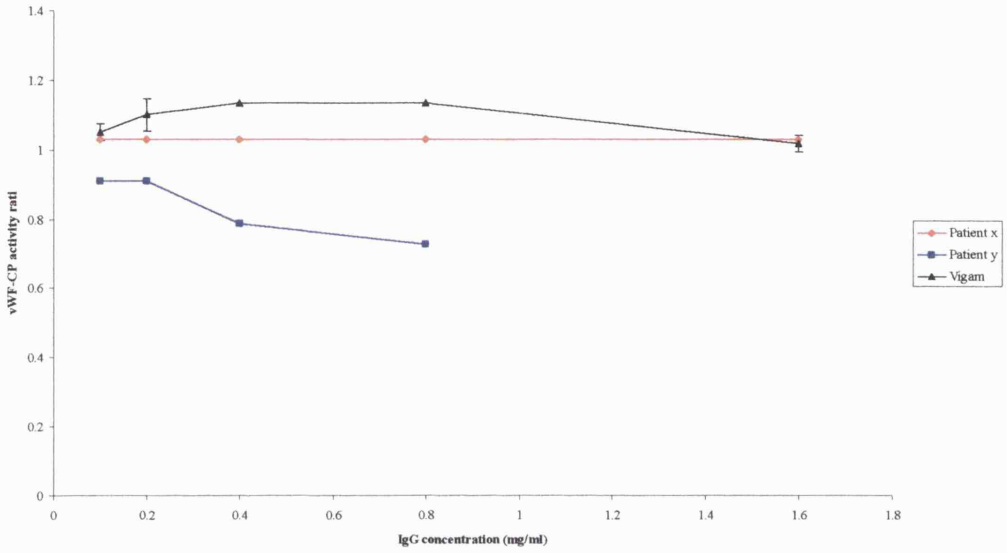


Figure 6.11 *The in vitro effect of IgG purified from two PAPS patients on intrinsic VWF-CP activity of PNP. A commercial IgG preparation serves as a negative control.*

Seven patients with a diagnosis of SLE, five of whom had well documented secondary antiphospholipid syndrome were studied. None had reduced VWF-CP activity.

6.4 Discussion

Both congenital and adult idiopathic TTP have been associated with deficiency of VWF-CP activity (Furlan *et al*, 1998b;Tsai & Lian, 1998) and this was demonstrated in chapters 4 and 5. Congenital TTP responds promptly to simple plasma infusion (Chintagumpala *et al*, 1992) whilst adult onset idiopathic disease typically requires multiple plasma exchange procedures before remission is attained (Rock *et al*, 1991). This difference may be attributable to the underlying cause of functional protease deficiency. Congenital TTP has been linked to a constitutional deficiency of VWF-CP

activity (Furlan *et al*, 1997;Furlan *et al*, 1998b) and has recently been linked to mutations within the *ADAMTS13* gene (Levy *et al*, 2001). In contrast, adult-onset idiopathic TTP appears to be secondary to the acquisition of an autoantibody of IgG subclass (Furlan *et al*, 1998b;Tsai & Lian, 1998). Yet in this study of 17 episodes of idiopathic TTP, only ten (59%) had evidence of a circulating inhibitor to VWF-CP on mixing studies with PNP. Similar findings have been reported elsewhere. In one study of 24 adult-onset TTP patients, 20 (83%) had a circulating inhibitor (Furlan *et al*, 1998b) whilst in another, 26 of 39 samples (67%) had inhibitory activity versus the protease (Tsai & Lian, 1998). A slightly lower incidence was found in a recent series of 39 patients with a diagnosis of TTP in which an inhibitor was present in only 17 (43%) (Veyradier *et al*, 2001c). Preliminary reports suggest that the inhibitor is an autoantibody of IgG subtype. Certainly in the four cases studied here IgG isolated from sera at presentation inhibited intrinsic protease activity of PNP.

Following these observations it has been suggested that TTP has an auto-immune pathogenesis. However the mere presence of autoantibodies does not necessarily imply causality. Antibodies may arise as a consequence of or be coincidental to clinical events and do not necessarily result in clinical complications. For instance APA are not always associated with clincal sequalae (Freyssinet & Cazenave, 1987;Creagh & Greaves, 1991) and large studies have indicated a prevalence of 1-3% for APA in normal populations (Shi *et al*, 1990;Lockwood *et al*, 1989). Causality requires demonstration that passive transfer of such antibodies generates the prototypic features of the TTP syndrome in animal models. Glomerular lesions reminiscent of those of TTP have been induced by passive transfer of a purified anti-gp70 autoantibody into non-immune mice. The antigenic target was apparently a platelet-bound protein of molecular mass 40 kDa (Hashimoto *et al*, 2001). However lysed human platelets have a lower protease activity than platelet poor plasma (Furlan *et al*, 1996) whilst ADAMTS13 has a non-glycosylated relative molecular mass of 154kDa (Levy *et al*, 2001).

If autoantibodies with inhibitory activity versus protease activity were central to the pathogenesis of TTP, inhibitory titre should fall with disease resolution. Although the inhibitory activity fell between presentation and remission in the three cases studied here there was still demonstrable inhibitory activity persisting into durable remission. Moreover IgG isolated from a remission sample taken from patient 27 had a more profound inhibitory effect on the intrinsic protease activity of PNP than that of the presentation samples investigated even though the patient was asymptomatic and remote from therapy other than aspirin. A similar phenomenon was seen in a patient with severe intermittent disease who relapsed several months later (data not shown). Tsai however found no evidence of inhibitory activity in 16 samples obtained during remission from the disease (Tsai & Lian, 1998). In contrast, Furlan reported that six of 15 patients (40%) had persistent inhibitory activity in remission. Of note in four of these patients the inhibitor titre was either unchanged or increased in comparison to the presenting sample (Furlan *et al*, 1998b). This throws some doubt on the pathogenic nature of these antibodies. Certainly there is little precedent in adults for transient expression of disease-causing autoantibodies nor is there evidence that plasma exchange permanently alters antibody synthesis. Perhaps a more likely explanation is that low levels of protease activity may be tolerated for relatively long periods of time in the absence of endothelial damage or other stimuli that cause the release of ULVWF. Immune modulation could also have a role to play. For example oestrogens exacerbate SLE in murine models of disease by altering the B cell repertoire in the absence of inflammation whilst inflammation itself can trigger polyclonal activation and autoreactivity. Similar mechanisms may be implicated in TTP since episodes appear to be triggered by a variety of factors including pregnancy, oral contraceptive pill and certain infections.

An auto-immune hypothesis for TTP also fails to explain the aetiology of adult onset disease in the absence of demonstrable inhibitory activity. There was no apparent inhibitor at presentation of seven (42%) of the episodes of TTP studied here. This may be the consequence of the relatively crude laboratory techniques which are currently utilised to screen for inhibitory activity. Inhibitors may have simple or complex

kinetics. In this study following preliminary studies an incubation time of 60 minutes was adopted since inhibition appeared to have plateaued by this time point. Nevertheless this may preclude identification of some time-dependent inhibitors. IgG was isolated from four cases with no inhibitory activity evident on mixing studies. Although inhibitory activity secondary to IgG antibodies was not apparent this may not necessarily exclude an auto-immune disease. IgG was added to a final concentration of 1.6mg/ml but has a serum concentration of approximately 13mg/ml. Thus a low titre antibody may not have been detected. Furthermore whilst the majority of auto-antibodies are of IgG subclass, they may also be of IgM or IgA subclass and these were not studied here.

Alternatively the failure to demonstrate inhibitory IgG auto-antibodies in all cases of TTP may be due to clinical heterogeneity. Whilst plasma exchange was demonstrated to be the treatment of choice in a landmark study, 20% in the infusion arm responded to plasma infusion alone (Rock *et al*, 1991). It is possible that some cases of adult-onset TTP represent delayed presentation of the congenital variant and a constitutional deficiency of protease activity. This is consistent with the observation that patient C in the recurrent TTP group had absent protease activity but no evidence of a circulating inhibitor. She certainly had a history of intermittent haemolytic episodes dating from childhood which have been prevented since the institution of prophylactic plasma infusion. Late presentation of congenital deficiency of VWF-CP activity is being increasingly recognised and appears independent of protease activity. In one family studied with documented *ADAMTS13* mutation, neonatal onset was seen in an individual with a protease activity of 0.06U/ml (6% normal) whilst a younger sibling with a protease activity of 0.02U/ml did not present until eight years of age (Levy *et al*, 2001). Futhermore, twenty three subjects with severe constitutional deficiency of VWF-CP were identified in a recent study. Parallel genetic mutation analysis was not performed. Although ten suffered their first TTP event during the first five years of life (median age 2 years), 11 had their first acute episode between 20 and 41 years of age (median 24 years). Two remained asymptomatic aged 37 and 44 years respectively. However once an initial acute bout of TTP had occurred all had had at

least one, but more commonly, frequent, relapses (Lämmle *et al*, 2001). In contrast of those seven samples studied here in whom an inhibitor could not be identified, relapses have only followed in three cases. It therefore seems unlikely that this entire group represents late presentation of congenital TTP although genetic analysis has yet to be performed. Instead this suggests, once again, that the pathogenesis of TTP is multifactorial.

Whilst the presence of inhibitory antibodies to protease may not entirely explain the pathophysiology of TTP their presence or absence may help to explain the variable response to plasmapheresis which is seen in clinical practice if they are a marker, surrogate or otherwise, of underlying pathological heterogeneity. A mean number of 15.8 plasma exchanges were required to attain remission in the Canadian Apheresis Trial but the range was wide (3 – 36) whilst some responded to simple plasma infusion (Rock *et al*, 1991). It may be that patients with undetectable or low inhibitory activity are those that respond either to simple infusion – since a sufficient excess of VWF-CP activity is present in FFP or cryosupernatant to adsorb any inhibitory antibody – or have a rapid response to plasmapheresis. In this study fewer plasmaphereses were required to attain CR in the subgroup of patients in whom there was no demonstrable inhibitory activity at presentation as compared to those in whom inhibitory protease activity was present (17 versus 26). Whether presenting inhibitory titre will transpire to be an effective prognostic indicator of response to therapy will require longitudinal prospective study. Although it is an attractive hypothesis clinical response may be influenced by multiple factors including complement activation and endothelial damage. Nevertheless if validated this might allow treatment to be tailored individually.

Up to 36% patients presenting with an index episode of TTP will relapse over the following ten years. There is currently no accurate means of identifying those at risk. Immunosuppressive therapy including cyclophosphamide (Bird *et al*, 1990) and more recently cyclosporin (Hand *et al*, 1998) have anecdotally been associated with a reduction in the frequency of relapse but prospective randomised studies are lacking.

Persistent inhibitory activity might represent those patients at risk of future relapse but as yet there are only limited case reports suggesting an association. For instance, deficient protease activity associated with an IgG inhibitor was found at presentation and subsequent relapse in a 34 year old man. Splenectomy performed one year after presentation resulted in disappearance of the inhibitor and complete clinical recovery which has been maintained for three years (Furlan *et al*, 1998a). Of those patients with a history of recurrent TTP were studied here, one appears to have a constitutional deficiency of protease activity whilst inhibitory IgG was demonstrated in the remainder. Although inhibitory activity was associated with subsequent relapse, this was not invariable - inhibitory activity spontaneously resolved in patient number 6 and patient number 33 remains in remission 18 months later. Relapse also occured in patients with a normal protease activity in durable remission as seen in chapter 4. Whether in fact inhibitory protease auto-antibodies do confer an increased relative risk of relapse will remain unknown until prospective studies with long-term follow-up are performed.

Four patients with congenital TTP were included in this study. In agreement with previous reports (Furlan *et al*, 1997;Furlan *et al*, 1998b) protease deficiency appeared to be secondary to a constitutional deficiency and there was no evidence of inhibitory activity. The mainstay of treatment is the infusion of plasma or plasma derived products containing protease activity. There is therefore a theoretical risk of the acquistion of inhibitory antibodies following exogenous exposure. However this is not a reported feature of the disease. A number of explanations are possible. Firstly VWF-CP may be of low antigenicity. Secondly antigenic protease may be present in congenital disease but dysfunctional. Certainly the spectrum of *ADAMTS13* mutations reported to date is notable for the relative paucity of obvious null alleles suggesting that that complete deficiency of ADAMTS13 may be lethal (Levy *et al*, 2001).

It thus appears that even when there is *in vitro* evidence of inhibitory VWF-CP activity and secondary protease deficiency, symptoms of TTP do not necessarily ensue. Further supportive evidence for this was provided by the findings of VWF-CP

activity in 30 patients with a diagnosis of either PAPS or SLE. Two of the patients with PAPS (patient numbers 18 and 23) had severe protease deficiency. The presence of inhibitory IgG was demonstrated in patient 18 who has never had any symptoms suggestive of TTP. Whether she is at risk of future events or whether this is an *in vitro* phenomenon cannot be determined. APA may interfere with phospholipid-dependent *in vitro* assays, but the results here do not support the latter hypothesis – two other patients (patient numbers 1 and 8) had a similar APA profile but normal protease activity. In contrast patient number 23 had a history of TTP, persisting severe protease deficiency after 32 months in remission but no demonstrable inhibitory IgG antibody. Whether this is an atypical presentation of constitutional deficiency of protease activity will require analysis of the *ADAMTS13* gene. Such a case is not unique: in a series of 61 patients with a diagnosis of collagen vascular disease, seven (11%) had a protease activity below 25% of the control plasma but only two of these had inhibitory IgG (Yasumuro *et al*, 2001).

The observation that severe protease deficiency may not necessarily lead to clinical sequalae is consistent with the hypothesis that other genetic and/or environmental modifying factors contribute to disease manifestations. As previously discussed these include endothelial activation, release of ULVWF from Wiebel-Palade bodies, reduced fibrinolysis and a prothrombotic state. For example, an increased prevalence of the factor V Leiden mutation has been noted in Caucasians presenting with thrombotic microangiopathy albeit in the context of normal protease activity (Raife *et al*, 2001b). APA have also been proposed to play a pathological role in the aetiology of TTP (Morita *et al*, 1996). This is because APA are prothrombotic: thrombosis was much more frequent in patients diagnosed with SLE who possessed APA (42%) than those without (24%) (McNeil *et al*, 1991). Moreover *in vitro* APA may induce endothelial activation, apoptosis and tissue factor expression, all of which have been implicated in TTP. However in this study there was no significant elevation of ACL, $a\beta_2$-GPI or anti-PT antibodies in 19 patients with a diagnosis of idiopathic or secondary TTP. This supports previous findings (Montecucco *et al*, 1987) but it should be remembered that other APA may be relevant in TTP: two recently reported

cases of chronic recurrent TTP were negative for ACL but positive for anti-phosphatidylinositol or phosphatidylserine antibodies (Trent *et al*, 1997).

In conclusion the pathogenesis of idiopathic adult-onset TTP appears multifactorial. Evidence of inhibitory protease activity was observed at presentation in only 59% cases of idiopathic adult-onset TTP and was shown in four of four patients tested to be secondary to the presence of an inhibitory IgG antibody. Demonstrable inhibitory activity was absent in the remaining cases. Whether such cases represent late presentation of constitutional deficiency of ADAMTS13 requires further investigation. Clinical disease appears to be modulated by hitherto unidentified factors since protease deficiency does not necessarily result in symptomatic disease. APA were not however implicated in this study. Whether inhibitory titre predicts disease severity or, if persistent, confers an increased risk of relapse requires prospective study.

7. Summary and conclusions

The primary aim of this thesis was to investigate the pathophysiology of TTP. Although the classical pentad of diagnostic features has been recognised for many years, the underlying pathophysiology remains elusive. Widespread microvascular thrombi composed almost entirely from platelets and incorporating abundant VWF are the characteristic histological finding in TTP tissue specimens. It has therefore been suggested that TTP may be a disease of primary platelet activation and aggregation. The extent of *in vivo* platelet activation was initially addressed in chapter 3 using flow cytometric techniques.

Results obtained from this investigation demonstrated platelet activation at presentation as shown by increased percentage positive CD62p and CD63 positive platelets. However, on an individual basis, only three (38%) had values above the normal range. Using alternative measurements of platelet activation: PMP's and platelet-monocyte aggregates, there was no significant evidence of platelet activation. However, these disparate results may be the consequence of the complex dynamics of cellular recruitment to microvascular thrombi under flow conditions. PMP's may mediate leucocyte-leucocyte interactions, leading to the accumulation of leucocytes on P-selectin positive surfaces, whilst preferential platelet-platelet adhesion may result in dissociation of PLA's. Furthermore, activated platelets have a half-life of one hour or less and may be preferentially recruited to microvascular thrombi. Alternatively, the failure to demonstrate profuse platelet activation may be secondary to the methods employed. Platelet P-selectin is rapidly lost to the plasma pool. Certainly in this study, sP-selectin was elevated in six of the eight patients at presentation. Platelets appeared to be the major source since plasma concentrations of sP-selectin correlated with percentage positive CD62p platelets. In contrast, there was no correlation with VWF:Ag concentration. However this observation could also be secondary to avid incorporation of VWF to the platelet thrombi.

Despite severe thrombocytopenia, TTP is rarely associated with haemorrhagic phenomena. This suggests that platelet function is maintained. In this study, however, agonist induced platelet surface expression of CD62p and CD63, surrogate markers of platelet function, were significantly reduced at presentation. This supports the notion that the most functional platelets have been lost from the circulation due to prior activation. It was considered elevated percentage reticulated platelets at presentation might maintain haemostasis since *in vitro* studies have suggested that reticulated platelets have superior functional capacities as compared to older circulating platelets. However percentage reticulated platelets were not elevated at presentation. Since grossly abnormal coagulation profiles are not a feature of TTP, there may be no need to invoke additional haemostatic mechanisms. Nevertheless, it was an interesting observation that the highest sP-selectin value was seen in one of the patients who died. P-selectin has recently been proposed to have pro-coagulant activity and may contribute to thrombosis. The hypothesis that it may represent a poor prognostic marker in TTP secondary to its procoagulant activity warrants further investigation.

Desite the apparently conflicting results, the results presented are consistent with *in vivo* platelet activation in TTP. Validation of the hypothesis that there is preferential recruitment of activated platelets to microthrombi might be achieved through immunohistological examination of tissue samples from patients with a diagnosis of TTP for the presence of CD62p and CD63 positive platelets and could be addressed in a future study. However whether platelet activation is a primary feature of TTP or a secondary phenomenon remains unclear. There was no significant difference between values of CD62p expression in those patients in whom VWF-CP was within the normal range as compared to those in whom it remained reduced. Nor was there a significant correlation between platelet CD62p expression and VWF-CP activity. Reduced protease activity is now thought to be central to the pathogenesis of TTP. Thus the findings do not therefore support the former hypothesis.

A platelet aggregating factor has long been postulated to play a role in TTP and attention has focussed on VWF, particularly ULVWF. At the outset of this thesis,

VWF-CP deficiency had recently been described in patients presenting with TTP and appeared to be both sensitive and specific, allowing differentiation from HUS. In chapter 4, having established an assay for VWF-CP activity, protease activity was assessed at presentation of idiopathic adult-onset TTP, secondary TTP, HUS and a range of miscellaneous haemolytic/thrombocytopenic conditions. In this manner it was demonstrated that severe VWF-CP deficiency had a sensitivity of 97% and a specificity of 80% for discrimination between acute idiopathic TTP and HUS diagnosed according to conventional clinical criteria. Furthermore, it was shown that protease deficiency was not a universal finding in cases of secondary TTP, suggesting that this diagnosis encompasses a heterogeneous group of conditions with distinct underlying pathologies. This is consistent with the failure of BMT-associated TTP to respond to plasmapheresis.

Serial protease measurements were performed in 16 patients with adult-onset idiopathic TTP to determine the correlation, if any, between protease activity and clinical course. A pivotal role of VWF processing in the aetiology of TTP would be supported by a significant positive correlation between protease deficiency and disease severity. Thrombocytopenia at presentation may be used as a surrogate marker of disease severity. No significant correlation with the severity of protease deficiency was demonstrated although this may reflect assay limitations, both in the measurement of platelet count and protease activity. Furthermore, four of 17 (24%) durable remissions were achieved despite persistent protease deficiency and circulating ULVWF multimers, supporting a "multi-hit" hypothesis for the aetiology of TTP. It was speculated that persistent protease deficiency in remission might be be a surrogate marker for those patients with a past history of intermittent disease and thus allow identification of those at high risk of future relapse. This could not be demonstrated, but may be a reflection of the relatively short duration of follow-up. Consequently, it remains unclear whether restoration of normal protease activity should be a therapeutic goal. It was an interesting observation that cyclosporin therapy was temporally associated with recovery and maintenance of normal protease activity. Moreover, in the two patients in whom it was discontinued, relapse ensued and in one

a prior progressive reduction in protease activity was observed, suggestive of causation.

Similar findings were reported in chapter 5, a study of VWF-CP activity in congenital TTP. Despite variations in clinical phenotype, all four patients studied had severe protease deficiency. This was consistent with the hypothesis that other genetic/environmental factors influence clinical phenotype. Nevertheless, the central role of protease deficiency in the pathogenesis of TTP was supported by the study of intrinsic protease activity of plasma and plasma-derived products. This demonstrated that *in vitro* protease activity correlated with, and also predicted, clinical efficacy: a factor VIII concentrate, *BPL 8Y* with intrinsic protease activity at least equivalent to that of PNP, was identified and has been successfully used as prophylaxis in patient number 2 for six months. However a number of questions remain unresolved. Protease activity remained severely depressed in post treatment samples and the increment in activity documented appeared insufficient to account for clinical effect, notwithstanding the long biologic half-life of VWF-CP. Uptake of protease onto endothelial cells or circulation of VWF-CP as an inactive precursor that is slowly, but continuously, activated may be an explanation. Alternatively, this phenomenon may be a consequence of assay limitations. That employed is a semi-quantitative since it is based on multimeric degradation which is used as a surrogate marker for cleavage of the Tyr 842-Met 843 bond. Complete multimeric degradation is not a physiological phenomenon. Instead, proteolysis to multimers of a range of molecular weights from 500 to 20,000kDa is seen. This may require a much lower protease activity than that necessary for complete loss of high and intermediate molecular weight multimers *in vitro*. The evolution of more sensitive protease assays will allow this question to be addressed.

Measurement of protease activity of first degree relatives identified five individuals with moderately reduced levels of VWF-CP, consistent with a carrier state. Such a phenomenon was not seen in the parents of patients 3 and 4 who were less severely affected. Again, this may be secondary to the assay employed which is relatively

insensitive at higher protease activities and could be confirmed by identification of *ADAMTS13* mutations in presumptive carriers. Whether such heterozygote carrier individuals themselves have an increased susceptibility to thrombosis or idiopathic or secondary TTP is currently a matter of conjecture and will require prospective study.

The findings from chapters 4 and 5 supported the theory that VWF-CP deficiency was pivotal to the pathophysiology of adult-onset and congenital TTP. Circulating protease inhibitors of IgG subtype have been reported in the former, whilst the latter appear to be secondary to a constitutional deficiency of VWF-CP. This is consistent with the observation that plasmapheresis results in superior survival, as compared to FFP infusions, in adult-onset TTP, whilst an exchange procedure is unnecessary in congenital cases. The disparate response to plasmapheresis seen in clinical practice is not consistently explained by presenting platelet count or severity of clinical symptoms and it seemed reasonable to hypothesise that this might be attributable to presenting inhibitory titre. Surprisingly there was no demonstrable inhibitor at presentation of seven (42%) of the episodes of idiopathic TTP studied. It is conceivable that at least some of these cases might represent delayed presentation of the congenital variant and a constitutional deficiency of protease activity. This could be investigated by mutation analysis of *ADAMTS13*. Whilst failure to detect time-dependent inhibitors cannot be excluded, IgG purified from four such selected patients had no effect on intrinsic protease activity of PNP over the concentration range 0.1 – 1.6mg/ml. Although this does not preclude a low titre antibody, it does demonstrate heterogeneity amongst this group of patients. When IgG was purified from four patients shown to possess a protease inhibitor at presentation, there was a concentration dependent reduction in the intrinsic protease activity of PNP. This may be of prognostic importance: a median of 17 plasmaphereses were required to attain CR in the former group as compared to 27 in the latter.

It was observed however, that clinical symptoms do not necessarily result from the simple presence of inhibitory IgG. This finding cannot be explained by inhibitor titre since IgG isolated from two patients in durable remission had a more profound

inhibitory effect on intrinsic protease activity of PNP than that of any of the presentation samples investigated. This throws some doubt on the pathogenic nature of these antibodies. Certainly there is little precedent in adults for transient expression of disease-causing autoantibodies nor is there evidence that plasma exchange permanently alters antibody synthesis. It is possible that low levels of protease activity may be tolerated for relatively long periods of time in the absence of endothelial damage or other stimuli that cause the release of ULVWF. Immune modulation could also have a role to play. Whilst it is an attractive hypothesis that the presence of inhibitory protease autoantibodies should confer an increased relative risk of relapse this was not demonstrated. However the numbers studied are too small and the period of follow-up remains too short to draw any firm conclusion. Instead, large-scale prospective studies with long-term follow-up are required.

In each study of protease activity in TTP, evidence was provided suggesting that protease deficiency does not invariably lead to clinical sequalae and that the pathogenesis of TTP is multifactorial. Possible candidates include endothelial modulation, additional VWF processing pathways, complement activation and immune regulation. It was hypothesised that antiphospholipid antibodies might play a role, firstly because a patient with a prior episode of TTP was identified who had persistent protease deficiency despite durable remission and had a concomitant diagnosis of PAPS. Secondly, TTP has been associated with auto-immune conditions such as SLE. However there was no significant elevation of ACL, aβ_2-GPI or anti-PT antibodies in 19 patients with a diagnosis of idiopathic or secondary TTP. Other factors must therefore be implicated in the pathogenesis of TTP. An additional patient was identified with a diagnosis of PAPS who had severe protease deficiency. There was no prior history of TTP. Whether she is at risk of future events or whether this is an *in vitro* phenomenon cannot be determined. APA may interfere with phospholipid-dependent *in vitro* assays, but the results here do not support the latter hypothesis – two other patients had a similar APA profile but normal protease activity.

In conclusion, the work performed in this thesis provides evidence of limited *in vivo* platelet activation and supports the theory that deficiency of VWF-CP activity, whether constitutional or acquired, is a major pathophysiological factor in the development of TTP. Many further questions are raised, beyond the scope of this thesis. For example, it appears that the clinical consequences of protease deficiency may be modulated by genetic/environmental factors. The recent identification of VWF-CP as ADAMTS13 will facilitate research in this area. Unless alternative splicing of ADAMTS13 results in the synthesis of proteins with distinct abilities to interact with cofactors, connective tissue, platelets and VWF, it is still difficult to explain the predilection of platelet microthrombi formation for the renal and cerebral vasculature. Endothelial heterogeneity may therefore be crucial to the pathogenesis of TTP and warrants further study.

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Appendix 1 Manufacturers cited in the thesis

Aventis Behring Ltd	Haywards Heath, RH16 1DB
Axis-Shield Diagnostics Ltd	The Technology Park, Dundee. DD2 1SW
Baxter Healthcare Ltd	Cexton Way, Thetford, Norfolk, IP24 3SE
Bayer plc	Bayer House, Strawberry Hill, Newbury, Berkshire, RG14 1JA
Beckman Coulter	Kingsmead Business Park, High Wycombe, Buckinghamshire. HP11 1JU
Becton-Dickinson UK Ltd	Cowley, Oxford. OX4 3LY
Bio Products Laboratory	Dagger Lane, Elstree, Herts. WD6 3BX
Centeon Ltd	Haywards Heath, RH16 1DB
Dade Behring Ltd	Division of Hoechst UK Ltd., Hoechst House, 50 Salisbury Road, Houndslow, Middx. TW4 6JH
Dako Ltd	High Wycombe, Buckinghamshire. HP13 5RE
Enzyme Research Laboratories	Pen Yr Heol Drive, Sketty, Swansea. SA2 9JT
Fisher Scientific	Loughborough, Leicestershire. LE11 5RG
Grifols UK	St. Andrew's Road, Cambridge CB4 1DP
Hyland Immuno, Baxter AG	Arctic House, Rye Lane, Dunton Green, Nr Sevenoaks. TN14 5HB
Life Technologies Ltd	Inchinnan Business Park, Paisley. PA5 9RF
Laboratoire Français du Fractionnement et des Biotechnologies	3, Avenue des Tropiques, BP 305 91958 Les Ulis, France

Merck (BDH) Ltd	Magna Park, Lutterworth, Leicestershire. LE17 4XN
Millipore (UK) Ltd.	11-15 Peterborough Road, Harrow HA1 2YH
Pharmacia Biotech	St. Albans, Hertfordshire. AL1 3AW
R&D Systems	Barton Lane, Abingdon, Oxon. OX14 3YS
Scheider & Schuell (Anderman & Co Ltd)	10, Optical Avenue, Keene, N.H. 03431 USA
Scottish National Blood Transfusion Service	Protein Fractionation Centre, 21 Ellen's Glen Road, Edinburgh. EH17 7QT
Sigma-Aldrich Chemical Co	Fancy Road, Poole, Dorset. NH17 7NH
Whatman 3MM	St Leonard's Road, Maidstone, ME16 0LS