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ROLE OF VON WILLEBRAND FACTOR GLYCANS IN MODULATING SUSCEPTIBILITY TO ADAMTS13 PROTEOLYSIS

A thesis submitted to The University of Dublin, Trinity College

for the degree of Doctor of Philosophy in the Department of Medicine.

by

Rachel McGrath

Haemostasis Research Group,

Institute of Molecular Medicine,

Trinity Centre for Health Sciences,

St. James's Hospital, Dublin 8

September 2010

DECLARATION OF ORIGINALITY

I hereby declare that this thesis is the result of my own work that has been carried out between September 2007 and August 2010 in the Haemostasis Research Group, Department of Medicine, Trinity Centre for Health Sciences, St. James's Hospital, Trinity College Dublin. All experimental work and analysis of the results were performed by myself, unless otherwise specified in the text.

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SUMMARY

von Willebrand factor (VWF) is a large plasma sialoglycoprotein that mediates platelet tethering at sites of vascular injury. VWF adhesive function is dependent upon multimeric composition, which is regulated in plasma by ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 motif, member 13). VWF is synthesised exclusively by endothelial cells and megakaryocytes, resulting in two distinct pools of circulating protein; plasma- and platelet-derived VWF. Previous studies have demonstrated that plasma-VWF N-linked glycans and blood group antigenic carbohydrate structures play a critical role in modulating susceptibility to ADAMTS13 proteolysis. In addition, the glycosylation profile of platelet-VWF differs from that of circulating plasma-VWF.

In this study, we investigated whether terminal sialic acid residues expressed on the N- and O-linked glycans of plasma-VWF may also regulate proteolysis by ADAMTS13. Furthermore, we also sought to determine the rate of ADAMTS13 cleavage of platelet-VWF, and whether modification of platelet-VWF glycosylation affects susceptibility to ADAMTS13 proteolysis.

VWF was purified from human plasma by cryoprecipitation, followed by gel filtration. Platelet-VWF was isolated from lysed platelets and purified using immuno-affinity chromatography. Following purification, VWF carbohydrate expression was modified using specific exoglycosidases and subsequent changes in glycosylation were assessed by lectin-binding ELISA. Sialic acid levels were quantified using definitive HPLC analysis.

Susceptibility of wild type untreated VWF and glycan modified VWF to ADAMTS13 proteolysis was next determined.

Enzymatic desialylation of plasma-VWF markedly impaired the rate of ADAMTS13-mediated proteolysis, as determined by residual VWF collagen binding activity over time ($p < 0.01$). Despite this, asialo-VWF exhibited increased susceptibility to proteases other than ADAMTS13 ($p < 0.05$). VWF sialylation is therefore a specific modulator of ADAMTS13 proteolysis. Consequently, quantification and molecular distribution of VWF sialylation was examined by sequential digestion and HPLC analysis. Total sialic acid expression on plasma-VWF was 167nmol/mg, of which the majority (133.4nmol/mg or 80.1%) was present on its N-linked glycan chains. Interestingly, despite the resistance to ADAMTS13 proteolysis observed upon complete desialylation, digestion of plasma-VWF with α2-3 neuraminidase to remove predominantly O-linked sialic acid did not influence the rate of ADAMTS13 proteolysis. Moreover, removal of sub-terminal galactose residues after reduction of sialic acid levels ablated the observed impairment in cleavage, suggesting that removal of sialic acid alone is sufficient to mediate this effect.

In comparison to endothelial derived plasma-VWF, platelet-VWF demonstrated marked resistance to ADAMTS13 proteolysis ($p < 0.001$). Glycan analysis demonstrated that terminal sialic acid expression on platelet-VWF was significantly reduced (~55%) compared to plasma-VWF. In vitro modification of platelet-VWF N- and O-linked carbohydrate structures significantly attenuated the ADAMTS13-resistant phenotype.

These novel data demonstrate that although sialic acid protects plasma-derived VWF against proteolysis by non-specific proteases, it also specifically enhances susceptibility to proteolysis by ADAMTS13. Moreover, due to different post-translational modification, platelet-VWF is specifically resistant to ADAMTS13 cleavage. Therefore, quantitative variation in VWF glycosylation represents a key regulator of VWF multimeric composition, and as such, is likely to be of clear pathophysiological significance.

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Finally I would like to dedicate this thesis to my parents Aidan and Bernie and to my grandparents Denis, Teresa and Patricia, for their unwavering support and constant guidance. They have taught me that patience, tolerance and understanding not only make a good person, but a great scientist.

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LIST OF ABBREVIATIONS

ADAMTS13 .. A Disintegrin And Metalloprotease with ThromboSpondin type 1repeats, member 1

ADP..... Adenosine DiPhosphate

APTT.....Activated Partial Thromboplastin Time

ASGPR..... Asialo GlycoProtein Receptor

Azyme. ..... alpha-N-Acetylgalactosaminidase

Az-..... Azyme treated

BaCl2. ..... Barium Choloride

BSA.... ..... Bovine Serum Albumin

Bzyme. ..... alpha-D-galactosidase

B_2 GPI .....B_2 GlycoProtein I

CnBr.. .....Cyanogen Bromide

CUB.... .....Complement c1r/c1s, Urchin epidermal growth factor and Bone morphogenic-1 like

Cys..... ..... Cysteine

DDAVP ..... 1-desamino-8-D-arginine vasopressin

EC..... .....Endothelial Cells

EDTA.. .....EthyleneDiamineTetraacetic Acid

ELISA..... Enzyme-Linked ImmunoSorbent Assay

ER..... .....Endoplasmic Reticulum

FPLC... .....Fast Protein Liquid Chromatography

FRET... ..... FluoRescence Energy Transfer

FT..... .....Flow Through

FVIII..... Factor VIII

GP..... ..... GlycoProtein

HEK.... ..... Human Embryonic Kidney

HEPES ..... (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HCL.... .....HydroChloric Acid

HPLC.. .....High Pressure Liquid Chromatography

HRP.... ..... HorseRadish Peroxidase

HUVEC .....Human Umbilical Vein Endothelial Cell

H_2O.... ..... Water

H_2SO_4..... Sulfuric Acid

IE..... Infected Erythrocyte

KCl..... Potassium Chloride

Met.... Methoinine

$MgCl_2$ ..... Magnesium Chloride

Mr..... Molecular Weight

NaCl..... Sodium Chloride

$NaHCO_3$ ..... Sodium Bicarbonate

Neu-... ..... Neuraminidase treated

O-Gly.. ..... O-Glycosidase treated

OPD.... ..... o-Phenylenediamine dihydrochloride

PBS-T.. ..... Phosphate buffered saline with Tween-20

pd-VWF ..... Plasma-derived VWF

PL-VWF..... Plasma-derived VWF

PNG-... ..... PNGase F treated

PT-VWF ..... Platelet-derived VWF

rADAMTS13..... Recombinant ADAMTS13

RiCoF.. ..... Ristocetin CoFactor Activity

RT-PCR..... Reverse Transcription-Polymerase Chain Reaction

rVWF.. ..... Recombinant VWF

SA (Neu5Ac) ..... Sialic Acid

SDS..... ..... Sodium Dodecyl Sulfate

SDS-PAGE ..... Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis

SEM.... ..... Standard Error of the Mean

TBS-T... ..... Tris Buffered Saline with Tween-20

TC Buffer ..... Tris-Citrate Buffer

THP-1..... Human acute monocytic leukaemia cell line

TSP1..... ThromboSPondin 1

TTP..... Thrombotic Thrombocytopenic Purpura

Tyr..... Tyrosine

ULVWF ..... Ultra Large VWF

VWD.....von Willebrand Disease

VWF.....von Willebrand Factor

VWFpp.....VWF ProPeptide

VWF:Ag.....VWF Antigen Level

VWF:CB.....VWF Collagen Binding Activity level

VWF:RCo.....VWF Ristocetin CoFactor Activity level

WPB.....Weibel-Palade Body

WT.....Wild Type

CHAPTER 1:

INTRODUCTION

1.1 von Willebrand Factor

von Willebrand factor (VWF) is a large adhesive glycoprotein that exerts crucial functions in primary haemostasis. It plays an important role in mediating the interaction between platelets and the blood vessel wall. Vascular injury results in the exposure of sub endothelial collagen, to which VWF binds. Following this, VWF acts as a molecular bridge and tethers platelets to the site of injury, resulting in formation of a temporary platelet plug and the cessation of bleeding (Franchini and Mannucci 2008, Sadler 1998a). VWF is also the physiological carrier protein for the procoagulant cofactor factor VIII (FVIII); this stabilises and protects FVIII from proteolysis and premature clearance from plasma (Lollar 1991). In the normal population, plasma VWF concentration levels vary over an extremely wide range. von Willebrand disease (VWD) is defined as a quantitative or qualitative deficiency of VWF, and is the most common inherited bleeding disorder in humans (Sadler, *et al* 2006), whereas elevated levels of VWF are associated with an increased risk of myocardial infarction (Chion, *et al* 2007, Crawley, *et al* 2008), ischemic stroke (Bongers, *et al* 2006) and venous thromboembolism (Koster, *et al* 1995, Nossent, *et al* 2006).

1.2 VWF structure

The primary translation product predicted from the cloned cDNA of *VWF* is known as *pre-pro-VWF* and consists of 2813 amino acids. This comprises a 22 amino acid signal peptide, an unusually large 741 amino acid propeptide (VWFpp), and a 2050 amino acid mature VWF monomeric subunit (Figure 1-1) (Titani *et al,* 1986).

The propeptide and the mature VWF subunit are almost entirely composed of four types of internally homologous repeating domains (A to D) in the order NH_2-D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-COOH (Figure 1-1) (Shelton-Inloes, *et al* 1986). The propeptide is made of the D1 and D2 domains only, while the mature subunit includes a truncated D' domain and a complete D3 domain, as well as the D4 domain. Each D domain is approximately 360 residues in length and contains 32-36 cysteine residues. The cysteine residues in the D3 domain participate in intermolecular disulphide bond formation.

The three A domains span residues 497 - 1111 of the mature VWF subunit (Figure 1-1). The A1 and A3 domains contain intra-molecular disulphide bonds which generate loops of 185 amino acid residues. The crystal structures of the A1, A2 and A3 domains of VWF have been elucidated (Emsley, *et al* 1998, Huizinga, *et al* 1997, Huizinga, *et al* 2002, Zhang, *et al* 2009). The A2 domain is similar to A1 and A3 at the primary sequence level but lacks the disulphide bonded loop. A domains are also present in other proteins including integrins, components of the complement system, and collagens (Colombatti and Bonaldo 1991).

The C domains of VWF have sequence similarity to thrombospondin and alpha-procollagen types I and III (Hunt and Barker 1987).

Cysteine residues are abundant within mature VWF (169 residues; there are 234 Cys residues in full length VWF). These cysteines are mainly found clustered in the *N*- and *C*-terminal regions of the mature subunit where they are involved in inter-molecular disulphide bonds. There are no detectable free sulphydryl groups in VWF, so it is assumed that those cysteines not forming inter-molecular disulphide bridges are matched in intra-molecular bonds.

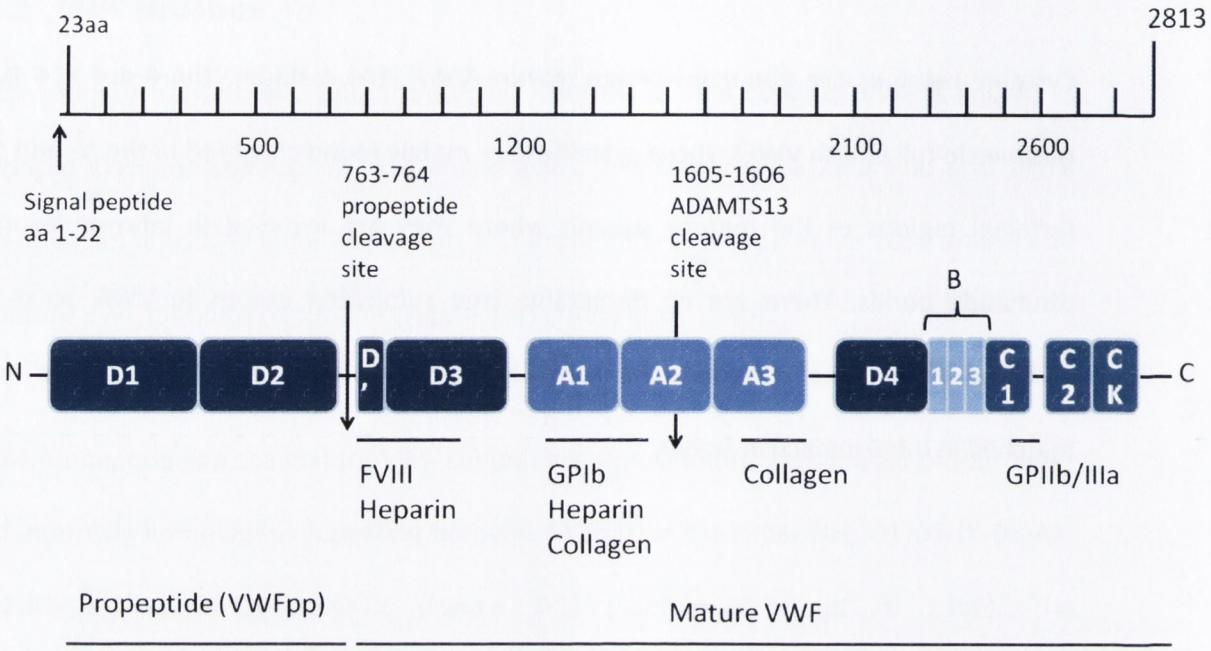


Figure 1-1 VWF structure-function

The domain structure of pre-pro VWF and the mature VWF monomeric subunit are represented, with corresponding functional binding sites indicated. Cleavage sites for generation of mature VWF and regulatory proteolysis by ADAMTS13 are also highlighted.

1.3 Structure - Function relationship

VWF circulates in a globular 'ball of string' conformation at low levels of shear stress (10-1000s^{-1}). This compact conformation, of ~2μm in size, does not bind to platelets or the endothelium readily. At critical shear rates of 2000-5000s^{-1} tethered VWF elongates as it travels through various fluid layers (Reininger 2008). Elongated or 'open' VWF is now haemostatically active and can participate in interactions with the vessel wall, followed by platelet binding. These conformational changes are reversible and occur over a very short timescale (Schneider, *et al* 2007).

VWF mediates platelet adhesion by acting as a bridge between vessel wall components and specific receptors on the platelet surface (Figure 1-2). Although VWF constitutively present in subendothelial tissues can support platelet adhesion by itself, optimal initiation of haemostasis also requires plasma- and platelet-VWF in order to increase local concentrations at sites of vascular injury (Sadler 1991).

1.3.1 Subendothelial matrix binding

Upon exposure of the subendothelial matrix, VWF binds to several types of collagen (collagen I, collagen III, and collagen VI) (Denis, *et al* 1993a, Pareti, *et al* 1987). Two binding sites located in the A1 and A3 domains of VWF have been identified as mediating the collagen interaction (Roth *et al,* 1986). These domains both contain an intra-chain disulphide bonded loop which may be important in maintaining the necessary conformation to allow VWF binding to collagen. Studies carried out using VWF A1 and A3 deletion mutants have demonstrated that the A3 domain contains the major physiologically active binding site for fibrillar collagen (Lankhof, *et al* 1996). Moreover,

amino acid residues in the lower half of the collagen-binding site, located at the front face of the VWF-A3 domain were found to be critical for binding to collagen III (Romijn, *et al* 2003). VWF strings can also interact with the endothelium through binding to intergrin $\alpha V\beta_3$, which is abundantly expressed on activated endothelial cells (Huang, *et al* 2009).

Heparin-like glycosaminoglycans are abundantly expressed on cell surfaces and in the extracellular matrix (Sasisekharan and Venkataraman 2000). VWF has at least two distinct binding sites for heparin. The A1 domain contains a heparin-binding site located in the loop region between residues Cys1272 and Cys1458 (Fujimura, *et al* 1987). Furthermore, this heparin binding-site was further localised to a 20-30 amino acid sequence from Tyr1328 – Ala1350 (Sobel, *et al* 1992). A second heparin-binding site of lower affinity exists within the first 272 residues of the mature subunit, adjacent to the FVIII binding site (Mohri *et al,* 1989). VWF binding to unfractionated heparin inhibits platelet binding via obstruction of the GPIbα binding site in the VWF A1 domain (Sobel, *et al* 1991). In contrast, Perrault et al showed that heparin can modulate and promote VWF-platelet binding through promoting the VWF interaction with GPIbα (Perrault, *et al* 1999).

1.3.2 Platelet binding

Following vessel injury, VWF tethers platelets to the site of injury, supports platelet-platelet aggregation, and ultimately thrombus formation (Reininger 2008). Platelets express two distinct receptors for VWF: the GPIb-IX-V complex and the GPIIb/IIIa complex, respectively. Under high shear rate conditions, the former is important in

initial platelet tethering to the damaged vessel wall (Ruggeri, 1993). Reversible, low-affinity bond formation between platelet GPIb and VWF occurs rapidly, reducing the velocity of the tethered platelets. GPIIb-IIIa binds VWF after tethering and platelet activation. This binding is more secure, and mediates spreading of platelets on the surface, and further platelet aggregation (Ikeda *et al,* 1991). Under low shear rate conditions, VWF is a less important mediator of platelet adhesion, as the VWF A1-GPIb bind has a fast dissociation rate and does not provide irreversible adhesion (Savage, *et al* 1996). However, above a critical shear stress value, VWF tethers to exposed collagen and adopts an extended chain conformation, with consequent exposure of intra-molecular GPIb binding sites (Siedlecki, *et al* 1996). Platelet microparticles are small spherical procoagulant particles that are released following platelet activation. These microparticles express many platelet surface receptors, and can bind to fibrinogen (Holme, *et al* 1998). Studies suggest that VWF may play a role in microparticle generation (Miyazaki, *et al* 1996, Ruggeri, *et al* 2006).

1.3.3 Glycoprotein Ib-IX-V

VWF-collagen binding results in conformational changes within the VWF A1-A3 domains and subsequent exposure of the GPIb binding site in the A1 domain (Ruggeri 2003). Similarly, under conditions of high shear stress and in the presence of modulators such as ristocetin or botrocetin, the A2 and surrounding domains adopt an active conformation, such that GPIb can gain binding site access (Berndt, *et al* 1988, Hulstein, *et al* 2005). Two short binding sequences in the A1 domain of VWF participate in the interaction with GPIb. Residues 474-488 (located at the boundary between the D3 and A1 domains) and 1457-1471 (in the A1 domain) are essential (Mohri *et al*, 1989). These

sequences interact with the *N*-terminal portion of the GPIb α chain (Vicente *et al*, 1988). Additional information about the VWF A1 domain-GPIbα interaction has come to light since the crystallisation of this domain while bound to the receptor. Part of the GPIbα molecule aligns with a strand of the VWF A1 domain comprising residues 1325-1329, resulting in the formation of a continuous β-sheet between both molecules (Huizinga, *et al* 2002). A second site of interaction within the VWF A1 domain includes a stretch of 6 non-continuous amino acids, in particular residues R1334 and E13762 which form direct contacts with GPIbα (Dumas, *et al* 2004). These residues are essential as mutation of R1362 totally abolished GPIb binding (Marx, *et al* 2008).

1.3.4 Glycoprotein IIb/IIIa

Glycoprotein IIb/IIIa (GPIIb/IIIa; integrin $\alpha_{IIb}\beta_3$) is a calcium-dependent integrin receptor complex found on the surface of platelets. Upon platelet activation, GPIIb/IIIa reorganises and undergoes conformational changes resulting in a high affinity state for binding to VWF (Shattil 1999). The VWF binding site for the GPIIb/IIIa complex has been localized to a tetrapeptide sequence near the carboxy-terminal end of the C1 domain (Denis, *et al* 1993b, Ruggeri, *et al* 2006). This sequence (Arg-Gly-Asp-Ser; known as an integrin binding or RGDS sequence) exists in several other proteins involved in cell adhesion (fibronectin, fibrinogen, vitronectin) (D'Souza, *et al* 1991). VWF binding to GPIIb/IIIa leads to platelet accumulation and formation of a stable platelet plug under conditions of high shear stress. The multivalent structure of VWF allows binding to several receptor units expressed on two or more platelets and ultimately leads to platelet aggregation (Coller 1997).

1.3.5 FVIII binding

Factor VIII (FVIII) is an essential component of the coagulation cascade. Deficiency of the FVIII molecule leads to haemophilia A, a severe hereditary bleeding disorder (Lillicrap 1991). FVIII is synthesised as a single chain glycoprotein, but circulates in the plasma as a heterodimer composed of two polypeptide chains, namely a light and heavy chain. In plasma, FVIII is stabilized by hydrophobic and hydrophilic interactions with a 50-fold molar excess of VWF (Vlot, *et al* 1995). VWF promotes the association of the light and heavy chains of FVIII upon secretion, whereas in the absence of VWF the heavy and light chains become rapidly degraded.

VWF binds FVIII with high affinity, with a dissociation constant (K_d) of 0.2- 0.4nM. The formation of the VWF:FVIII complex occurs rapidly, with an association rate (K_{on}) of 5.9 x 10^6 $M^{-1}s^{-1}$ (Vlot, *et al* 1995). Rapid complex formation prevents FVIII from interacting with other ligands such as FIXa (K_d = 15nM/L). The site of this interaction between VWF and FVIII is still unclear, since VWF biosynthesis is restricted to vascular endothelium and megakaryocytes, while the major site of FVIII synthesis is the liver. However, more recent evidence has demonstrated that FVIII is synthesised and secreted from lung endothelium, and that FVIII and VWF are co-expressed in human pulmonary artery endothelial cells and pulmonary microvascular EC (Jacquemin, *et al* 2006, Shovlin, *et al* 2010).

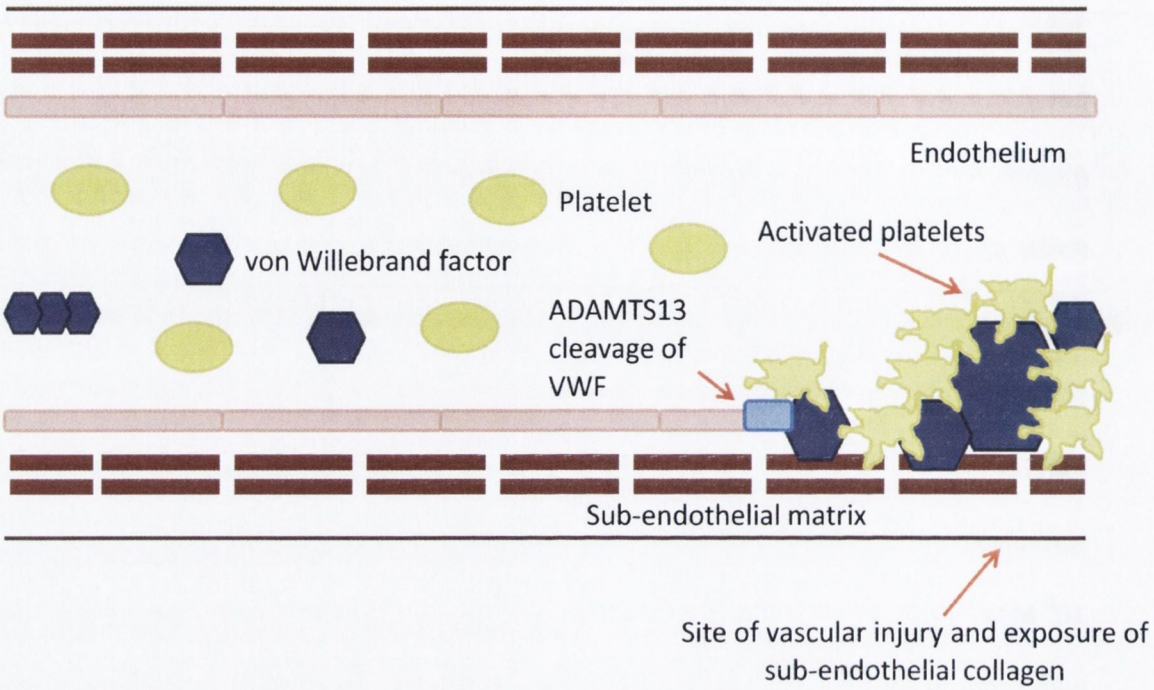


Figure 1-2 VWF function

Overview of the functional activity of VWF at areas of endothelial injury.

1.3.6 Additional VWF interactions

More recent studies have demonstrated that VWF binds to several other plasma and matrix proteins, eliciting diverse adhesive functions in the bloodstream (Figure 1-3). Thrombospondin-1 (TSP1) is an adhesive matrix glycoprotein that has many functions including participation in cell-cell and cell-matrix interactions, tumourigenesis, angiogenesis and platelet aggregation (Bonnefoy, *et al* 2008, Sid, *et al* 2004). TSP1 binds to the A2 and A3 domains of VWF. This interaction competitively obstructs regulatory proteolysis of VWF by ADAMTS13 (Bonnefoy and Hoylaerts 2008, Wang, *et al* 2010). VWF also binds to osteoprotegerin, a member of the tumour necrosis-factor receptor superfamily, that has an important role in bone remodelling as well as being involved in several vascular diseases (Shahbazi, *et al* 2007). Although VWF-osteoprotegerin complexes are present in the circulation, the functional significance of this interaction remains to be identified (Zannettino, *et al* 2005). P-selectin, expressed on activated endothelium, can tether newly released ULVWF to the cell surface (Padilla, *et al* 2004). However, in contrast to these findings, Huang et al demonstrated that neither an anti P-selectin antibody nor P-selectin itself reduced the number of VWF- or VWF-platelet-strings in vitro. They suggest that $\alpha V\beta_3$ integrin is the major physiological ligand for VWF binding to endothelium subsequent to secretion (Huang, *et al* 2009). In addition, roles for VWF in inflammation have been described, as VWF can promote leukocyte tethering and rolling via binding to both P-selectin glycoprotein ligand-1 and β2-integrins (Pendu, *et al* 2006). Another protein that binds to VWF via the A1 domain is β_2-Glycoprotein I (β_2 GPI). This interaction inhibits VWF-induced platelet aggregation, as β_2 GPI competitively binds to the same area of VWF as GPIbα, preferably when the A1 domain is in its active platelet-binding conformation (Hulstein, *et al* 2007). Anti-β_2 GPI autoantibodies

neutralize the inhibitory effect of β_2 GPI on VWF-platelet binding activity, which could therefore lead to a prothrombotic state. VWF has a critical function in platelet-mediated infected-erythrocyte adhesion to activated endothelium, which contributes to the progression of cerebral malaria (Bridges, *et al* 2010). Binding of *Staphylococcus aureus* protein A to VWF via the A1 domain has been hypothesized to facilitate pathogen intravascular colonisation (Hartleib, *et al* 2000, O'Seaghdha, *et al* 2006). Finally, magnesium ions have been highlighted as modulators of VWF activity as physiological and/or treatment levels of Mg^{2+} led to a partial inhibition of VWF collagen binding and ristocetin-induced platelet aggregation (Dong, *et al* 2008).

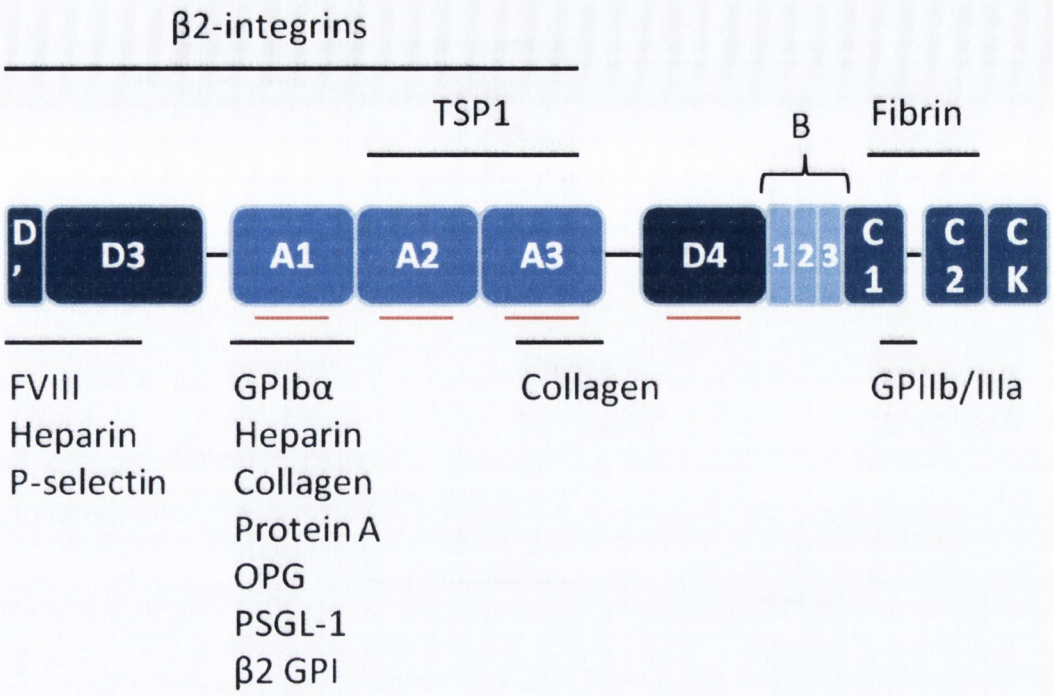


Figure 1-3 VWF binding interactions

The domain structure of the mature VWF monomer is shown, with corresponding sites for VWF binding partners indicated. The red lines designate sites of VWF-ADAMTS13 interaction. β2 GPI; β_2 Glycoprotein I, OPG; osteoprotegerin, PSGL-1; P-selectin glycoprotein ligand-1, TSP1; thrombospondin-1.

1.4 VWF biosynthesis

1.4.1 Expression of *VWF* - tissue distribution

VWF is present within endothelium, megakaryocytes, plasma and the subendothelial matrix. However, in vivo biosynthesis of VWF is limited to endothelial cells and megakaryocytes (Sadler 1998b). There is accumulating evidence that *VWF* gene expression may vary in endothelial cells derived from different vascular beds. Immuno-histochemistry performed on porcine blood vessels has demonstrated that VWF is consistently present in venous endothelial cells, with the exception of the pulmonary vein, and was absent from the majority of porcine arterial endothelial cells (Rand, *et al* 1987).

Yamamoto et al used quantitative RT-PCR (reverse transcription-polymerase chain reaction) and in-situ hybridisation analysis to study murine VWF gene expression in adult CB6 mice (Yamamoto, *et al* 1998). Although *VWF* mRNA was identified in endothelial cells from all tissues examined, there was a wide range in the levels detected. High *VWF* mRNA levels were present in endothelial cells derived from spleen, aorta, lung and brain. Lowest levels were found in liver, gut and kidney endothelial cells. *VWF* expression was again significantly higher in venous than in arterial endothelial cells.

1.4.2 Post-translational modification

Following mRNA translation, pre-pro-VWF undergoes extensive post-translational processing to produce multimeric VWF (Kaufman, 1998). This is achieved sequentially as it passes from the endoplasmic reticulum (ER) to either the extracellular domain or to Weibel-Palade bodies/α-granules (Figure 1-4).

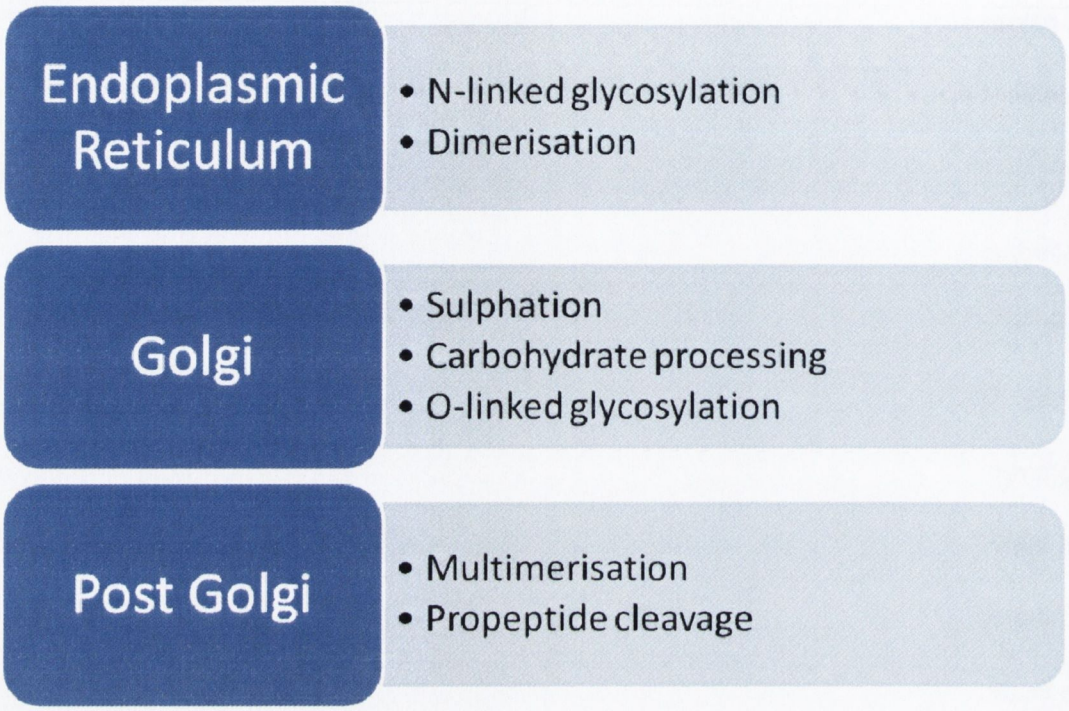


Figure 1-4 Schematic representation of VWF post-translational modifications.

Various post-translational modifications of VWF and where they take place within endothelial cells and megakaryocytes are described above, in order of occurrence.

1.4.2.1 N-linked glycsoylation

The primary translation product of pre-pro-VWF passes into the rough endoplasmic reticulum (ER), where the *N*-terminal 22 amino acid signal peptide is cleaved. Within the ER, high-mannose containing oligosaccharide side chains are added to twelve asparagine (*N*-linked) sites on each VWF subunit and to four N-linked sites on the propeptide (Figure 1-5) (Titani, *et al* 1986). N-linked glycosylation sites are most abundant at the *C*-terminal end of the VWF monomer, with seven sites located between residues 2223 and 2789 (Figure 1-5). N-linked glycosylation is essential before dimerisation of VWF can take place. Treatment of endothelial cells with the antibiotic Tunicamycin, which prevents N-linked glycosylation, results in the accumulation of pro-VWF monomers in the ER (Wagner and Marder 1984) and also prevents efficient secretion of VWF (McKinnon, *et al* 2010).

Within the ER, the enzyme oligosaccharyltransferase (OST) transfers a preformed 14-saccharide core unit onto the emerging VWF polypeptide chain (Kornfeld and Kornfeld 1985). Glucosidases rapidly remove terminal glucose residues from this unit, and the resulting structure becomes a ligand for the resident ER lectin chaperones calreticulin and calnexin (Allen, *et al* 2000, Spiro, *et al* 1996, Ware, *et al* 1995). When the protein is correctly folded, the final glucose residue is removed, which allows release from chaperones. This also signals that the protein is ready to continue its transit through the ER to the Golgi. Next, ER and Golgi mannosidases further process the carbohydrate structure. Following this, Trans-Golgi glycosyltransferases add GlcNac and galactose sugars to produce complex type carbohydrates (Moremen, *et al* 1994). These glycan chains may be capped by sialic acid to produce the fully processed N-linked

oligosaccharide structures. Fucosyl transferase (FUT1) competes with sialyl transferases in the endothelium to add fucose to the end of the chain, thus creating the H-antigen of blood group O. Following addition of the H-antigen, blood group A and B glycosyltransferases add a single terminal sugar moiety (GalNAc or galactose, respectively) resulting in blood group specific VWF (Matsui, *et al* 1992).

Blood group is a known determinant of VWF levels in plasma, with type O individuals possessing ~20% lower levels of VWF in their plasma than non-O persons (in the order of O < A = B < AB) (Jenkins and O'Donnell 2006). Blood group determinants do not affect levels of VWF synthesis or secretion (O'Donnell and Laffan 2001); the mechanism responsible for differing levels of VWF in individuals of various blood groups is hypothesized to be based on VWF catabolism and clearance from the circulation (Gallinaro, *et al* 2008).

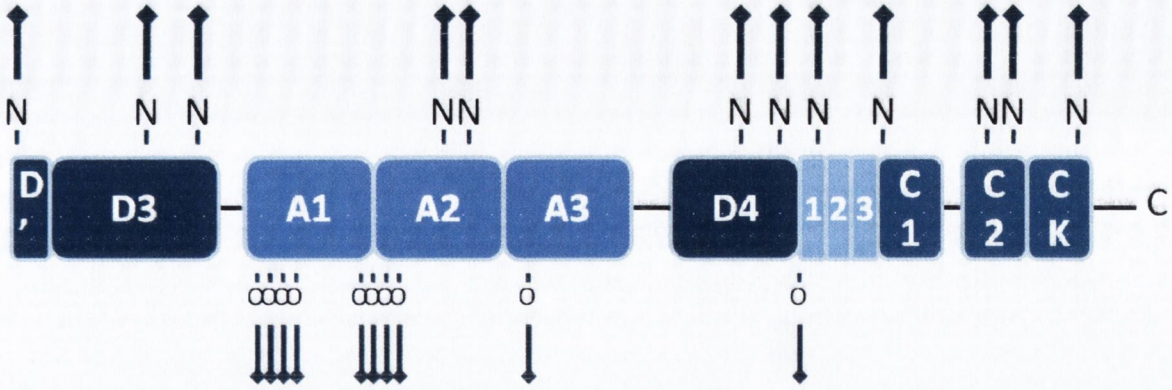


Figure 1-5 Position of N- and O-linked glycan sites throughout the VWF monomer.

Mature VWF contains 12 putative sites for the addition of N-linked glycan structures, distributed through the monomeric structure. 10 sites of O-linked glycosylation are present, with clusters of 4 O-glycans apparent in the A1 and A2 domains.

1.4.2.2 Dimerisation

VWF is unique in that it undergoes two distinct intracellular polymerization steps which are compartmentalized into two different organelles (Figure 1-4). Dimerisation occurs within the ER, whereas the later multimerisation of the dimers occurs in the Trans- or post-Golgi. Dimerisation occurs through the formation of an unknown number of intersubunit C-terminal disulphide bonds. These bonds are all located within the last 150 residues of each subunit, which follow the C2 domain (Dong, *et al* 1994). Several studies have shown that only dimeric VWF molecules are transported from the ER to the Golgi (Wagner, *et al* 1986, Wise, *et al* 1988).

1.4.2.3 O-linked glycosylation

In addition to the N-linked glycosylation sites, the mature VWF subunit also has ten O-linked glycan sites present on serine and threonine residues (Figure 1-5). Oligosaccharide side chains are added to these within the Golgi. Eight of the O-linked glycosylation sites are clustered in two small areas within the A1 and A2 domains (residues 485-500 and 705-724) of the mature VWF monomer (Titani, *et al* 1986). Recently, the presence of ABO(H) blood group antigenic structures on a small proportion of VWF O-glycans has been demonstrated (Canis, *et al* 2010).

1.4.2.4 Polymerization

Multimerisation of pro-VWF dimers occurs in the Trans or post-Golgi by further disulphide bonding. Inter-dimer disulphide bond formation involves cysteine residues at the *N*-terminal ends of the VWF D3 domain; Cys1222 and Cys1227. Furthermore, two more essential cysteine residues have been described in the VWF D' D3 domains, which

facilitate the oxidoreductase mechanism of VWF multimerisation and form interunit disulphide bonds (Purvis, *et al* 2007). Intra-organelle conditions play an important part in the formation of these disulphide bonds. Reduced cysteine residues are required for polymerization as the presence of N-ethylmaleimide prevented the binding reaction from occurring (Mayadas, *et al* 1989).

Multimerisation studies utilizing both in vivo and in vitro experiments have demonstrated that a low pH is required (optimal 5.8 in vitro) (Mayadas and Wagner, 1989). This is in keeping with the pH usually found in the Trans-Golgi compartment and within secretory vesicles, both of which are sites of active proton pumping (Glickman *et al,* 1983). Formation of VWF multimers is also a calcium dependent process, as inhibition can be achieved by the use of chelating agents, such as EDTA. This requirement correlates well with the high calcium concentration observed within secretory vesicles.

VWF polymerization is also dependent upon the large VWF propeptide. The VWF propeptide acts as an endogenous pH dependent chaperone that promotes VWF disulphide formation within the Golgi (Vischer and Wagner 1994). It is not necessary for the propeptide to be a contiguous part of the proVWF structure for effective polymerization.

1.4.2.5 Propeptide cleavage

Cleavage of the large VWF propeptide is a very late processing event and appears to occur in both the trans-Golgi and secretory granules. Cleavage is mediated by the enzyme furin, and occurs between the arginine and serine residues in the sequence

Lys762 - Arg763 - Ser764 present at the C-terminal end of the VWF D2 domain (Rehemtulla and Kaufman 1992, van de Ven, *et al* 1990). Approximately 1% of plasma-VWF contains uncleaved propeptide (Fay *et al*, 1986), and propeptide removal is essential before VWF can effectively bind FVIII (Bendetowicz, *et al* 1998, Leyte, *et al* 1991).

1.4.3 VWF propeptide

The VWF propeptide (VWFpp) is present in normal plasma, after both constitutive and regulated secretion from endothelial cells. VWFpp is released into the plasma in equimolar amounts with VWF. However, VWFpp displays a shorter half-life than VWF (2 versus 12 hours, respectively) (Borchiellini, *et al* 1996). By exploiting this property, VWF and VWFpp plasma levels have been used as a measure of acute endothelial cell activation in several conditions, including vascular disorders and diabetes mellitus. Moreover, comparisons of VWF and propeptide levels can distinguish between acute and chronic endothelial cell perturbation (van Mourik, *et al* 1999). In addition, VWFpp levels are elevated in thrombosis patients (Nossent, *et al* 2006) and VWFpp levels have been used as a tool to study decreased VWF survival in type VWD 1 (Haberichter, *et al* 2006).

VWFpp is an essential component of VWF storage organelles within endothelial cells (Weibel-Palade bodies; WPBs), as absence of propeptide ablates WPB formation (Haberichter, *et al* 2005). Moreover, VWFpp has been shown to be critical for the targeting of VWF to WPBs, and is also essential for trafficking of an unrelated protein (C3α) to WPB storage (Haberichter, *et al* 2002). Whether VWFpp retains a biological function after cleavage is unknown. However, it contains an active collagen-binding

domain, and has been shown to inhibit collagen-induced platelet aggregation (Takagi, *et al* 1991, Takagi, *et al* 1989).

1.4.4 Mature VWF

As a result of the processes detailed above, mature VWF has a multimeric structure and exists as a series of oligomers containing a variable number of subunits, ranging from a minimum of 2 to a maximum of 100. The monomeric size of VWF, taking into account both N- and O-linked glycosylation, is ~250kDa. Thus, mature VWF multimers vary in size from 500kDa to ~10,000kDa (Ruggeri and Zimmerman 1981).

1.5 VWF secretion

Several pathways of VWF secretion from the endothelium have been described. Constitutive secretion of VWF from endothelial cells requires no cellular stimulation and maintains circulating VWF levels at ~10μg/ml in healthy individuals (Sadler 2009). Acute stimulated release of VWF entails activation of endothelial cells through the action of a variety of agonists including histamine, interleukin-1 and thrombin, among others. VWF stored within Weibel-Palade bodies is exocytosed in response to endothelial stimulation (van Mourik, *et al* 2002). Recently, a third pathway of VWF secretion was identified, namely, basal secretion (constitutive-like secretion), in which elements of both the constitutive and stimulated pathway were reported (Giblin, *et al* 2008).

1.5.1 Constitutive secretion

After synthesis within endothelial cells, steady levels of VWF are spontaneously and continuously released. Approximately 90% of circulating VWF present in plasma is as a result of the constitutive pathway (Sporn, *et al* 1986). The mulitmeric distribution of this pool of VWF includes a range of molecular weight multimers. Constitutively secreted VWF is not directed for storage within endothelial cells, and is targeted for immediate release; the mechanisms responsible for this phenomenon however remain to be determined.

1.5.2 Stimulated secretion

Upon activation of endothelial cells, hyperactive ULVWF multimers are released into the bloodstream (Wagner and Bonfanti 1991). This releasable pool of VWF constitutes ~10% of VWF secreted from the endothelium. Endothelial activation can result from stimulation by inflammatory mediators, haemostasis/coagulation specific molecules, or though vascular injury. Several other agonists for stimulated secretion of VWF have been recently identified including vasopressin (Kaufmann, *et al* 2000) and shiga toxin (Huang, *et al* 2010, Nolasco, *et al* 2005). In contrast, other conditions and molecules that reduce VWF secretion have also been reported. For example, endothelial derived nitric oxide was shown to limit stimulated secretion of VWF from Weibel-Palade bodies (Qian, *et al* 2010).

1.5.2.1 Weibel-Palade bodies

VWF storage organelles within endothelial cells are known as Weibel-Palade bodies (WPBs), which were first identified in 1964 (Weibel and Palade 1964). WPBs originate from vesicles that bud off from the Golgi, and are elongated tubular/rod-like structures

that are ~0.2μm wide and up to 5μm in length (Sadler 2009). WPBs undergo exocytosis and expulsion of their cargo into the bloodstream upon endothelial cell activation. VWF stored in WPBs is of the largest mulitmeric species and is rapidly proteolysed by ADAMTS13 upon secretion (Dong, *et al* 2002, Ewenstein, *et al* 1987).

Densely packed VWF is present in tightly coiled tubules within WPBs. Other constituents of WPBs include mediators of inflammation including P-selectin (Bonfanti, *et al* 1989), chemokines such as interleukin-8 and eotaxin-1, protein growth factors including angiopoietin-1, the cytokine osteoprotegerin and a vasoconstrictor, endothelin-1 (Rondaij, *et al* 2006). The elongated rod shape of WPBs is dependent on VWF. Furthermore, VWF propeptide is essential for the formation of WPBs (Wagner, *et al* 1991). VWF is essential for WPB formation as VWF deficient mice do not contain WPBs in their endothelial cells (Denis, *et al* 2001).

1.5.3 Basal secretion

The basal secretory pathway of VWF from endothelial cells was identified by Giblin et al in 2008 (Giblin, *et al* 2008). By using a model for kinetic analyses of VWF storage and secretion, they noted that a significant proportion of VWF that was not targeted for constitutive release exhibited delayed secretion, in the absence of endothelial cell activation. Moreover, by disrupting the constitutive pathway, it was found that the majority of VWF is not constitutively released but is subject to basal secretion from a post Golgi organelle, assumed to be the WPB. This basal secretion does not require stimulation of endothelial cells and is described as regulated or spontaneous release of VWF from storage organelles.

1.6 VWF synthesis in megakaryocytes

VWF was first identified in platelets in the 1970's. Immunoelectrophoresis was used to show the presence of the FVIII-related antigen (FVIIIR:Ag; later known as VWF) in platelets (Howard, *et al* 1974). Subsequently, Sporn et al showed that VWF was biosynthesised by human megakaryocytes, and this VWF was produced and processed in a similar way to endothelial derived VWF (Sporn, *et al* 1985). To date, de novo platelet biosynthesis of VWF has not been demonstrated, however significant levels of VWF mRNA have been detected in platelets (Ginsburg, *et al* 1989).

1.6.1 Platelet α-granules

Platelet-VWF is stored in the platelet α-granules (Harrison and Cramer 1993, Wencel-Drake, *et al* 1984). α-granules are the most abundant secretory organelles found in platelets, comprising ~10% of the total platelet volume (Blair and Flaumenhaft 2009). Within the α-granules, VWF colocalises with other molecules that have primary functions in haemostasis and inflammation such as adhesive proteins, cytokines, protease inhibitors and coagulation factors (Harrison and Cramer 1993). Many studies by Cramer et al revealed that VWF is present in tubule like structures in the peripheral electronluscent zone of α-granules, and these granule structures are absent from porcine VWD megakaryocytes (Cramer, *et al* 1988, Cramer, *et al* 1986, Cramer, *et al* 1985, Wilbourn, *et al* 1993). These tubules are similar in shape and size to the VWF tubules present in endothelial Weibel-Palade bodies (Cramer, *et al* 1985). An overview of platelet-VWF biosynthesis is shown in Figure 1-6.

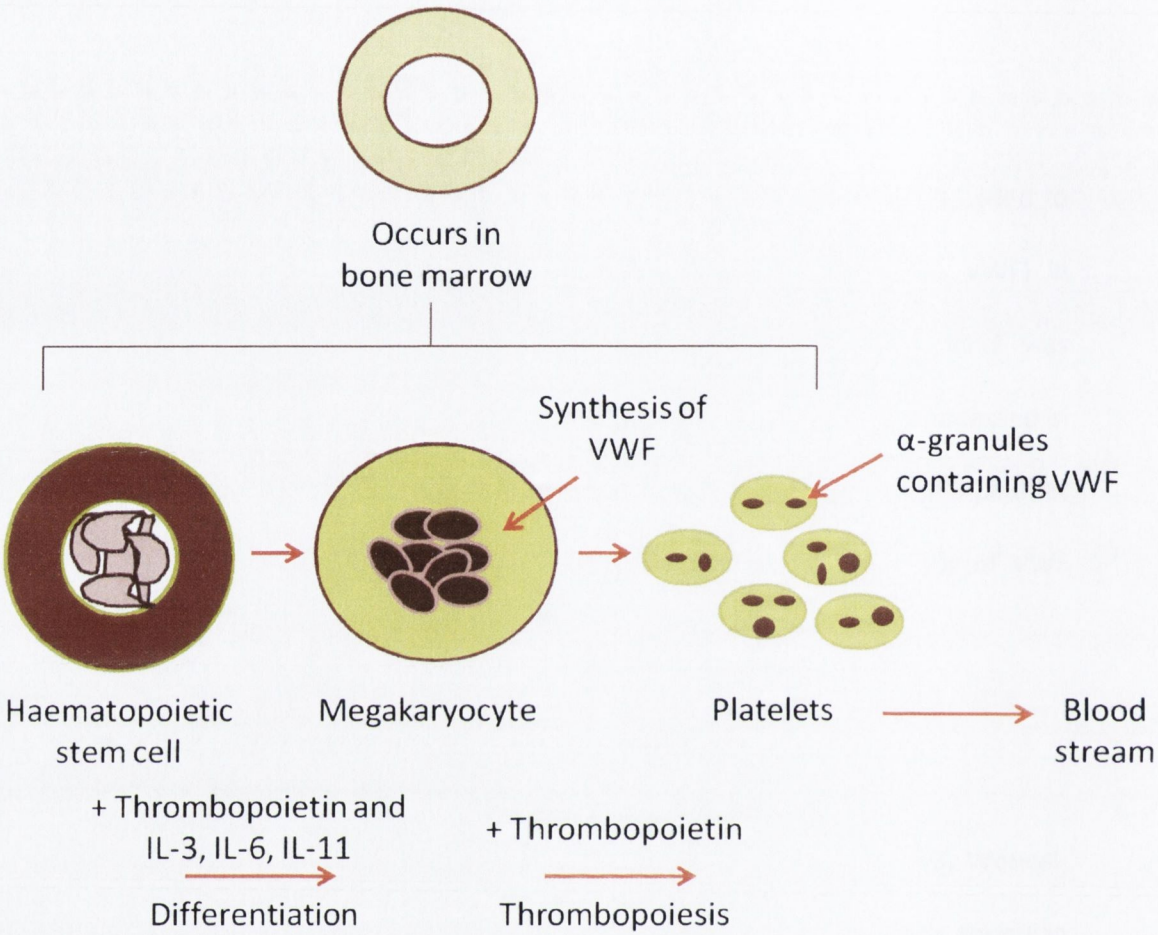


Figure 1-6 Synthesis of VWF within megakaryocytes.

The steps involved in biosynthesis of VWF within the bone marrow and megakaryocytes is outlined above.

1.6.2 Platelet-derived VWF function

Numerous studies have demonstrated that VWF is released from platelets upon stimulation with collagen, thrombin, thromboxane A2 or ADP (FitzGerald 1991, Koutts, *et al* 1978, Zucker, *et al* 1979). Platelet-VWF constitutes ~15–20% of the total VWF circulating in plasma. Several studies have shown that platelet-VWF is composed of higher molecular weight multimers than circulating endothelial-derived plasma-VWF (Fernandez, *et al* 1982, Gralnick, *et al* 1985). Moreover, platelet-derived VWF has a higher prothrombotic activity than plasma-VWF (Fernandez, *et al* 1982). Platelet-VWF preferentially binds to the platelet receptor GPIIb/IIIa complex upon secretion from α-granules, thereby supporting platelet aggregation. Furthermore, it was observed that 60-70% of released platelet-VWF bound to GPIIb/IIIa after platelet stimulation (Parker and Gralnick 1986). Platelet-VWF binding to GPIIb/IIIa is dependent on the RGDS integrin binding sequence in the VWF C1 domain as GPIIb/IIIA binding was inhibited by a synthetic RGDS peptide. Moreover, a fibrinogen γ-chain peptide also inhibited the VWF GPIIb/IIIa interaction, indicating that platelet VWF and fibrinogen both bind to this receptor complex in a similar way (Parker and Gralnick 1989). Platelet-VWF also competes with fibrin for occupancy of the GPIIb/IIIa complex on thrombin-stimulated platelets (Hantgan, *et al* 1990).

1.6.3 Platelet-VWF levels in von Willebrand disease

In view of the significant heterogeneity in bleeding phenotype associated with VWD, platelet-VWF antigen and activity levels have been investigated in patients with different subtypes of VWD. In a study of 13 different kindred with type 1 VWD, Mannucci *et al* described three different subgroups based upon platelet-VWF levels (Table 1-1)

(Mannucci 1995). *'Platelet-low'* had reduced platelet VWF:Ag and VWF:RCo; *'platelet-normal'* showed platelet-VWF:Ag and VWF:RCo within the normal range; and *'platelet-discordant'* where platelets contained normal concentrations of VWF:Ag and normal VWF multimers, but VWF:RCo was disproportionately reduced. Another study that included patients with VWD again demonstrated significant heterogeneity in platelet-VWF levels (Weiss, *et al* 1983). Once again, *platelet-low* and *platelet-normal* phenotypes were observed in patients with type I VWD.

Platelet-VWF levels have also been determined in patients with type 2 and type 3 VWD (Weiss, *et al* 1983). Unsurprisingly, platelet-VWF was not detected in homozygous type 3 patients (Bouma, *et al* 1975, Ruggeri, *et al* 1978, Sultan, *et al* 1978). In addition, *platelet-normal* and *platelet-low* phenotypes were again observed. Consequently, it is clear that quantitative platelet- and plasma-VWF levels can vary independently. The underlying molecular mechanisms and translational implications of this variation remain to be defined.

In addition to its role in VWD, a reduction in platelet-VWF levels may also contribute to the bleeding diathesis of Grey platelet syndrome. Furthermore, reduced platelet-VWF activity has also been implicated in mediating the bleeding tendency associated with myeloproliferative disorders, including essential thrombocythaemia (Castaman, *et al* 1995, Meschengieser, *et al* 1987).

Type 1 VWD platelet subtype	VWF:Ag level	VWF:RCo level	Response to DDAVP
Platelet-low	Within normal range	Within normal range	Normal
Platelet-normal	Reduced	Reduced	Low
Platelet-discordant	Within normal range	Reduced	Low

Table 1-1 An overview of platelet-VWF levels and function in type 1 VWD.

1.6.4 Differences between plasma- and platelet-VWF

A number of important differences have been described between platelet-VWF and plasma-VWF. In particular, multimer gel analyses have demonstrated that VWF stored within the α-granules is enriched in haemostatically active UL-VWF multimers (Fernandez, *et al* 1982, Ruggeri, *et al* 1982, Ruggeri and Zimmerman 1980). Despite the fact that ADAMTS13 has recently been identified in platelet lysates (Liu, *et al* 2005, Suzuki, *et al* 2004), platelet-VWF multimers do not demonstrate a triplet pattern after electrophoresis (Fernandez, *et al* 1982), suggesting that platelet-VWF does not undergo proteolysis by ADAMTS13. In addition, platelet-VWF also differs from plasma-VWF in that it is not bound to procoagulant FVIII (Wion, *et al* 1985, Yarovoi, *et al* 2003).

Accumulating evidence suggests that the glycosylation profile of platelet-VWF also differs significantly from that of plasma-VWF. Although the N- and O-linked glycosylation profiles of platelet-VWF have not been described in detail, preliminary data suggest that these structures differ significantly from those expressed on plasma VWF (Kagami, *et al*

2000, Williams, *et al* 1994). Despite the fact that ABO blood group antigens are expressed on a number of different platelet membrane glycoproteins (GP) such as GPIb, IIb, and IIIa (Ogasawara, *et al* 1993, Santoso, *et al* 1991), blood group A and B determinants are *not* present on platelet-VWF (Brown, *et al* 2002, Matsui, *et al* 1999). The absence of AB expression on platelet-VWF may have functional importance, since ABO expression on plasma-VWF has been shown to significantly influence ristocetin-induced platelet aggregation activity (Sarode, *et al* 2000). Furthermore, despite the fact that ABO blood group significantly influences plasma-VWF levels, it has no effect on platelet-VWF concentrations (Rodeghiero, *et al* 1992, Sweeney and Hoernig 1992).

The glycosylation profiles of platelet- and plasma-VWF may also differ in other respects (Kagami, *et al* 2000, Williams, *et al* 1994). In particular, Williams et al found that expression of sialic acid was significantly reduced on platelet-VWF (56.7nmol/mg) compared to plasma-VWF (115.5 nmol/mg) (Williams, *et al* 1994). Furthermore, total galactose expression on platelet-VWF was also reduced approximately two-fold compared to plasma-VWF. The clinical significance of these findings remains unclear. However, sialic acid expression has previously been shown to play a key role in protecting VWF multimers against proteolysis by a number of serine and cysteine proteases (including trypsin, chymotrypsin and plasmin) (Berkowitz and Federici 1988, Federici, *et al* 1984, Sodetz, *et al* 1977). Further studies are required to determine whether these differences in platelet-VWF glycosylation, including absence of AB blood group antigen expression, have any direct effect upon the functional activities of platelet VWF, or influence susceptibility to ADAMTS13 proteolysis.

The functional properties of purified platelet-VWF have been compared to those of plasma-VWF (Williams, *et al* 1994). Both platelet- and plasma-VWF bound to type I collagen with similar affinities. Interestingly however, despite the presence of higher molecular weight multimers, platelet-VWF bound to GpIbα with markedly lower affinity than plasma-VWF. In contrast, platelet-VWF demonstrated significantly enhanced ability to bind to activated GpIIb/IIIa, and to unfractionated heparin. The molecular mechanism(s) responsible for mediating these differences in functional properties have not been established, but is likely to be mediated at least in part by variations in VWF glycosylation (Mannucci 1995, Williams, *et al* 1994).

	Endothelial cell origin	Megakaryocytic origin
1. 1. Constitutive secretion	Yes	No
2. 2. Storage organelles	Weibel-Palade bodies	α-granules
3. 3. Secretagogues	Thrombin, ADP, epinephrine, histamine	Thrombin, ADP, collagen, thromboxane A2
4. 4. Multimers	Range of multimers	UL-VWF
Glycosylation		
5. ABO antigen expression	Yes	No
6. Sialic acid expression	++	+
7. Galactose expression	++	+
Function		
8. Collagen binding	++	++
9. GPIb binding	++	+
10. GPIIb/IIIa binding	++	+++
11. Heparin binding	++	+++

Table 1-2 Comparison of endothelial-derived and platelet-derived VWF:- general characteristics, glycosylation and functional activities.

1.7 Clearance of VWF

After constitutive secretion from the endothelium, VWF circulates in plasma with a half life of ~12 hours, prior to clearance from the bloodstream (Denis, *et al* 2008). However, the exact mechanisms responsible for clearance of VWF have not yet been elucidated. Previous studies have indicated that VWF is predominantly removed from the circulation via the liver and spleen (Lenting, *et al* 2004). Regulation of VWF clearance is critical for haemostasis, as increased rates of clearance can lead to a bleeding phenotype and quantitative VWD (Brown, *et al* 2003, Casonato, *et al* 2002).

1.7.1 In vitro VWF clearance

Clearance pathways of VWF have been studied using cellular models of liver and spleen macrophages. FVIII/VWF complexes were taken up and subsequently degraded by differentiated THP-1 macrophages in culture (van Schooten, *et al* 2008).

1.7.2 In vivo VWF clearance

Administration of radiolabeled VWF to VWF-deficient mice showed that the majority of VWF was targeted to the liver for degradation (Lenting, *et al* 2004). VWF was also found to be distributed throughout the kidneys and spleen, suggesting these organs may also play a role in VWF clearance in vivo. Removal of VWF from the circulation in this model was found to be independent of VWF multimer size. Furthermore, deletion of several domains of VWF had an effect on the rate of clearance; deletion of the N-terminal D3-D' domains resulted in increased VWF clearance, whereas deletion of the C-terminal D4-CK domains led to significantly reduced clearance levels.

Animal studies have also been used to illustrate that VWF clearance is partially mediated through the asialo glycoprotein receptor (ASGPR; Ashwell receptor) expressed on hepatocytes (Braun, *et al* 1996). *ASGPR 1* knockout mice demonstrated an increased level of circulating VWF, however ablation of ASGPR expression did not completely inhibit VWF clearance, suggesting other receptors may be participating in this process (Grewal, *et al* 2008). Similarly, blockade of the reticulo-endothelial system (including phagocytes such as Kuppfer cells) of the liver of rabbits resulted in reduced clearance of the FVIII/VWF complex (Sodetz, *et al* 1977). Moreover, administration of the hepatic ASGPR inhibitor asialo-α_1-acid glycoprotein significantly increased FVIII/VWF survival time (Sodetz, *et al* 1977). Finally, a point mutation identifed in the D3 domain of VWF (R1205H) results in rapid clearance and represents the molecular mechanism responsible for type Vincenza VWD (Casonato, *et al* 2002).

1.7.3 Role of carbohydrate in VWF clearance

The glycan profile of VWF is an important modulator of clearance from the circulation. Enzymatic desialylation of VWF leads to an 50-fold decrease in VWF half life when studied in rabbits (Sodetz, *et al* 1977). Furthermore, sialic acid expression levels were highlighted as a critical determinant of VWF survival in mice displaying impaired sialylation due to absence of the ST3Gal-IV sialyltransferase (Ellies, *et al* 2002). Similarly, recombinant VWF devoid of O-linked glycosylation was cleared significantly faster than wild type recombinant VWF, or endothelial plasma-derived VWF (Stoddart, *et al* 1996). An animal model of VWD, the RIIIS/J mouse displays additional expression of GalNAc residues on VWF, due to the aberrant expression of a glycosyltransferase in the endothelium (Mohlke, *et al* 1999). Low levels of VWF in the RSIII/J mouse have been

attributed to increased clearance levels via the ASGPR. Further evidence to support this hypothesis is provided by the fact that infusion of the ASGPR inhibitor asialofetuin corrected VWF levels and prevented rapid clearance of VWF in these mice. Blood group determinants also appear to have an effect on the rate of VWF removal from the plasma. Gallinaro et al have shown that the elimination half-life of blood group O VWF is significantly shorter than that of non-O VWF after treatment of healthy subjects with DDAVP (Gallinaro, *et al* 2008).

1.8 Characterisation of VWF glycosylation

The post-translational modification of VWF includes abundant glycosylation (Wagner and Marder 1984). As previously discussed, mature VWF contains 12 putative sites for N-linked glycosylation and 10 O-linked sites (Titani, *et al* 1986). Several techniques have been used to determine the types of glycans expressed on VWF, their abundance and distribution throughout the VWF monomer.

1.8.1 N-linked glycosylation

The N-linked oligosaccharides chains of VWF are large, complex structures. These carbohydrates have been characterised using a combination of sequential exoglycosidase digestion in conjunction with methylation analysis (Matsui, *et al* 1992), gas liquid chromatography and liquid chromatography (Samor, *et al* 1982), NMR spectroscopy (Debeire, *et al* 1983) and lectin analyses (Matsui, *et al* 1991, Osawa and Tsuji 1987).

Monosialylated (56%) or disialylated (30%) bi-antennary and tri-antennary complex-type glycan chains were shown to constitute the predominant N-linked structures (Matsui *et al*, 1992). The primary N-linked glycan structure expressed on plasma-VWF is a monosialylated bi-antennary complex carbohydrate, in which the basic pentasaccharide core has two N-acetyllactosamine residues (Gal β1-4 GlcNAc) added, one of which is capped by terminal sialic acid (Debeire, *et al* 1983, Samor, *et al* 1982) (Figure 1-7). Unusually, the complex N-linked oligosaccharide of plasma-VWF also express covalently linked A (GalNAc α1→3 [Fuc α1→2] Galβ 1→4 GlcNAc β1→), and B (Gal α1→3 [Fuc α1→2] Galβ 1→4 GlcNAc β1→) blood group antigenic determinants (Matsui, *et al* 1999, Matsui, *et al* 1992). Besides VWF, the only other plasma glycoproteins previously shown to express covalently linked ABO blood group antigens are FVIII and α_2-macroglobulin (Matsui, *et al* 1999).

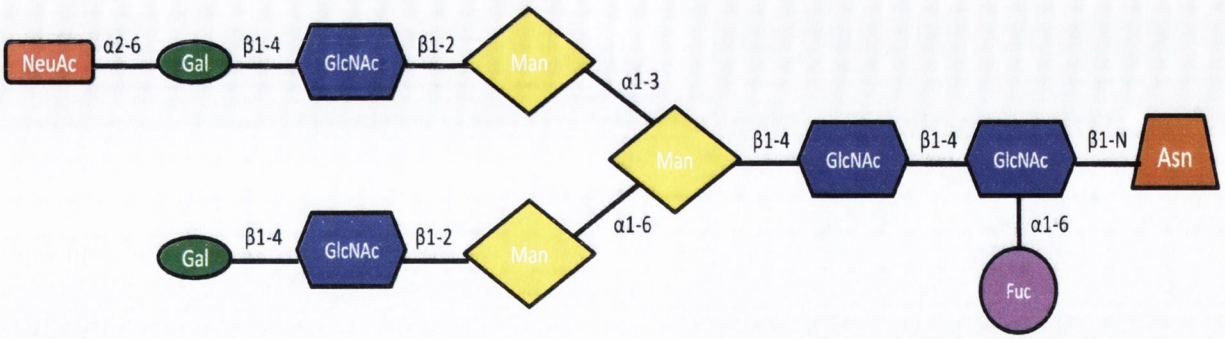


Figure 1-7 VWF N-linked glycan structure.

Primary structure of the major O-linked glycan chain present on endothelial derived plasma-VWF. NeuAc represents sialic acid, Gal represents galactose, GlcNAc represents N-Acetylglucosamine, Man represents mannose, Fuc represents fucose and Asn indicates that the oligosaccharide structure is attached to the nitrogen of asparagine residue side chains.

1.8.2 O-linked glycosylation

In contrast, the O-linked glycans of VWF are predominantly short mucin-type carbohydrates, with a single sialylated tetrasaccharide structure (NeuAc(2-3)Gal(β1-3)[NeuAc(α2-6]GalNAc-ol) accounting for more than 70% of the reduced O-linked glycans (Figure 1-8) (Samor *et al*, 1989). This carbohydrate structure is also referred to as the O-linked sialylated tumour-associated T-antigen, as it was first identified in studies on carcinoma cells (Springer 1984). Recently, the entire O-glycome of VWF was described in detail. Again, the predominant O-linked oligosaccharide structure expressed on VWF was found to be a core 1 sialylated structure ($NeuAc_2Hex_1HexNAc_1$) (Canis, *et al* 2010). Interestingly, the presence of blood group antigenic structures was demonstrated. The distribution of ABO(H) moieties was ~1% expression on O-linked sugar structures. An unusual tetra-sialylated core 1 O-glycan structure was also described; this structure includes a disialosyl motif, where two sialic acid resides are linked together (Canis, *et al* 2010).

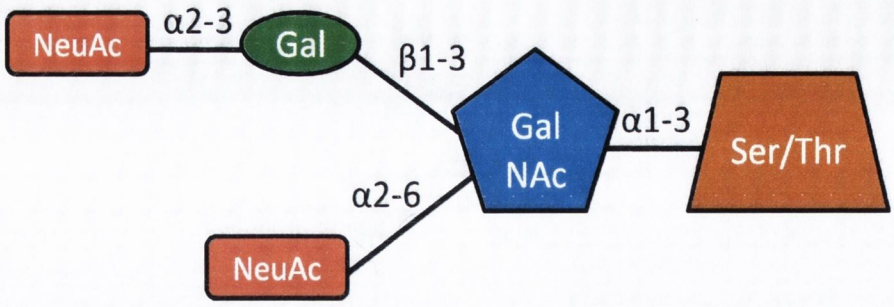


Figure 1-8 VWF O-linked glycan structure.

Primary structure of the major O-linked glycan chain present on endothelial derived plasma-VWF. NeuAc represents sialic acid, Gal represents galactose, GalNAc represents N-Acetylgalactosamine and Ser/Thr indicates indicates that the oligosaccharide structure is attached to the hydroxy oxygen of serine or threonine residue side chains.

1.9 Functional significance of VWF carbohydrate expression

Previous studies have shown that VWF oligosaccharide structures participate in the modulation of VWF function. Modification of VWF glycosylation through treatment with specific exoglycosidases or production of recombinant glycan variants of VWF has proven to be invaluable techniques for the characterisation of VWF carbohydrate function.

The function of asialo-VWF has been extensively studied. Desialylation of VWF was found to result in spontaneous platelet binding in the absence of ristocetin, suggesting sialic acid residues on VWF negatively regulate the VWF-platelet interaction (De Marco and Shapiro 1981). In contrast, other studies have indicated that asialo-VWF does not display increased affinity for platelets, regardless of the presence or absence of ristocetin (Goudemand, *et al* 1985, Rosenfeld and Kirby 1979). Removal of sialic acid does not affect VWF binding to collagen, nor does it directly affect multimer size. However, desialylation of VWF increases susceptibility to non-specific proteolysis, which leads to degradation of high molecular weight multimers (Berkowitz and Federici 1988, Federici, *et al* 1984).

Subsequent removal of penultimate galactose residues from VWF after treatment with neuraminidase affects platelet binding, such that asialo-agalacto-VWF displays reduced RiCoF and platelet aggregation levels in vitro (Gralnick 1978, Kao, *et al* 1980, Sodetz, *et al* 1978). Other functional studies have demonstrated that asialo-agalacto-VWF binds to collagen with a similar affinity to wild type VWF, and also displays a normal multimeric pattern (Gralnick, *et al* 1983). Similarly, N-linked deglycosylation of VWF does not affect

multimeric structure or collagen binding activity (McKinnon, *et al* 2008). Federici et al determined that removal of N-linked glycans via treatment with endoglycosidase F ablated increased platelet binding, that was previously seen for asialo- and asialo-agalacto-VWF (Federici, *et al* 1988). Studies on recombinant O-less VWF showed that loss of O-linked glycans did not increase VWF susceptibility to degradation in plasma (Carew, *et al* 1992). Moreover, O-less rVWF demonstrated a decreased interaction with fixed platelets in the presence of ristocetin, however no effect on platelet agglutination was observed in the presence of botrocetin. The effect of VWF glycosylation on heparin binding has not been studied.

VWF glycan modification	VWF:CB	RiCoF	Platelet aggregation	Multimeric structure	Non-specific proteolysis
Asialo	↔	↑	↑	↔	↑
Agalacto	↔	↓	↓	↔	↑
N-less	↔	↔	↔	↔	↑
O-less	↔	↓	↓	↔	↔

Table 1-3 Effect of glycan modification on VWF functional activity.

↑ denotes an increase in activity; ↔ indicates no effect/normal activity; and ↓ represents a reduction in function.

1.10 VWF proteolysis – ADAMTS13

Regulation of VWF activity in plasma occurs through proteolysis of large multimers into smaller less adhesive forms. Circulating plasma VWF undergoes limited proteolytic degradation in healthy individuals (Zimmerman *et al*, 1986). This degradation results in rapid removal of any unusually large VWF multimers (ULVWF) from the circulation, and ultimately leads to the generation of dimers of VWF (140kDa and 176kDa; (Berkowitz, *et al* 1987). In some forms of type 2A von Willebrand disease, VWF proteolysis is pathologically increased, leading to absence of HMWM of VWF and a consequent bleeding tendency (Goodeve 2010). Several other proteases, including plasmin, trypsin and chymotrypsin have also been shown to cleave VWF (Andersen, *et al* 1980, Batlle, *et al* 1987, Berkowitz and Federici 1988).

1.10.1 Identification and characterisation of ADAMTS13

Isolation of a plasma protease capable of cleaving VWF into smaller forms was achieved by two research groups in 1996 (Furlan, *et al* 1996, Tsai 1996). Further studies and N-terminal sequencing identified this enzyme as a member of the ADAMTS family of metalloproteases (Zheng, *et al* 2001). The 'VWF cleaving protease' was partially purified and determined to be ~200kDa in size. Purification and in vitro analysis showed that ADAMTS13 selectively cleaves VWF; no other substrate(s) have been identified to date.

ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 motif, member 13) was purified and further characterised by two groups in 2001 (Fujikawa, *et al* 2001, Zheng, *et al* 2001). ADAMTS13 is a zinc-metalloprotease that utilises divalent, positively charged metal ions (cations) for its catalytic activity (Ca2+, Ba2+ etc)

(Malemud 2006). It is a large (~190kDa) multidomain glycoprotein containing ten putative N-linked glycosylation sites (Zhou and Tsai 2009) (Figure 1-9). The protease is primarily synthesised in hepatic stellate cells and circulates in the plasma at a concentration of 0.5-1μg/ml (2-5nM) (Uemura, *et al* 2005, Zhou, *et al* 2005). More recently, ADAMTS13 antigen was detected in human endothelial cells in culture, and also in permeabilized platelets (Suzuki, *et al* 2004, Turner, *et al* 2006). Analysis by reverse transcription-polymerase chain reaction (RT-PCR) showed low levels of ADAMTS13 mRNA in endothelial cells and platelets, suggesting that synthesis of the protease also occurs here. Moreover, constitutive secretion of ADAMTS13 from human umbilical vein endothelial cells (HUVECs) has also been described (Turner, *et al* 2009b).

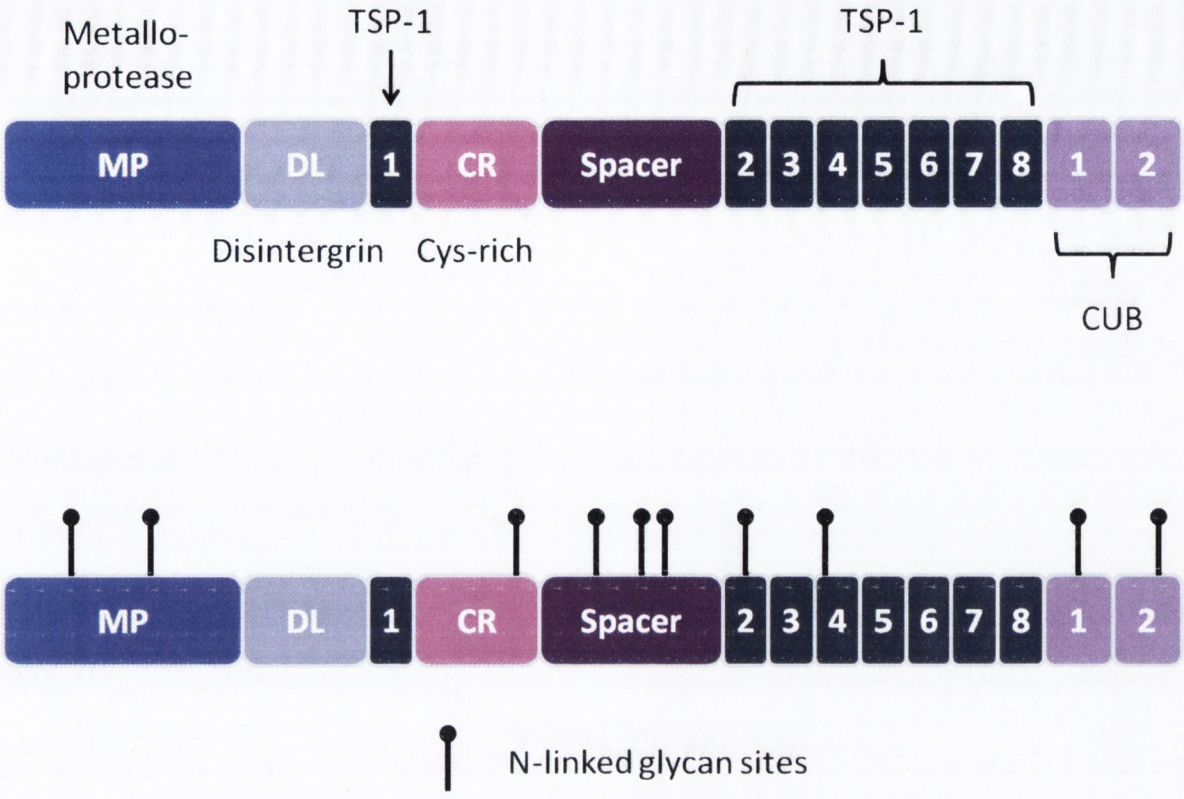


Figure 1-9 Multidomain structure of ADAMTS13.

The structure of ADAMTS13 incorporating the metalloprotease, disintergrin-like, thrombospondin type 1 repeats (TSP-1) 1-8, cysteine-rich, spacer and complement C1r/C1s, urchin epidermal growth factor, and bone morphogenic-1 like (CUB) domains are represented above. Putative sites of N-linked glycosylation are also highlighted.

1.10.2 VWF-ADAMTS13 interaction

ADAMTS13 proteolyses VWF at the Tyr1605-Met1606 scissile bond, found in the VWF A2 domain (Dong, *et al* 2003). Since the identification of ADAMTS13 in 2001, many studies have been carried out to determine the molecular mechanisms and conditions under which the protease regulates VWF activity. Newly secreted Weibel-Palade body (WPB) derived ULVWF is rapidly cleaved by ADAMTS13 on the endothelial cell surface (Arya, *et al* 2002). This suggests that ADAMTS13 regulates ULVWF activity as soon as it is secreted, and thus prevents hyperactive and prothrombotic ULVWF multimers from entering the bloodstream. Platelet VWF strings are cleaved by ADAMTS13 under flow (Donadelli, *et al* 2006). Moreover, platelet VWF complexes are the preferred substrates for ADAMTS13 in an in vitro model of shear stress (Shim, *et al* 2008). Binding of VWF to GP1bα on the surface of platelets allows the protein to adopt a conformation that is more permissive to cleavage by ADAMTS13 (Nishio, *et al* 2004). More recently, ULVWF derived from histamine stimulated HUVECs was shown to be cleaved by ADAMTS13 in the absence of shear stress, denaturants or platelets. This implies that rapid secretion from endothelial cells and successive binding to the endothelium allows ADAMTS13 to access the VWF A2 domain (Turner, *et al* 2009a).

Several exosites and amino acid residues present in ADAMTS13 are critical for proteolytic function and cleavage of VWF. The non-catalytic spacer domain of ADAMTS13 binds to an exosite found at the carboxy-terminal end of the VWF A2 domain (Gao, *et al* 2006). Furthermore, 3 amino acid residues in the ADAMTS13 spacer domain have been shown to be crucial for VWF-cleaving activity; Arg659, Arg660 and Tyr661 (Jin, *et al* 2010). Similarly, Zhou et al have identified 4 cysteine residues (C1192, C1213,

C1236 and C1254) which were highlighted as vital for efficient secretion of ADAMTS13, and also for the efficient proteolysis of VWF under static conditions (Zhou, *et al* 2010). Several other ADAMTS13 domains have been shown to provide contacts with exosites in the A2 domain of VWF; the first thrombospondin-1 repeat; the cysteine rich; and the spacer domain (Gao, *et al* 2008). Moreover, an essential functional role for the ADAMTS13 disintergrin-like domain has also been demonstrated (de Groot, *et al* 2009). Finally, susbsites within the metalloprotease domain are important for binding and cleavage of VWF (de Groot, *et al* 2010).

Important areas within VWF that contribute to the ADAMTS13 interaction have also been characterised. Visinical cysteine residues Cys1669 and Cys1670, found in the A2 domain of VWF, form a disulphide bond that is critical for regulation of ADAMTS13 access to the Tyr1605-Met1606 cleavage site (Luken, *et al* 2010). In addition, an ADAMTS13 binding site in the D4 domain of globular VWF has also been described (Zanardelli, *et al* 2009).

FVIII may act as a co factor for ADAMTS13. The presence of FVIII increased the rate at which ADAMTS13 was able to cleave VWF by approximately 10-fold (Cao, *et al* 2008). However, paradoxically, individuals with haemophilia A (absence of FVIII) do not exhibit high levels of ULVWF (Jacquemin, *et al* 2003). Recently, both FVIII and platelets were shown to synergistically increase VWF susceptibility to ADAMTS13 proteolysis under conditions of shear stress (Skipwith, *et al* 2010). Deglycosylation of ADAMTS13 does not affect its catalytic activity, suggesting that the carbohydrate portion of ADAMTS13 helps

to maintain stability and protect from proteolytic degradation in the plasma, and is not involved in protease function (Zhou and Tsai 2009).

To date, the mechanisms through which ADAMTS13 activity is regulated have not been elucidated. Both thrombin and plasmin can cleave and inactivate ADAMTS13, which may contribute to the regulation of VWF proteolysis (Crawley, *et al* 2005). Magnesium ions have been shown to enhance VWF cleavage by ADAMTS13, and therefore decrease platelet aggregation (Dong, *et al* 2008). Oxidation of tyrosine and methionine residues in VWF by neutrophil oxidants (hydrogen peroxide, superoxide radical and hypochlorous acid) inhibits ADAMTS13 proteolysis (Chen, *et al* 2010). Interleukin-6, free haemoglobin and thrombospondin-1 are known inhibitors of ADAMTS13 in vitro (Bernardo, *et al* 2004, Bonnefoy, *et al* 2006, Studt, *et al* 2005). Clearance mechanisms have not been defined for ADAMTS13.

1.10.3 VWF and ADAMTS13 in health and disease

Regulation of VWF adhesive activity is crucial for maintaining homeostasis in the blood. Deficiency of ADAMTS13 results in the life threatening microangiopathy, thrombotic thrombocytopenic purpura (TTP) (Levy, *et al* 2001). TTP is characterised by the consumption of platelets, as a result of the formation of platelet and VWF rich thrombi in the microvasculature. Most cases of TTP can be attributed to low ADAMTS13 antigen levels or decreased ADAMTS13 activity; due to a dysfunctional protein or acquired autoantibodies (Luken, *et al* 2005).

Several animal models have shown that deficiency of ADAMTS13 leads to a prothrombotic state, with the duration of VWF-platelet-endothelium interactions being increased (Banno, *et al* 2006). Furthermore, administration of recombinant ADAMTS13 to deficient mice promoted breakdown of thrombi in arterioles (Chauhan, *et al* 2008). Interestingly, ADAMTS13 deficient mice do not present with TTP like symptoms until challenged with shiga toxin (Motto, *et al* 2005).

Regulation of VWF adhesive potential by ADAMTS13 is not only crucial for the control of haemostasis, but also plays a major role in the regulation of VWF function in inflammation. VWF has been shown to participate in the progress of inflammation through adhesion to polymorphonuclear cells and monocytes, thereby contributing to leukocyte tethering and rolling (Pendu, *et al* 2006). Thrombocytopenia is also a major complication of inflammation, and VWF is an important mediator of this process (Wagner and Frenette 2008). ADAMTS13 levels have been documented to be low in both chronic and acute inflammation (Kremer Hovinga, *et al* 2007, Martin, *et al* 2007). Moreover, an inverse correlation has between high levels of ULVWF and low levels of ADAMTS13 been observed in systemic inflammation (Claus, *et al* 2009). Similarly, high and low levels of ULWVF and ADAMTS13 respectively have been found in ICU patients with systemic inflammation resulting from cardiac surgery or severe sepsis (Bockmeyer, *et al* 2008). The thrombotic state associated with endotoxemia may be due to an imbalance in the expression of both VWF and ADAMTS13; as high and low mRNA levels, as well as high and low blood antigen levels, were found in LPS challenged mice (Mimuro, *et al* 2008).

1.10.3.1 Malaria, VWF and ADAMTS13

Previous studies have demonstrated the presence of circulating ULVWF in the plasma of malaria patients, with a correlation between levels of ULVWF and severity of disease (de Mast, *et al* 2007, Groot, *et al* 2007, Hollestelle, *et al* 2006). Endothelial dysfunction occurs in malaria, as high levels of VWF propeptide have been reported (Hollestelle, *et al* 2006). Moreover, elevated levels of hyperactive ULVWF have been described in *P. falciparum* infection, exceeding those reported in both acute septicaemia and TTP (van Mourik, *et al* 1999).

Both cerebral and severe malaria patients have decreased ADAMTS13 antigen and activity levels. An inhibitory factor present in malarial plasma was found to reduce ADAMTS13 activity in vitro (Larkin, *et al* 2009). Similarly, deficiency of ADAMTS13 antigen and activity levels in plasma from patients infected with *P. vivax* and *P. falciparum* malaria was also shown; the molecular mechanism responsible for decreased ADAMTS13 levels in this instance remains to be elucidated (de Mast, *et al* 2009). More recently, it was reported that platelet accumulation at microvascular sites in the brain is mediated through a novel bridging mechanism, where ULVWF strings, decorated with platelets, sequester infected erythrocytes (IE) on activated endothelium. This IE adhesive surface can be rapidly removed upon treatment with ADAMTS13 (Bridges, *et al* 2009).

1.10.4 VWF glycosylation and ADAMTS13

Modulation of ADAMTS13 activity upon VWF in plasma has not been fully characterised. However, VWF carbohydrate structures, and modification of these glycan moieties, have shown they are critical for regulating VWF susceptibility to proteolysis. Removal of N-

linked glycan chains via enzymatic deglycosylation leads to a four-fold increase in the rate of cleavage by ADAMTS13. In particular, the sugar structure present at N1574 in the VWF A2 domain was found to be crucial for protecting VWF from ADAMTS13 degradation (McKinnon, *et al* 2008). Single terminal carbohydrate structures, namely ABO(H) blood group antigenic structures have also been shown to modulate ADAMTS13 cleavage of VWF, with blood group O VWF being significantly more susceptible to cleavage than blood group A, B or AB (Bowen 2003, O'Donnell, *et al* 2005). Bombay type VWF, where the H-antigenic structure is missing, due to a deficiency in H transferase (*FUT 1*), displays increased susceptibility to ADAMTS13 proteolysis compared to blood group AB or O VWF (O'Donnell, *et al* 2005). Removal of complete oligosaccharide chains and the presence or absence of terminal glycan residues appears to alter VWF conformation, such that ADAMTS13 can gain access to the cleavage site and/or binding sites present in the A2 and surrounding domains of VWF. The roles of O-linked glycans, terminal sialic acid expression, and sub-terminal galactose residues in mediating VWF proteolysis by ADAMTS13 remain to be elucidated.

1.11 Purpose of investigation:

In light of the role that VWF carbohydrate structures play In modulating susceptibility to ADAMTS13 proteolysis, we hypothesized that terminal sialic acid expression levels may also be exerting an influence. Modification of endogenous sialic acid levels on VWF alters susceptibility to non-specific proteolysis. Moreover, removal of complete N-linked glycan structures increases the rate of ADAMTS13 proteolysis ~4-fold. Therefore, the aim of part of the work detailed in this thesis was to establish the contribution of sialic acid to the regulation of plasma-derived VWF proteolysis by ADAMTS13.

In addition, platelet-VWF displays variable glycosylation to plasma-VWF, and this could subsequently impact on the rate of ADAMTS13 proteolysis. Many functional comparisons have been drawn between plasma- and platelet-derived VWF; however susceptibility to ADAMTS13 cleavage remains to be addressed. In the second part of this thesis, I describe how platelet-VWF susceptibility to ADAMTS13 was investigated, and also how the glycan structures present on platelet-VWF are a key modulator of this process.

CHAPTER 2:

MATERIALS AND METHODS

2.1 Purification of VWF by fast protein liquid chromatography (FPLC)

2.1.1 Purification of VWF from Haemate P®

Haemate P® (ZLB Behring, Germany) is a commercial concentrate used to treat patients with von Willebrand disease (VWD). Haemate P® contains high concentrations of VWF, FVIII, albumin and excipients. Heparin sepharose affinity chromatography followed by size exclusion chromatography was used to purify plasma-derived VWF (pd-VWF) from Haemate P®.

2.1.2 Heparin-Sepharose affinity chromatography

VWF contains a heparin binding domain; this property is exploited for purification utilising immobilised heparin. A SK-16 chromatography (Amersham Pharmacia, UK) was packed with 30ml of Heparin-Sepharose 6 fast flow (Amersham Pharmacia, UK) according to manufacturer's instructions. The column was equilibrated with 2 column volumes of buffer A (100mM NaCl, 20mM Tris-HCl, pH 7.4). Haemate P®, resuspended in 10ml dH_2O was passed over the column at a flow rate of 0.4ml/min. After sample loading was complete, the column was washed with buffer A until the UV baseline returned to 0 (~10 column volumes). VWF was eluted from the column with elution

buffer (300mM NaCl, 20mM Tris-HCl, pH 7.4; Figure 2-1). One 5ml fraction was collected and high salt levels removed using a HiTrap™ desalting column (GE Healthcare, Buckinghamshire, UK; Figure 2-2). These columns are pre-packed with Sephadex™ G-25 Superfine and remove salt from protein samples by size exclusion chromatography. Following desalting/buffer exchange, VWF was further purified by gel filtration.

2.1.3 Gel filtration

Gel filtration (size exclusion) chromatography separates proteins on the basis of size. Larger proteins elute from the column through a stationary phase at a faster rate than smaller proteins. Therefore the highest molecular weight VWF mulitmers are present in the first fractions collected from the column (Figure 2-3). Consequently, this methodology can be exploited for the separation of high, intermediate and low molecular weight multimers of VWF.

To isolate high molecular weight VWF, a Sepharose 2B-CL gel filtration column (Amersham Pharmacia, UK) (600mm x 26mm, 318.5ml volume) was pre-equilibrated overnight with 2 column volumes of Tris-citrate (TC) buffer (20mM Tris, 10mM sodium citrate, pH 7.4). 5-10 ml of VWF, present in cryoprecipitate (2.3.1), or in eluted fractions from heparin sepharose chromatography (2.1.2), was applied to the column at a flow rate of 0.5ml/min, then eluted with TC buffer at a flow rate of 1ml/min. Eighteen 5ml fractions were collected (fractions B4-C9; Figure 2-3) and assessed for purity and VWF content by SDS-PAGE (2.5.1) followed by Coomassie® blue staining (2.5.2), and VWF:Ag ELISA (2.4.1) respectively.

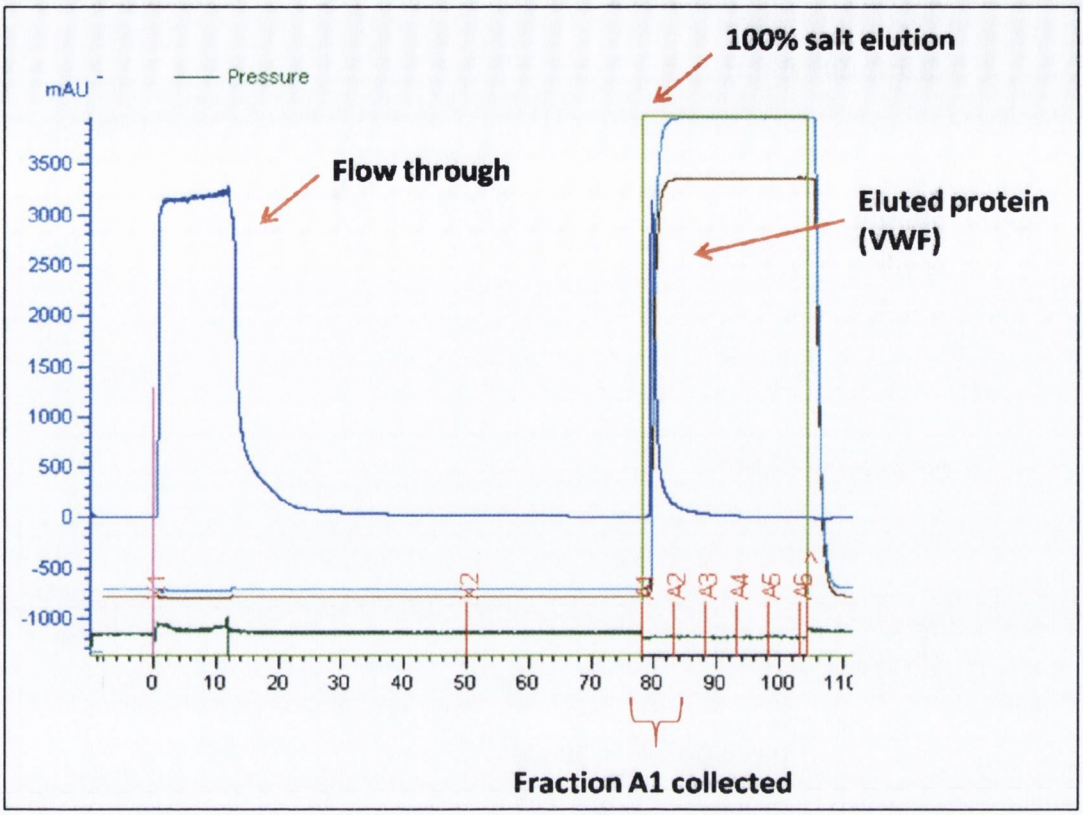


Figure 2-1 Purification of pd-VWF: - heparin sepharose chromatography.

The majority of proteins found in Haemate P® or plasma do not bind to heparin, and are present in flow through fractions (first peak). VWF binds to heparin and is therefore present in the eluate obtained after passing buffer containing 300nM NaCl (100% salt condition) over the column (second peak; corresponding to fraction A1).

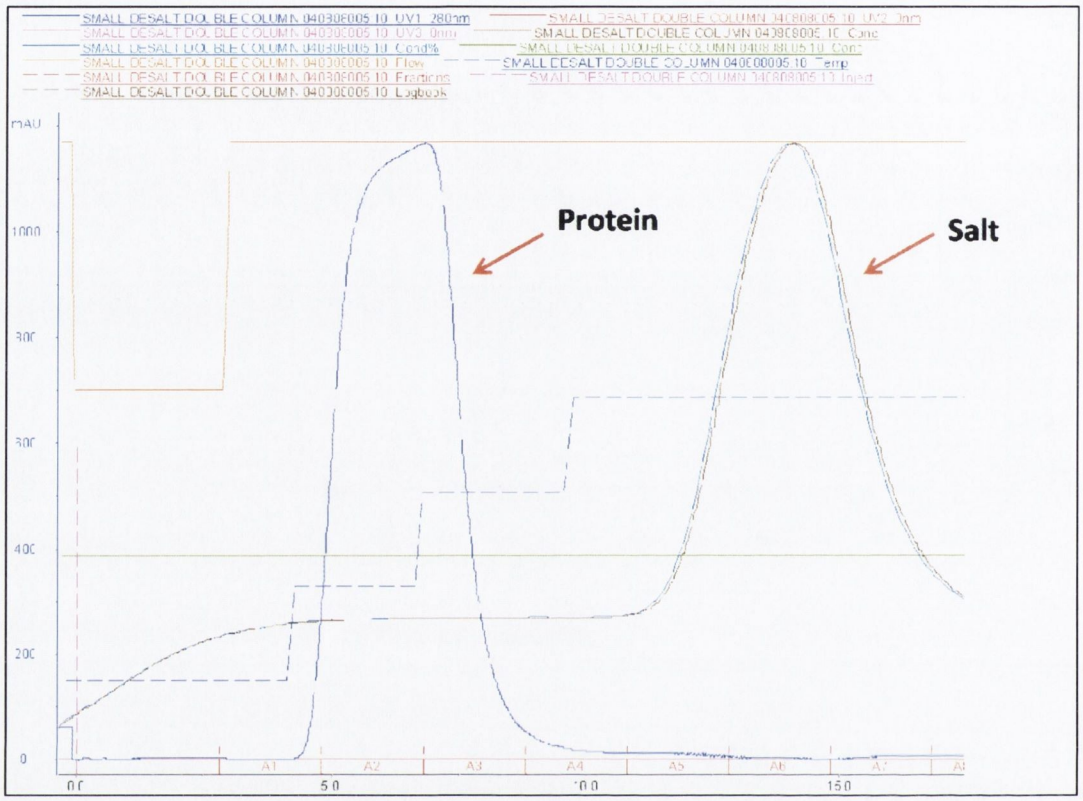


Figure 2-2 Purification of pd-VWF: - desalting step.

The UV spectrum shows proteins eluting from the column (first peak) prior to salt (second peak), as this chromatography step is based on size exclusion. Desalting using FPLC is a convenient and quicker way to carry out buffer exchange than dialysis.

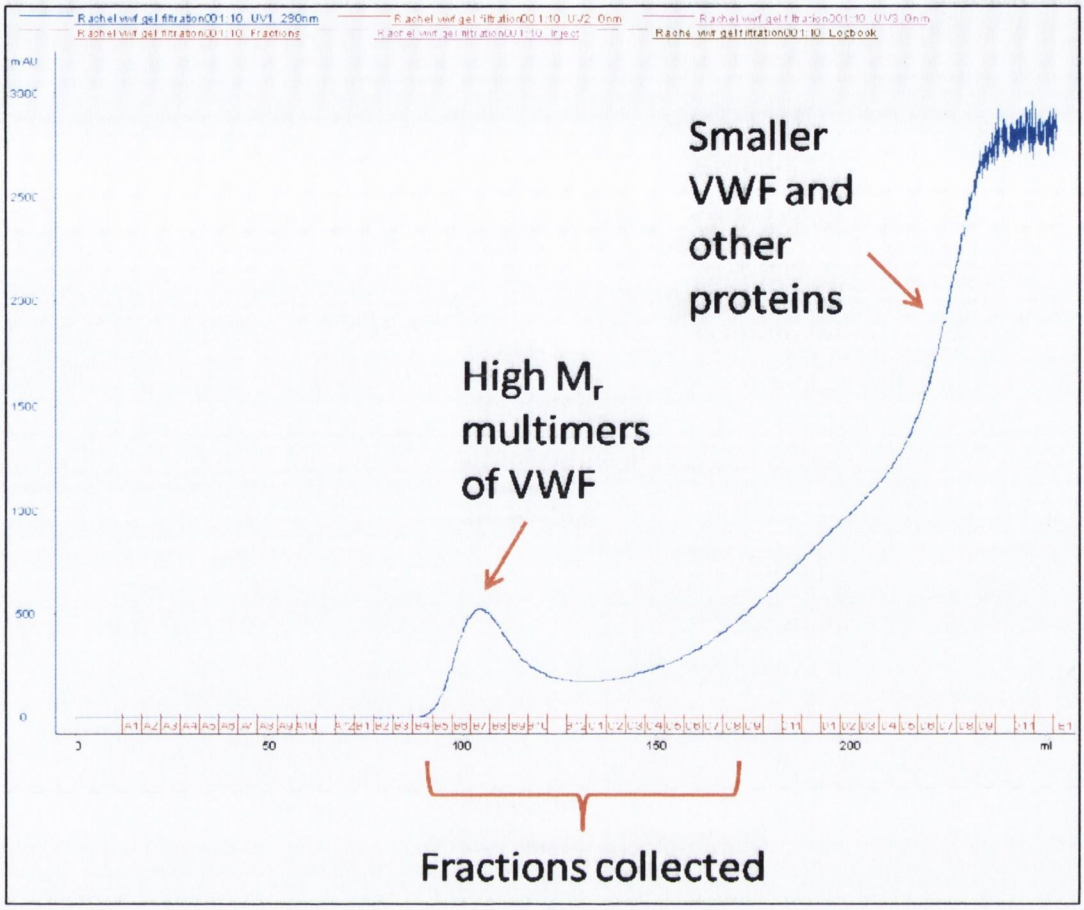


Figure 2-3 Purification of pd-VWF: - size exclusion chromatography.

VWF present in reconstituted Haemate P®; fractions from prior purification steps; or cryoprecipitate was applied to a Sepharose 2B-CL gel filtration column (Amersham Pharmacia, UK).

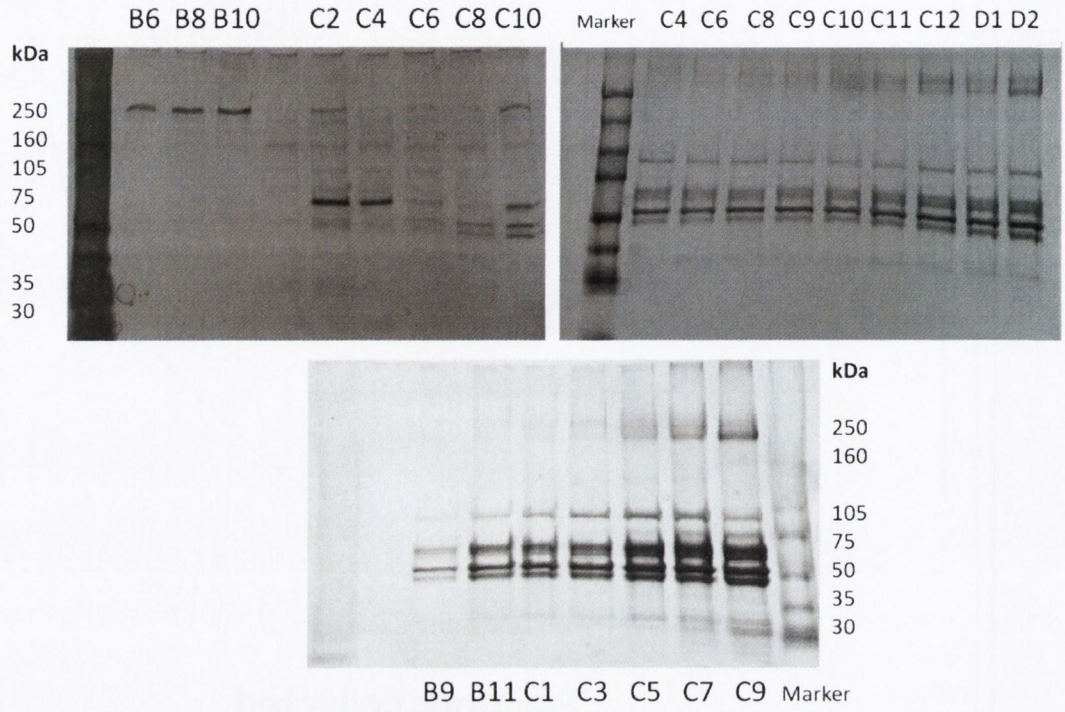
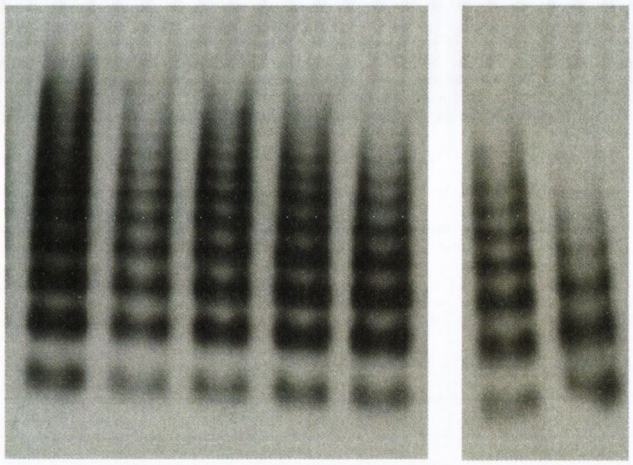


Figure 2-4A Purification of pd-VWF: - assessment of purity by SDS-PAGE.

Purity of fractions collected after heparin sepharose and size exclusion chromatography were analysed by SDS-PAGE followed by silver staining. 3 representative purifications are shown. The purity of VWF, present at ~250kDa decreases with increasing fraction number.



B6 B8 B10 B12 C2 C6 C10

Figure 2-4B Purification of pd-VWF: - assessment of multimer distribution.

Multimer analysis of VWF obtained from purification protocol involving heparin sepharose chromatography and gel filtration. VWF multimer size decreases with increasing fraction number corresponding to separation based on size.

FPLC Fraction Number	VWF:Ag (µg/ml)	FPLC Fraction Number	VWF:Ag (µg/ml)
B5	48	**B12**	155
B6	114	**C1**	138
B7	155	**C2**	123
B8	151	**C3**	69
B9	168	**C4**	62
B10	166	**C5**	42
B11	159	**C6**	32

Table 2-1 Yield of VWF in fractions collected after size exclusion chromatography as determined by VWF:Ag ELISA.

2.2 Purification of platelet-derived VWF

2.2.1 Isolation of platelets

Expired platelets were obtained from the Components Department, National Blood Centre and Irish Blood Transfusion Service, St. James's Hospital, Dublin 8. Two units of pooled platelets in leukocyte-depleted plasma (~460ml) were decanted into 50ml tubes (~30ml per tube). Platelets were obtained from plasma by centrifugation at 1500g for 10 min at room temperature. The plasma supernatant was discarded and the platelet pellet retained for preparation of washed platelets.

2.2.2 Platelet washing

Platelet pellets were gently resuspended in buffer A (130mM NaCl, 9mM $NaHCO_3$, 6mM dextrose, 10mM trisodium citrate, 0.9mM $MgCl_2$, 3mM KCl, 10mM Tris pH 7.4) containing protease inhibitors (Protease Inhibitor Cocktail I, Calbiochem, Merck, UK). This protease inhibitor cocktail contains selective inhibitors of serine-, cysteine- and metallo-proteases - AEBSF (500μM) and Aprotinin (150nM); E-64 (1μM) and Leupeptin (1μM); and EDTA (0.5mM) respectively. Following resuspension, platelets were centrifuged at 1000g for 10 min. The supernatant was decanted and the platelet washing step was repeated a further three times.

2.2.3 Platelet lysis by snap freeze-thawing

Platelet pellets were resuspended in buffer A. Platelet lysis was carried out by repeated snap freeze-thaw cycles. The platelets were immersed in liquid nitrogen to ensure rapid freezing, followed by rapid defrosting at 37°C in a water bath. Subsequently, platelet lysate was vortexed on a bench top mixer (Vortex Genie® 2, Scientific Industries Inc, USA) set to speed 10 (~2500rpm) for 3 min. Freeze-thaw cycles were repeated 3 times. After sufficient lysis, the platelet mixture was centrifuged at 10,000g for 30 min twice to remove cellular debris and microvesicles (Polgar, *et al* 1998). Platelet proteins present in lysate were stored at -80°C until further use.

2.2.4 Platelet releasate

Platelet releasate was obtained as described elsewhere (Coppinger, *et al* 2004). In brief, washed platelets were stimulated with 5μg/ml thrombin in a BioData (Malvern, PA, USA) PAP-4 aggregometer under constant stirring (1100 rpm). Aggregations were performed for 3 min and the reactions stopped by the addition of 10mM benzamidine. The supernatant was collected and analysed for VWF content as described in section 2.1.3.

2.2.5 Purification of platelet-VWF by immunoaffinity chromatography

Immunoaffinity chromatography was carried out in collaboration with Dr. Maartje van den Biggelaar and Dr. Jan Voorberg in the Department of Plasma Proteins, Sanquin Research, Amsterdam, The Netherlands as previously described (van den Biggelaar, *et al* 2007). Briefly, VWF was purified from platelet lysate by immunoaffinity chromatography

employing the monoclonal antibody (mAb) CLB-RAg20 (Stel, *et al* 1984) coupled to CNBr sepharose 4B (Amersham Biosciences, Buckinghamshire, UK). Multimeric VWF was eluted using 0.1 M NaCl and 50 mM HEPES (pH 7.4). VWF containing fractions were dialyzed against 50mM Tris-HCl containing 150 mM NaCl (pH 7.4) and stored at -80°C.

2.3 Purification of blood group specific VWF from plasma

2.3.1 Cryoprecipitation

Various circulating proteins precipitate out of solution at low temperatures of 4°C or lower. This phenomenon is utilised to extract VWF from plasma. Human plasma not required for patient treatment was obtained from the Irish Blood Transfusion Service (IBTS, St. James's Hospital, Dublin 8) and was frozen overnight at -80°C. Two units of blood group specific fresh frozen plasma (~300ml per unit) were thawed for 24 hours at 4°C. Thawed plasma was pooled in a pre-chilled glass beaker and stirred slowly. 50% (v/v) ethanol, pre-chilled at 4°C was added drop wise to the stirring plasma to a final concentration of 8% (v/v). The plasma was stirred at 4°C for 30 mins and then decanted into 50ml centrifuge tubes in an ice bucket. The resulting precipitate was harvested by centrifugation; 3500rpm for 30 mins at 4°C. The cryo-supernatant was decanted off and a 2ml aliquot was kept and stored at -80°C. Cryo-supernatant can be used as a source of ADAMTS13. The VWF rich pellets were resuspended in 12ml of Tris-Citrate (TC) buffer (50mM Tris, 10mM sodium citrate, pH 7.4) and centrifuged at 3500rpm for 30 mins at room temperature. The VWF-containing supernatant was removed and VWF was further purified by size exclusion chromatography as in 2.1.3.

2.4 Analysis of VWF

2.4.1 VWF:Ag ELISA

VWF antigen (VWF:Ag) levels in platelet lysate, FPLC fractions and citrated plasma samples were measured by sandwich enzyme-linked immunosorbent assay (ELISA). Flat-bottomed 96-well MaxiSorb plates (Nunc, Loughborough, UK) were coated with rabbit polyclonal anti-human VWF antibody (A0082; Dako, Glostrup, Denmark) at a final concentration of 6.2μg/ml (1:500 dilution), in 50mM carbonate buffer (pH 9.6), overnight at 4°C. After washing with phosphate buffered saline containing 0.05% Tween pH 7.4 (PBS-T; BioUltra, Sigma, Ireland), the plates were blocked for non-specific binding with PBS-T containing 3% bovine serum albumin (BSA; Sigma, Ireland) for 1 hour at room temperature. After three further washings, test samples were added to the wells. Blanks used were PBS-T or blocking solution. Coagulation reference plasma (containing 10μg/ml VWF; Technoclone, Surrey, UK) diluted 1:80-1:2560 was used to construct standard curves for calibration. Test samples were usually diluted 1:200-1:1600. The samples were incubated on the plate for 2 hours at 37°C. The plates were then washed three times with PBS-T and subsequently were incubated with rabbit polyclonal anti-human VWF peroxidase conjugate, (P0226; Dako, Denmark) diluted 1:500 in PBS-T (final concentration 2.2μg/ml), for 1 hour. After a further three washes, 190μl of HRP substrate were added (Sigma colour fast *o*-Phenylenediamine dihydrochloride; OPD) and the reaction was stopped after 3-10 min by the addition of 100μl of 1M H_2SO_4. Absorbance was read at 492nM using a VERSAmax microplate reader (Molecular Devices, CA, USA).

All ELISA samples were tested in duplicate, using three replicate wells for each set of test conditions. The intra-assay and inter-assay coefficients of variation were both less than 5% and the lower limit of VWF detection was 0.3µg/ml.

A standard curve was generated by plotting VWF concentration in µg/ml against optical density (OD) 492nM for the corresponding samples.

2.4.2 VWF collagen binding assay (VWF:CB)

The largest molecular weight multimers of VWF bind to collagen with the highest affinity. Therefore, a collagen binding assay can be used to determine the multimeric composition of VWF in a test sample. VWF:CB was determined by using an ELISA based collagen binding assay. 96-well MaxiSorb (Nunc, Loughborough, UK) plates were coated with 5µg/ml of recombinant human collagen III (BioVision, CA, USA) diluted in 50mM carbonate buffer pH 9.6 overnight at 4°C. The wells were washed with PBS-T 3 times and blocked with PBS-T containing 3% BSA for 1 hour at room temperature. Wells were then washed as before and test samples were diluted 1:200-1:1600 in PBS-T. Coagulation reference plasma (Technoclone, Surrey, UK) was diluted 1:80-1:2560 and used to generate a standard curve. Samples were incubated on the plate for 2 hours at room temperature. Following washing, anti-VWF-HRP pAb (Dako, Glostrup, Denmark) diluted 1/500 in PBS-T was added to the wells and incubated for 1 hour at room temperature. The wells were washed and colour was developed as described above (2.4.1). A standard curve and log-log plot were also constructed as for VWF:Ag ELISA (2.4.1) and VWF:CB for each of the test samples was determined in µg/ml.

To determine the VWF:CB/VWF:Ag ratio, VWF:CB was divided by VWF:Ag. Values of 0.7-1.3 are considered within the normal range.

2.4.3 VWF multimer gel

The multimeric pattern of VWF can be visualised by analysis in agarose gels under non-reducing conditions. Low resolution agarose gels (1.8%; 1.5mm diameter) were prepared by dissolving 0.5g of agarose (SeaKem® HGT(P) Agarose, Lonza, ME, USA) in 50ml of electrophoresis buffer (200mM Tris, 100mM glycine (Fisher Scientific, Ireland), 0.1% sodium dodecyl sulphate (SDS; Sigma, Ireland) pH 9.0). The agarose was dissolved by heating the solution to ~70°C. The gels were cast in pre-warmed glass plates and allowed to set at room temperature. The gels were stored overnight at 4°C to ensure uniformity and settling.

Samples were diluted 1:10 with multimer gel sample buffer (10mM Tris, 1mM EDTA, 2% SDS, 8M urea, 0.01% bromophenol blue, pH 8.0) and were incubated for 30 min at 60°C. The gels were set up in a Bio-Rad mini-gel electrophoresis tank and the tank was filled with electrophoresis running buffer. 10µl of each sample were applied to each well and the gels were electrophorised for ~3 hours at 8-10mA. After electrophoresis, transfer of protein to PVDF membranes (Immobilon-FL, Millipore, MA, USA) was carried out overnight at 30V in the cold room (to maintain the gels and buffer at 4°C). The membranes were then rinsed with TBS-T (Tris-buffered saline containing 0.05% (v/v) Tween-20, Sigma, Ireland) and blocked for 1 hour at room temperature with TBS-T containing 3% skimmed milk powder. Finally, the membranes were incubated with anti-VWF-HRP pAb (Dako, Glostrup, UK) diluted 1:2000-1:10000 for 1 hour. After thorough

washing, bound antibody was detected with the SuperSignal® west pico chemiluminescent substrate kit (Pierce, Thermo Fisher, Ireland), the membranes were exposed to autoradiography (x-ray) film (Fujifilm, Bedford, UK) and the films were developed using the AGFA CP1000 automatic film-developing system (AGFA, Germany).

2.5 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

5% SDS-polyacrylamide gels were used for detection of VWF. These gels were cast using a Mini-Protean®II casting stand (Bio-Rad, Alpha Technologies, Wicklow, Ireland), according to the manufacturer's instructions. Alternatively, pre-cast gradient gels were used for analysis of VWF purity and cleavage (4-15% gradient gels, Bio-Rad, Alpha Technologies, Ireland). Samples containing the optimum concentration of VWF required for analysis by SDS-PAGE (see Table 2-2) in 20μl, were mixed with 5μl of 5X reducing SDS-PAGE loading buffer (50% (v/v) glycerol, 8% (w/v) SDS, 0.4% (w/v) bromophenol blue, in 0.5M Tris-HCl, pH 6.8; mix 3 parts of this buffer with 1 part β-mercaptoethanol for reducing conditions) and boiled for 5-30 min. A pre-stained high range molecular weight marker (ColorBurst, Sigma, Ireland or HiMark™ Pre-stained HMW protein marker, Invitrogen, Paisley, UK) was electrophoresed in parallel with all test samples for protein identification based on size. Electrophoresis was carried out at 25mA per gel for 60-120 min. Detection of proteins was performed by silver staining, Coomassie® blue staining and western blotting.

Method of analysis after SDS-PAGE	Optimal [VWF]
Silver Staining	500ng
Coomassie® Blue Staining	1-2μg

Western Blotting	50ng

Table 2-2 Optimal loading concentrations for analysis of VWF by SDS-PAGE.

2.5.1 Silver staining

Silver staining is a technique used to visualise non-specific protein bands on a gel. Silver staining of SDS-PAGE gels was carried out using the SilverXpress® Silver Staining Kit (Invitrogen, Paisley, UK) according to the manufacturer's instructions. This kit is based on the chemical reduction of silver ions to metallic silver on a protein band. Briefly, gels were fixed with 50% (v/v) methanol and 10% (v/v) acetic acid, and sensitised by treatment with 50% (v/v) methanol containing 5% (v/v) sensitizer. Following this, gels were stained with silver nitrate, and protein bands were developed with H_2O containing formaldehyde and citric acid.

2.5.2 Coomassie® blue staining

Coomassie® (Coomassie® Brilliant Blue, Sigma, Ireland) blue staining is another method for detection of proteins. Coomassie® blue staining is less sensitive than silver staining; higher concentrations of protein are required for visualisation of bands on the gel (Table 2-1). After SDS-PAGE, gels were washed with dH_2O and incubated in Coomassie® blue staining solution (0.1% (w/v) brilliant blue (Sigma, Ireland), 20% (v/v) methanol, 10% (v/v) acetic acid) for 40 minutes. Gels were then rinsed in dH_2O and incubated in destain (50% (v/v) methanol, 10% (v/v) acetic acid) for 1 hour.

Alternatively, Simply Safe Stain (Invitrogen, Paisley, UK) was used according to the manufacturer's guidelines. Gels were washed in dH_2O 3 times for 5 min, before incubation in Simply Safe Stain for one hour at room temperature with gentle agitation. Gels were de-stained with dH_2O for 1 hour, followed by another 1 hour water washing step.

2.5.3 Western blotting

After electrophoresis, proteins were transferred onto a PVDF membrane that had been pre-activated by immersion in methanol, using an electroblot system (Mini-Protean®II; BIO-RAD). Transfer was performed overnight on ice at 30V in transfer buffer (25mM Tris 25mM, 192 mM glycine and 20% (v/v) methanol). Following protein transfer, the membrane was blocked for 1 hour using a 5% solution of fat-free milk in TBS-T. The membrane was washed in TBS-T, and then incubated with a conjugated primary antibody (rabbit polyclonal anti-human VWF-HRP conjugate, Dako, Glostrup, Denmark) diluted 1:5000 in 5% milk protein. Following 1 hour of incubation in antibody, the membrane was washed, and the blot was visualized and developed as before (section 2.4.3).

2.6 Glycosidase digestions

The endogenous glycan pattern of VWF was modified using various exo-glycosidase enzymes. Specific glycosidases remove particular carbohydrate structures depending on their position and linkage to the underlying glycan chain. In all glycosidase digestion experiments, VWF was present at a final concentration of 65µg/ml. The reactions were

incubated overnight (16-24 hours) at 37°C. Table 2-3 outlines the different glycosidases used, their source, and which sugar structures they preferentially remove.

Glycosidase	Source Organism	Commercial Source	Specifically removes
PNGase F	*Flavobacterium meningosepticum*	New England Biolabs, UK	N-linked glycans
O-glycosidase	*Streptococcus pneumoniae*	Sigma, Ireland	O-linked glycans
α2-3,6,8,9 neuraminidase	*Arthrobacter ureafaciens*	Calbiochem, Merck, UK	α2-3 and α2-6 linked sialic acid
α2-3 neuraminidase	*Streptococcus pneumoniae*	Sigma, Ireland	α2-3 linked sialic acid
β1-3,4,6 galactosidase	*Streptococcus pneumonia* and *Xanthamonas sp.*	Calbiochem, Merck, UK or Sigma, Ireland	Galactose
α-N-Acetylgalactosaminidase (A-Zyme)	Chicken Liver	Sigma, Ireland	A antigen
α-D-galactosidase (B-Zyme)	Green Coffee Beans	Sigma, Ireland	B antigen
α-L-fucosidase	Bovine Kidney	Sigma, Ireland	H antigen

Table 2-3 List of exoglycosidases used to alter carbohydrate expression levels on glycoproteins.

2.7 Analysis of VWF glycan composition

2.7.1 Lectin plate binding assays

Following each glycosidase digestion, quantitative changes in VWF carbohydrate expression were assessed using modified sandwich enzyme-linked immunosorbent assays (ELISA). Untreated wild-type VWF and glycan modified VWF were diluted in PBS, coated onto 96-well MaxiSorb plates (Nunc, Loughborough, UK) and incubated at 37°C for 2 hours. Biotinylated conjugated lectins (Table 2-4; all from Vector Laboratories, UK) diluted to a final concentration of 1µg/ml in PBS-T were then added to the plates and incubated for 1 hour. The plates were washed with PBS containing 0.05% (v/v) Tween, (PBS-T), and then incubated with streptavidin-HRP (R and D systems, UK), diluted 1:200 in PBS-T for 1 hour. After a further three washes, the plates were incubated with the HRP specific substrate Ortho-phenylenediamine-dihydrochloride (Sigmafast OPD, Sigma, Ireland). The reaction was stopped with 1M H_2SO_4, and the optical density measured at a wavelength of 490nm. Dilutions of purified pooled pd-VWF were used to construct standard curves for calibration, and levels of specific glycan expression pre- and post-glycosidase treatment were expressed as a percentage of wild-type untreated controls.

Lectin	Specificity
Concanavalin A (Con A)	N-linked glycans
Jacalin	O-linked glycans (mono- and di-sialylated T-antigen)
Sambucus nigra	Terminal α2-6 and to a lesser degree α2-3 linked sialic acid
Maackia amurensis II	α2-3 sialic acid
Ricinus communis agglutinin (RCA)	D-galactose
Ulex europaeus	α-fucose

Table 2-4 Lectins used to quantify glycan structures on VWF and their specificity.

2.7.2 Analysis of blood group antigen expression levels on VWF

Terminal blood group ABO(H) antigenic carbohydrate structures on both the N- and O-linked glycans of VWF were quantified using a modified ELISA. 96-well MaxiSorb plates (Nunc, Loughborough, UK) were coated with rabbit polyclonal anti-human VWF antibody (A0082; Dako, Glostrup, Denmark) diluted 1:500 in 50mM carbonate buffer (pH 9.6) overnight at 4°C. The wells were blocked with PBS-T containing 3% BSA and washed with PBS-T thrice. Untreated and glycan modified blood group specific pd-VWF were incubated on the plate for 2 hours at 37°C. Test samples were diluted to a final concentration range of 2-0.252μg/ml. Following another round of washing, antibodies specific for each terminal blood group antigen were added as described in Table 2-5. The plates were again washed with PBS-T and goat anti-mouse conjugated to HRP (IgM) was added at a final concentration of 2μg/ml (1:1000 dilution). Excess secondary antibody was removed by washing (x3), the plates were developed and absorbance was read at 490nM as described in 2.4.1. Quantification of histo-blood group antigens on glycan modified VWF was established by expressing the values obtained as a percentage of wild-type untreated controls.

Antibody	Source	Carbohydrate structure detected	Working dilution
Anti-A	BioClone® Ortho-Clinical Diagnostics Inc. NJ, USA	GalNAc α1-3 - H	1:50
Anti-B	BioClone® Ortho-Clinical Diagnostics Inc. NJ, USA	Gal α1-3 - H	1:10
Anti-H	IBGRL Research BRIC39	Fucose α1-2 - Gal β1-R	1:20

Table 2-5 Antibodies used to detect histo-blood group antigen expression levels on VWF glycan chains.

2.7.3 Quantification of sialic acid by high pressure liquid chromatography (HPLC)

Sialic acid levels on VWF were measured by HPLC analysis in collaboration with Dr. Barry Byrne and Prof. Richard O'Kennedy in the Applied Biochemistry Group and School of Biotechnology, Dublin City University.

For the preparation of samples for HPLC-based quantitation of sialic acid, pd-VWF was incubated with or without α2-3,6,8,9 neuraminidase, PNGase F, or O-glycosidase as previously described (section 2.6). Following glycosidase treatment, liberated glycan chains (i.e. N-linked glycans following PNGase F; O-linked glycans after O-glycosidase; and sialic acid following neuraminidase digestion) were separated from VWF by centrifugation (2 min at 15000g) through 50kDa molecular weight cut-off columns (Vivaspin 500 columns; GE Healthcare Life Sciences, UK). Liberated glycans were collected in the flow-through (FT), whilst undigested oligosaccharides remained attached to VWF (Bound). Sialic acid moieties were liberated from the terminal ends of FT and Bound oligosaccharide chains respectively by mild acid hydrolysis (0.1N HCl; 80°C for 60 minutes), and then desiccated in a speed-vacuum drier (Particular Sciences, Ireland) at room temperature. After re-constitution in 100μl of HPLC-grade water, this process was repeated to ensure that all traces of HCl were completely removed. Liberated sialic acid (Toronto Chemicals; Ontario, Canada) was derivatised with 100μl of OPD solution (20mg/ml prepared in 0.25M sodium bisulfate) at 80°C for 40 minutes. OPD, sodium bisulphate, fetuin, asialofetuin and all mobile phase constituents were of analytical grade and purchased from Sigma Aldrich (Dorset, U.K.).

All HPLC-based analysis was performed using a Shimadzu Prominence HPLC system (Mason Technology, Ireland) and a TSK-C18 stationary phase (Supelco), whose temperature was maintained at 10°C at all times by a column oven. OPD derivatives of sialic acid were eluted with two mobile phases; the primary mobile phase (A) consisted of butylamine (0.15%, v/v), ortho-phosphoric acid (0.5%, v/v) and tetrahydrofuran (1.0%, v/v). The secondary mobile phase (B) consisted of a mixture of mobile phase A and acetonitrile (50:50). All assays were performed at a flow rate of 1.0ml/minute using isocratic conditions (A:B = 87:13). Eluants (10μl) were monitored for fluorescence at wavelengths of 340nm and 420nm for excitation and emission, respectively. All assays were performed in triplicate and analytical samples were interspersed with water samples.

2.8 Expression and purification of ADAMTS13

2.8.1 Expression of ADAMTS13

Recombinant human ADAMTS13 (cDNA kind gift of Dr R. Montgomery, Medical College of Wisconsin, USA) was stably expressed in HEK293 cells. ADAMTS13 producing HEK293 cells in MEMα medium (Gibco, Invitrogen, UK) were seeded in a T25 flask (Corning®, Sigma, Ireland) at ~$1x10^5$ cells/cm^2. The cells were allowed to grow for 48 hours and were subsequently scaled up to confluence (~$1.75x10^7$ cells per cm^2) in 10 individual T175 flasks (Corning®, Sigma, Ireland). A sample of medium was taken and ADAMTS13 expression and secretion into the culture medium was assessed by western blotting (Figure 2-4). Subsequently, the cells were trypsinised and transferred into HYPERflask™

cell culture vessels (Corning®, Sigma, Ireland). After 24 hours the culture medium was aspirated, the cells were washed with sterile PBS (Sigma, Ireland) and then re-incubated in 560 ml of serum free medium (Opti-MEM® reduced serum media, Invitrogen, UK). After 48 hours, the medium was collected for concentration and purification of ADAMTS13 by FPLC.

2.8.2 Purification of rADAMTS13

Serum free media containing rADAMTS13 was concentrated from ~560ml to ~50ml using a Millipore Labscale™ TFF System with a Pellicon® XL 10K Filter (Millipore, MA, USA). Following concentration, high levels of salt were removed from the media using double-HiTrap™ desalting columns (GE Healthcare, Buckinghamshire, UK). Desalting is an essential step at this point in the purification process, to ensure the protein samples are present in the correct buffer conditions for subsequent FPLC steps. Following deslating, anion-exchange chromatography using a HiTrap™ IEX column (pre-packed with Q Sepharose™ High Performance; GE Healthcare, Buckinghamshire, UK) was carried out to partially purify ADAMTS13. Ion exchange chromatography is a purification step based on charge-charge interactions. Anion exchange chromatography utilises a positively charged binding resin to which ADAMTS13 binds as it possesses a net negative charge at neutral pH. ADAMTS13 was eluted from the column using 50mM Tris-HCl pH 7.8 containing 1M NaCl (Figure 2-5). The salt in the elution buffer releases proteins from their bound state as it competes for binding to the immobilised matrix. Finally, NaCl was removed from the eluted fractions by another desalting step (Figure 2-6).

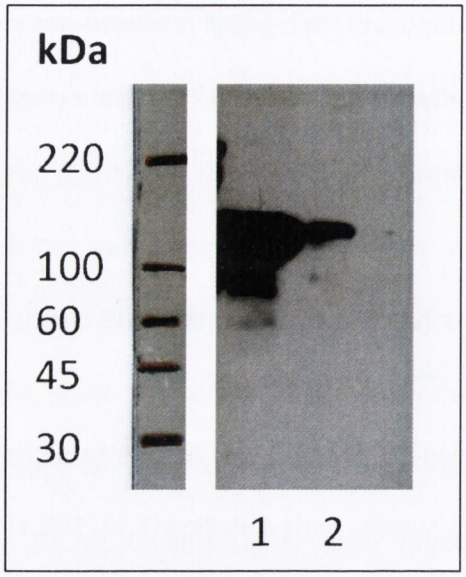


Figure 2-5 Expression of recombinant human ADAMTS13 by HEK293 cells.

The presence of ADAMTS13 in cell culture medium was determined by western blot. Following transfer of proteins and blocking, ADAMTS13 was detected using a primary polyclonal rabbit anti-human ADAMTS13 antibody (ab71550; Abcam, Cambridge, UK) and a secondary goat anti-rabbit IgG peroxidase conjugate antibody (ab97190; Abcam, Cambridge, UK). A large band representing ADAMTS13 present in HEK293 culture medium is visible at ~190kDa in lane 1. A positive control (previously purified ADAMTS13) was used and is also present at ~190kDa (lane 2).

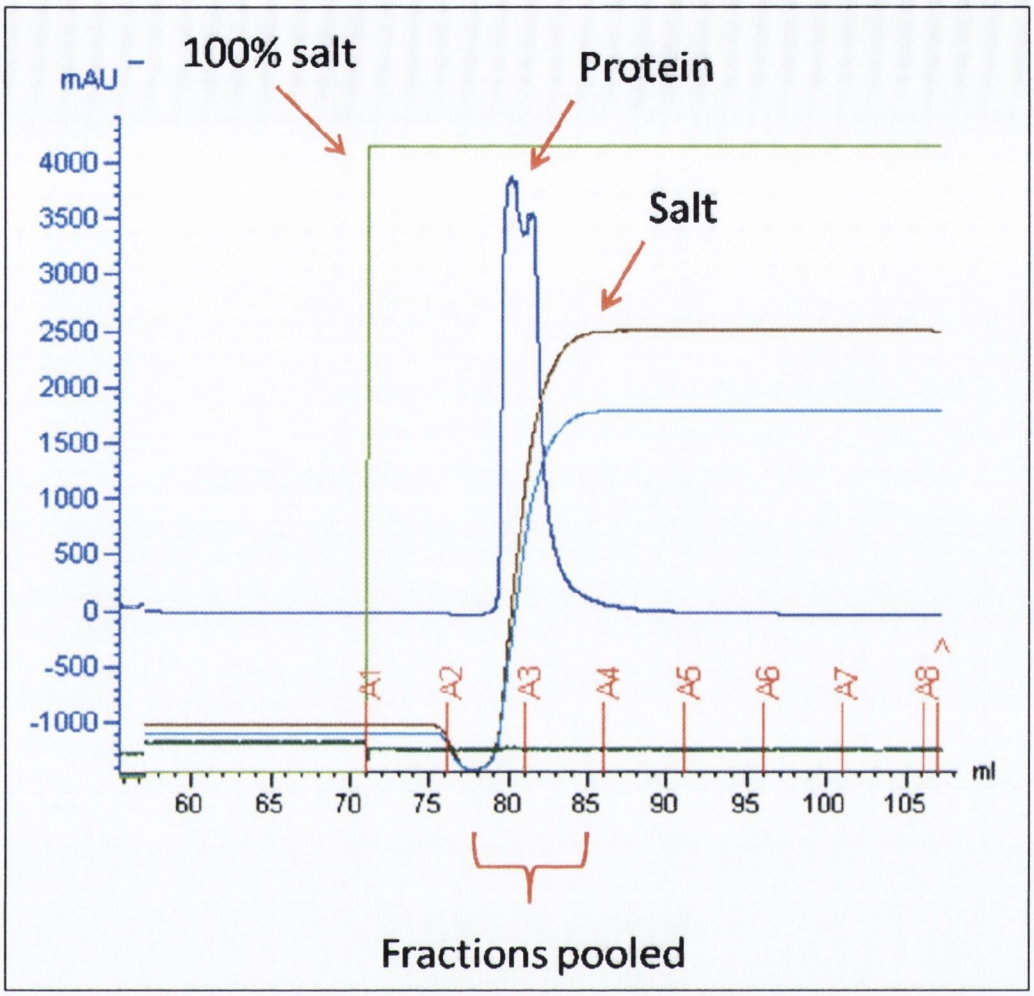


Figure 2-6 Purification of ADAMTS13:- anion exchange chromatography.

ADAMTS13 possesses a net negative charge at neutral pH and therefore binds to the anion exchange column. 100% salt conditions (green line) resulted in elution of proteins from the column. The salt conditions passing over the column are indicated by the brown line. Fractions A2 and A3 were pooled for desalting and further analysis.

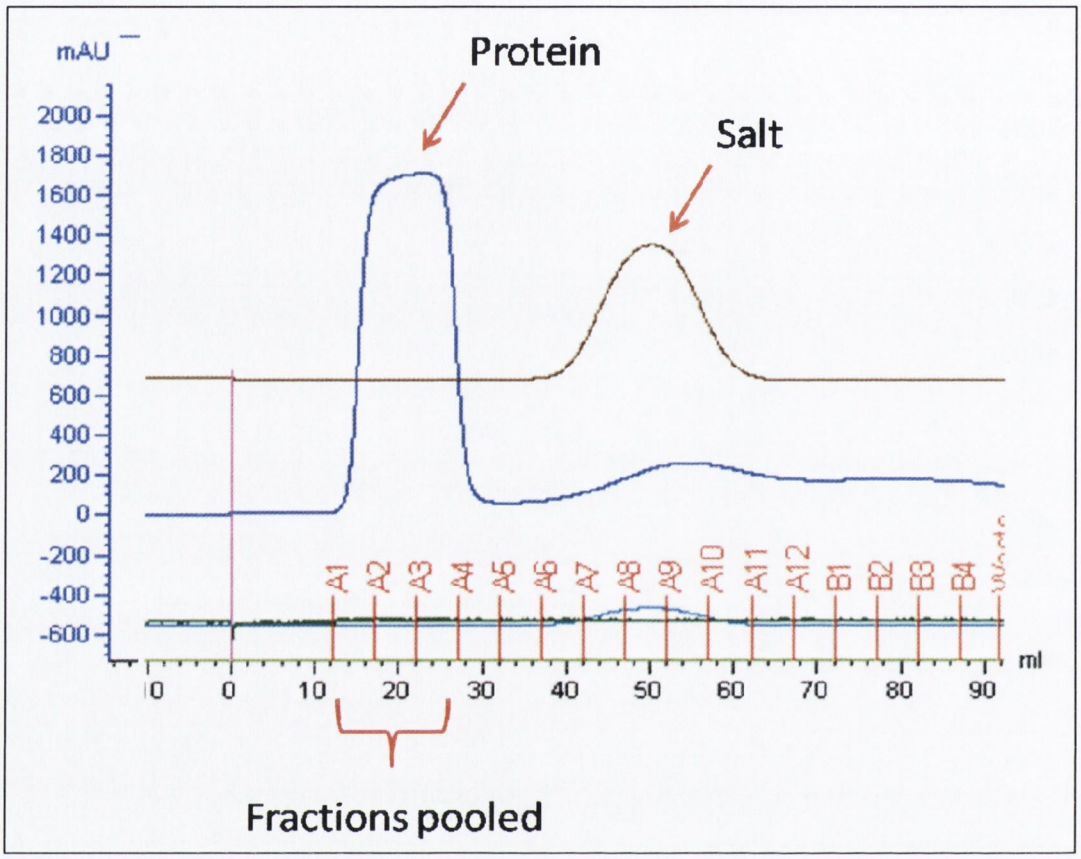


Figure 2-7 Purification of ADAMTS13:- desalting step.

Salt was removed from fractions containing ADAMTS13 using a HiTrap™ desalting column (GE Healthcare, Buckinghamshire, UK). Proteins are represented by the first peak, the second peak indicates salt. Fractions A1-A4 were pooled and analysed for ADAMTS13 purity and activity. The activity of ADAMTS13 in test samples is determined (as a value in nM) in comparison to normal pooled plasma; inferred from the plasma level of 2-5nM.

2.8.3 Analysis of ADAMTS13 activity - FRETS-VWF73 assay

FRETS-VWF73 (Peptides International, KY, USA) is an ADAMTS13 specific fluorescence quenching substrate. FRET (fluorescence resonance energy transfer) is a mechanism describing the transfer of energy between a donor chromophore to an acceptor chromophore, typically when the donor chromophore is excited. FRETS-VWF73 is a 73 amino acid peptide comprising the ADAMTS13 cleavage site and surrounding amino acids in the VWF A2 domain. The excitation and emission spectra of the chromophores are within the 340-450nm range. Fluorescence is measured every 5 min for 1 hour to determine ADAMTS13 enzymatic activity levels.

The FRETS-VWF73 assay involves construction of a standard curve by using serial dilutions of normal pooled plasma, according to the manufacturer's guidelines. The reaction rate (slope) for each sample is calculated by linear regression. Unknown ADAMTS13 activity in plasma/test samples is quantified by calculation of the slope and comparing to the standard curve.

2.9 ADAMTS13 cleavage assay

To assess VWF susceptibility to ADAMTS13 cleavage, 3nM rADAMTS13 was pre-incubated with 10mM $BaCl_2$ for 10 minutes at 37°C. The activated ADAMTS13 was then incubated at 37°C with 6-10μg/ml VWF in a reaction mix containing urea (1-2M), 10mM $BaCl_2$, 5mM NaCl, and 15mM Tris-HCl (pH 7.8). At specific time points (0, 30, 60, 90, and 120 minutes), subsamples were removed, and 1mM EDTA was added to stop the

cleavage reaction. VWF proteolysis was analyzed and assessed by the reduction in residual VWF:CB over time.

2.10 Non-specific proteolysis assays

To determine VWF susceptibility to non-specific cleavage by serine or cysteine proteases, chymotrypsin (30-60 U/mg VWF), cathepsin B (4 U/mg VWF) and carboxypeptidase Y (19U/mg VWF) digestions were carried out at 37°C for 90 min in physiological buffer (50mM Tris-HCl, pH 7.8). All proteases were purchased from Sigma, Ireland. The extent of proteolysis was determined by residual VWF:CB after 90 min as detailed in 2.4.2.

2.11 VWF-ADAMTS13 plate binding assay

To assess binding of VWF and ADAMTS13, a plate binding assay was used. This assay was optimised and based on the protocol described by Zanardelli *et al* in 2009.

VWF diluted to 12nM in 50mM sodium carbonate buffer (pH9.6) was immobilized onto the wells of a 96-well microtiter plate (MaxiSorb, Nunc, UK). The plate was washed with PBS-T and the wells were blocked with 150 µl of PBS-T with 3% BSA for 1 hour at room temperature. After repeated washing, increasing concentrations of wild-type ADAMTS13 (0-50 nM) in 50mM Tris-HCL pH 7.8, in the presence of 1 mM EDTA, were applied for 2 hours at 37°C. Bound ADAMTS13 was detected by first adding polyclonal rabbit anti-human ADAMTS13 antibody (ab71550; Abcam, Cambridge, UK) diluted 1:1000 in PBS-T for 1 hour. A secondary goat anti-rabbit IgG peroxidase conjugate antibody (ab97190; Abcam, Cambridge, UK) was applied before adding Sigma ColorFast OPD substrate to the

wells. Binding curves were fitted to the one-site specific binding model using GraphPad Prism 5 software, to determine K_d(app) values.

2.12 Analysis of factor VIII (FVIII) activity

FVIII activity levels in test samples were established by an activated partial thromboplastin time (APTT) based one-stage clotting assay as described previously (Lattes, *et al* 2010). The reagents used were from the HemosIL™ APTT Lyophilized silica kit (IL Company, MA, USA) and the assay was performed using an Amelung KC4 Micro Clinical Coagulation Analyzer (Amelung, Trinity Biotech, Ireland).

Briefly, 25µl of test samples diluted 1/10 in working buffer solution were incubated with 25µl of FVIII deficient plasma and 50µl of APTT reagent (containing phospholipids and silica as an activator) at 37°C for 5 min. Coagulation was triggered by adding 50µL of 25mM $CaCl_2$ solution and clot detection was performed by recording changes in optical density read at 671 nm. A calibration curve was generated by using serial dilutions of the reference plasma into buffer solution (1:5 to 1:400). Test samples were usually diluted 1:20 and 1:40. Linear regression analysis using GraphPad Prism 5 software was used to convert clotting times in seconds into FVIII activity.

2.13 Data analysis and statistics

Data were analyzed with the use of the GraphPad Prism program (GraphPad Prism Version 5.0 for Windows; GraphPad Software Inc.). Experiments were performed in triplicate. All data are expressed as mean ± SEM. To assess statistical differences, the

data were analyzed with Student's unpaired 2-tailed *t* test. Statistical significance was assigned at $p < 0.05$.

CHAPTER 3:

OPTIMISATION OF EXPERIMENTAL TECHNIQUES

3.1 Lectin plate binding assays

Lectins are highly specific sugar-binding proteins that are ubiquitously expressed in microorganisms, plants and animals. Modification of VWF glycosylation can be measured by a reduction/increase in lectin binding affinity. I used a series of ELISA based lectin plate binding assays to characterise VWF glycosylation pre- and post-oligosaccharide removal, as outlined in Chapter 2, section 2.7.1.

To optimise the lectin plate binding assays, several variables and parameters were altered, to obtain the best assay conditions and to ensure high levels of reproducibility. Initially, a polyclonal anti-human VWF antibody was used to coat the plates, as for the VWF:Ag ELISA detailed in Chapter 2, section 2.4.1. However, this led to non-specific lectin binding and a high level of background noise. Optimum coating conditions were found to be VWF alone in PBS for 2 hours at 37°C. Direct binding of VWF to the plate surface also facilitates unwinding of the VWF globular structure, and may allow lectins further access to partially hidden or buried glycan structures. Furthermore, several blocking conditions were also tested; the most suitable was found to be 3% bovine serum albumin (BSA) in PBST. BSA is routinely used blocking protein and is not glycosylated. Details of coating and blocking conditions used are described in Table 3-1.

Test conditions used	Level of background noise
Coating	
Anti-VWF pAb	High
Lectin	High
VWF	Low
Blocking	
3% milk	High
1% BSA	Low-medium
3% BSA	Low

Table 3-1 Lectin plate binding assay:- optimal coating and blocking conditions.

Several variations of coating and blocking steps were tested to optimise lectin plate binding assays. Optimal conditions were found to be direct coating of VWF onto the plate, and blocking with 3% BSA. Low background noise was defined as less than 10% non-specific binding of lectins in blank wells.

For each different lectin, a range of VWF concentrations were used for the construction of standard curves. A linear relationship for each individual lectin was achieved by titrating VWF concentration against optical density at 492nm. For example, to quantify N-linked glycan structures using the lectin Concanavalin A, 0.0125-0.8μg/ml of VWF were coated onto the plate and this resulted in OD492nm levels within the range of 0.015-1. Similarly, to assess levels of sialic acid on VWF using *sambucus nigra* lectin, the following VWF concentrations were used: 0.05-0.335μg/ml; which gave OD492nm readings of 0.1-1.5.

Construction of linear standard curves depended on the level of expression of a given carbohydrate structure on VWF, and also on the sensitivity of the lectin used. This is evident for assessment of blood group antigen levels on VWF, as higher concentrations of VWF are required for accurate detection of these particular sugar structures due to their low frequency on VWF glycan chains. Table 3-1 indicates the range of VWF concentrations required per lectin, to obtain optimum linear standard curves for quantification of glycans on test samples.

Lectin	Glycan detected	VWF concentration range	OD range obtained (492nM)
Concanavalin A	N-linked	0.0125 – 0.8 µg/ml	0.015 - 1
Sambucus nigra	Sialic acid	0.05 - 0.335µg/ml	0.1 - 1.5
Maackia amurensis II	Sialic acid	0.04 - 12.5µg/ml	0.05 - 1.2
Ricinus communis agglutinin	Galactose	0.03 - 0.675µg/ml	0.14 - 1.45
Anti-A	A antigen	0.03 - 1µg/ml	0.12 - 1.6
Ulex europaeus	H antigen	0.05 - 2µg/ml	0.2 – 1.35

Table 3-2 List of optimum concentrations of VWF used to construct lectin based standard curves for the assessment of carbohydrate expression levels pre- and post-glycosidase digestion.

3.2 Glycosidase digestions

Exoglycosidases are specific enzymes that can be used to modify the oligosaccharide chains of glycoproteins. Several glycosidases were used in this study to change VWF carbohydrate expression levels (Figure 3-1 and Figure 3-2). Other methods of altering glycoprotein carbohydrate profiles are available (chemical modifications, hydrolysis), however glycosidase digestion maintains the functional and structural integrity of the protein, and allows further functional characterisation to be carried out. Due to the quantitative differences in individual glycan structures on VWF, and also the availability/accessibility of certain oligosaccharide structures in comparison to others, optimisation of individual glycosidase digestions was essential in order to ensure the correct parameters were being used in each assay.

Optimal concentrations of enzyme required to remove ~50% or >90% of the detectable sugar structures were determined after titration of both VWF levels and glycosidase activity. As VWF possesses a large amount of carbohydrate structures, and is composed of many polymers, the recommended units of glycosidase to use in each digestion were initially increased ~2-fold. Once a large decrease in lectin binding was observed, enzymatic units used per digestion were reduced to the minimum amount of activity required for a reproducible quantitative change in VWF carbohydrate expression.

Incubation times were also varied to ascertain the best conditions for glycan removal. Incubation of VWF with glycosidases for 1, 2, 4, 6 or 12 hours did not result in sufficient removal of sugar structures. However, overnight digestions for a minimum of 16 hours resulted in sufficient carbohydrate digestion, and incubation at 37°C for this length of time had no effect on VWF structure or function (as determined by VWF:Ag and

VWF:CB; data not shown). The optimum concentrations of glycosidase used to remove the indicated amount of glycan are detailed in Table 3-3. All glycosidase reactions were carried out overnight (16-24 hours) at 37°C unless otherwise stated.

To determine the percentage difference in carbohydrate levels, control samples were incubated (in the absence of glycosidase) in reaction buffer, at the same temperature, and for the same length of time as treated samples. Loss of glycan expression was determined by the reduction in residual lectin binding for treated samples versus undigested wild type controls.

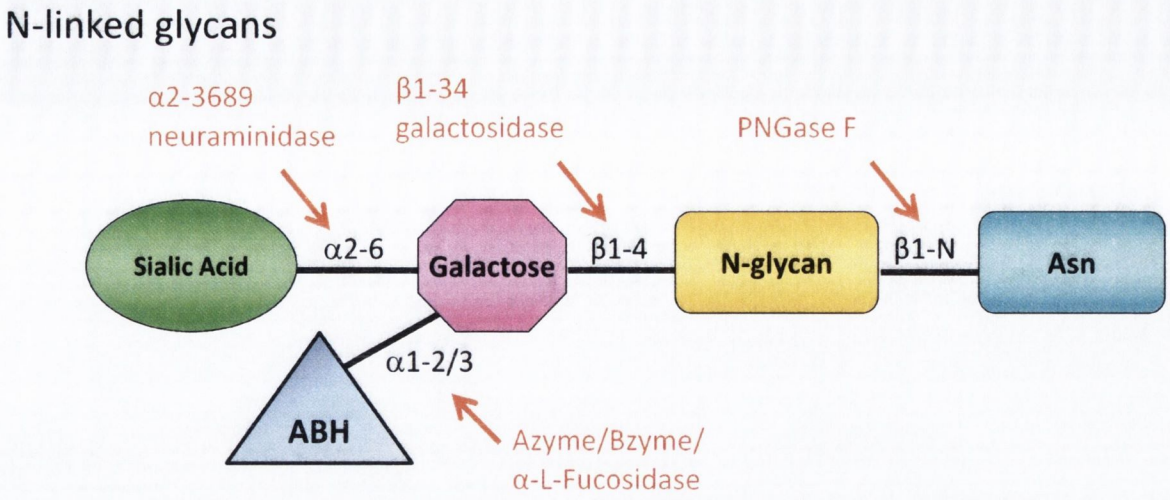


Figure 3-1 Overview of glycosidases used to modify VWF N-linked glycan chains.

Various specific exoglycosidases were used to digest and remove VWF oligosaccharide structures. α2-3689 neuraminidase removes α2-6 linked terminal sialic acid. Azyme, Bzyme and α-L-fucosidase can be used to digest terminal blood group antigenic moieties from blood group specific VWF. β1-34 galactosidase digests sub-terminal galactose residues and PNGase F can be used to remove complete N-glycan structures.

O-linked glycans

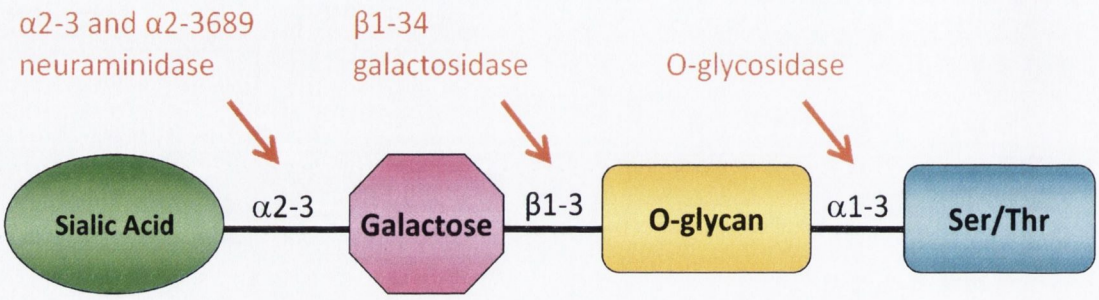


Figure 3-2 Overview of glycosidases used to modify VWF O-linked glycan chains.

Various specific exoglycosidases were used to digest and remove VWF oligosaccharide structures. α2-3 neuraminidase specifically removes α2-3 linked terminal sialic acid from O-glycans, whereas α2-3689 treatment results in complete desialylation of both N-and O- carbohydrate chains. β1-34 galactosidase digests sub-terminal galactose residues and O-glycosidase can be used to remove complete O-glycan structures.

Glycosidase	Specifically removes	>90% reduction (all values per mg VWF)	~50% reduction (all values per mg VWF)	Detection method
PNGase F	N-linked glycans	500U	65U	Concanavalin A Anti-VWF
O-glycosidase	O-linked glycans	278U	-	Jacalin *Maackia amurensis* II
α2-3,6,8,9 neuraminidase	α2-3 and α2-6 linked sialic acid	1.25U	8mU	*Sambucus nigra*
α2-3 neuraminidase	α2-3 linked sialic acid	1.56U	95mU	*Maackia amurensis* II
β1-3,4,6 galactosidase	Galactose	3.75U	-	*Ricinus communis* agglutinin
α-N-Acetyl galactosaminidase (A-Zyme)	A antigen	2U	0.2U	Anti-A
α-D-galactosidase (B-Zyme)	B antigen	200U	50U	Anti-B
α-L-fucosidase	H antigen	19.38U	5U	*Ulex europaeus* Anti-H

Table 3-3 List of optimum concentrations of exoglycosidases used to alter carbohydrate expression levels on VWF. The source organism and commercial source of each enzyme are listed in Chapter 2, section 2.6.

3.3 Platelet lysis

Platelet-derived VWF is present in platelet α-granlues. Upon stimulation and activation, platelets release their granular cargo into the bloodstream, including significant levels of ULVWF. To obtain platelet-derived VWF, platelets were lysed under several conditions, and the lysate was tested for VWF:Ag and VWF:CB as described in Chapter 2, section 2.4. Platelets lysis results in the splitting open of the platelet surface membrane, and also lysis and exposure of the granule contents.

Platelets were isolated from plasma by centrifugation and washed to ensure removal of plasma proteins, including VWF. After washing, platelets are resuspended in ~30-50ml of appropriate buffer for ensuing lysis. Details of this methodology are described in Chapter 2, sections 2.2.3-2.2.4.

Several methods of lysis were used, as outlined in table 3-3. Triton X-100 is a non-ionic detergent routinely used for cell lysis. It permeabilizes the plasma membrane by disrupting the hydrophobic membrane surface and allowing the passage of soluble proteins into the water-based supernatant. Similarly, freeze-thaw cycles disturb the surface of cells through the formation of ice crystals. Cryoprecipitation is a technique based on differential melting temperatures, which is used to isolate certain proteins from plasma, including VWF. Interestingly, the constituents of platelet lysate did not behave in the same way as plasma proteins, thus, low levels of VWF were obtained from the cryoprecipitate, showing this protocol is not suitable for platelet-VWF isolation.

The optimum lysis conditions were found to be snap freeze thaw of platelets, from extremely cold temperatures in liquid nitrogen (~-200°C) to physiological temperature in

a water bath (37°C) followed by vortex mixing for 3 minutes at a high speed (~2500rpm). This process was repeated a further 3-4 times to ensure sufficient lysis of platelets and their α-granules. The presence of protease inhibitors and in particular, inhibitors of calpains, was shown to be vital to preserve VWF functional integrity, as loss of high molecular weight multimers due to proteolytic degradation was observed in the absence of inhibitors. Snap freeze thaw lysis plus inhibitors yields the maximum level of platelet-VWF maintaining high molecular weight multimers, as determined by VWF:CB. Therefore, this lysis technique was chosen for further experiments, and for obtaining platelet-VWF for subsequent purification steps.

Lysis Conditions	Starting volume of platelets	VWF:Ag	VWF:CB	VWF:Ag/ VWF:CB
0.5% Triton-X 100 5mM EDTA	1 unit (~320ml)	35µg/ml	32µg/ml	1
1% Triton-X 100 5mM EDTA	1 unit (~320ml)	42µg/ml	38µg/ml	1
Cryoprecipitation of platelet lysate	50ml	5µg/ml	3µg/ml	0.6
Slow Freeze-Thaw (-80°C → 37°C)	1 unit (~320ml)	50µg/ml	45µg/ml	1
Snap Freeze Thaw (-200°C → 37°C)	2 units (~640ml)	80µg/ml	42µg/ml	0.5
Snap Freeze Thaw in the presence of protease inhibitors	2 units (~640ml)	64µg/ml	115µg/ml	1.8
Activation of platelets with TRAP	10ml	15µg/ml	1µg/ml	<0.1

Table 3-4 List of various platelet lysis protocols tested.

Several lysis techniques were used to obtain platelet-VWF that retained functional activity. Snap freeze thaw of platelets in the presence of protease inhibitors were found to be the best conditions for optimum attainment of VWF. The table above indicates the levels of VWF obtained from lysis of 2 units of pooled platelets.

3.4 ADAMTS13 cleavage assay

The static ADAMTS13 cleavage assay can be used to assess the rate of VWF proteolysis in vitro, and can also be used to differentiate between VWF samples that present differential susceptibilities to cleavage. This assay utilises urea as a denaturant, which facilitates a conformational change within the A1-A3 domains of VWF and thereby allows ADAMTS13 access to the A2 domain cleavage site. The rate of VWF cleavage is determined by the reduction in residual VWF:CB over time, as decrease in VWF mulitmer size due to the extent of ADAMTS13 proteolysis is directly proportional to the decline in VWF collagen binding activity.

To optimise the cleavage assay, several concentrations of ADAMTS13 were incubated with VWF (Figure 3-3), which was present at a final concentration of 6μg/ml. 3nM ADAMTS13 is the optimal concentration for quantifiable proteolysis of VWF, as a steady reduction in residual VWF:CB is observed. 3nM is also within the physiological circulating range of ADAMTS13 (~2-5nM).

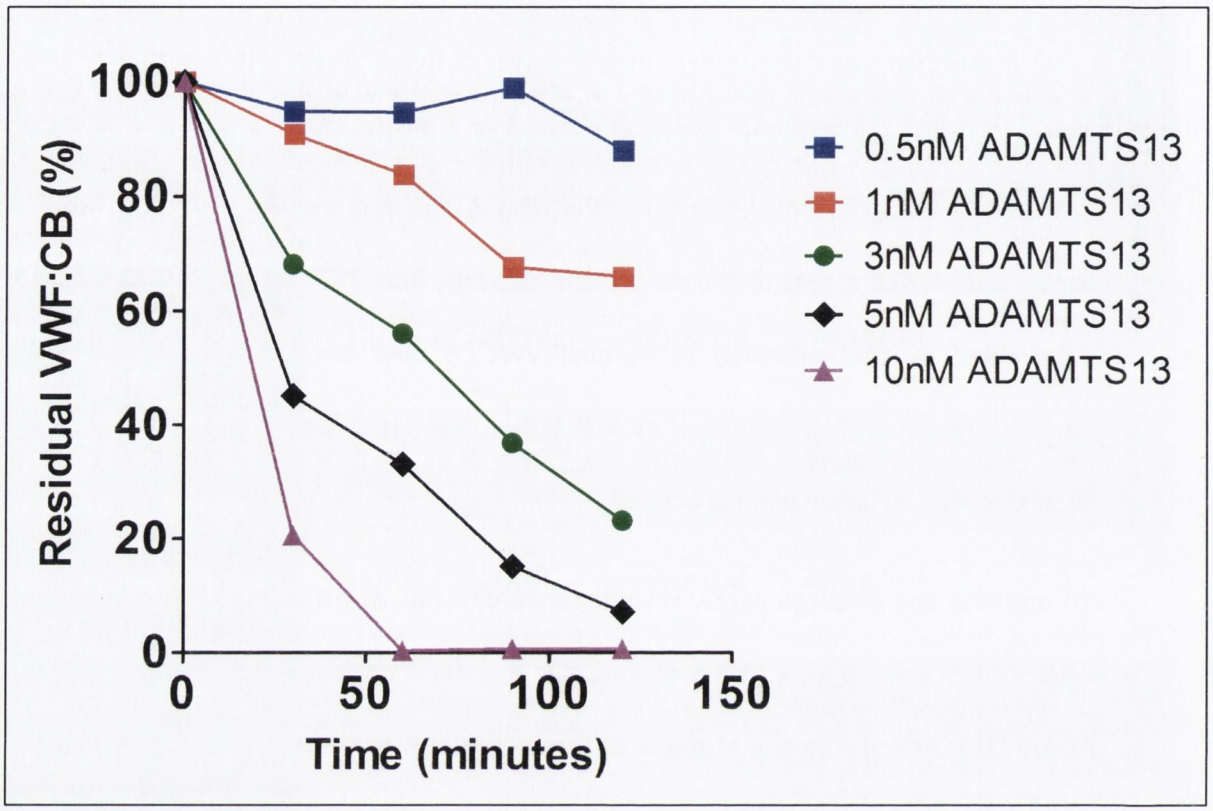


Figure 3-3 Optimisation of ADAMTS13 proteolysis assay.

Several concentrations of ADAMTS13 were tested to obtain optimal proteolysis conditions. 3nM ADAMTS13 was chosen as this amount of ADAMTS13 results in a steady fall off in residual VWF:CB and can be used to differentiate between VWF samples that display various susceptibilities to cleavage.

CHAPTER 4:

CHARACTERISATION OF SIALIC ACID EXPRESSION ON VWF AND THE EFFECT OF SIALIC ACID IN REGULATING VWF SUSCEPTIBILITY TO ADAMTS13

4.1 Introduction

In normal plasma, VWF multimer distribution is regulated by ADAMTS13, a metalloprotease which specifically cleaves VWF at the Tyr1605-Met1606 peptide bond within the A2 domain (Levy, *et al* 2005). As described earlier (Chapter 1, section 1.10), the post-translational modification of VWF within EC and megakaryocytes includes significant glycosylation, with each VWF monomer containing 12 N-linked and 10 O-linked glycosylation sites (Titani, *et al* 1986).

Several studies have demonstrated an important role for VWF carbohydrate structures in determining susceptibility to ADAMTS13 proteolysis (Bowen 2003, McKinnon, *et al* 2008, O'Donnell, *et al* 2005). In particular, ABO(H) blood group carbohydrate antigens expressed on the oligosaccharide chains of VWF have been shown to significantly influence rate of cleavage by ADAMTS13 (Bowen 2003, O'Donnell, *et al* 2005). These observations are interesting given that ABO(H) determinants differ only with respect to a single terminal monosaccharide moiety (O'Donnell and Laffan 2001). Furthermore, ABO(H) blood group determinants are only expressed on a minority (13%) of the

complex N-linked glycans of plasma VWF, and on an even smaller proportion of VWF O-glycans (~1%) (Canis, *et al* 2009, Matsui, *et al* 1992). In contrast, the majority of the N- and O-linked glycan chains of VWF are capped by negatively-charged sialic acid residues (Matsui, *et al* 1992, Samor, *et al* 1982, Samor, *et al* 1989).

Sialic acid (N-acetylneuraminic acid; Neu5Ac) is a negatively charged carbohydrate moietiy, that is typically found as a terminal monosaccharide attached to glycoconjugates (Varki 1999). Sialic acid is part of a family of derivatives of neuraminic acid; a monosaccharide unit with a 9-carbon backbone. An N-linked substitution of an acylamino group in neuraminic acid at the C-5 position gives rise to the most common derivative of the group; namely sialic acid or Neu5Ac. Sialic acids are important for cell-cell, cell-protein and protein-protein interactions, as well as being implicated in immunological processes, host-pathogen interactions and cancer progression (Angata and Varki 2002, Schauer 2009, Traving and Schauer 1998, Varki and Varki 2007).

It is well established that expression of sialic acid on many glycoproteins plays a critical role in protection from both intracellular and extracellular proteolytic degradation (Byrne, *et al* 2007). Moreover, previous studies have demonstrated that sialylation plays an important role in protecting VWF against a variety of plasma proteases (Federici, *et al* 1984). Consequently, I sought to determine whether terminal N-linked and/or O-linked sialic acid residues may also play a role in determining VWF susceptibility to proteolysis by ADAMTS13.

VWF was digested with various glycosidases to alter terminal sialic acid expression levels. Residual sialic acid on VWF was quantified by lectin plate binding assays, as well as by HPLC analysis. The effect of modification of endogenous VWF sialic acid levels on susceptibility to regulatory proteolysis by ADAMTS13 was then evaluated.

4.2 Characterisation of neuraminidase-treated von Willebrand factor

Plasma-derived VWF was purified from either human blood group-specific plasma, or from the commercial VWF/FVIII concentrate Haemate P (see Chapter 2, section 2.1), and sialic acid content was analysed using a variety of different glycan detection techniques. The glycan structures of purified VWF were modified using different exoglycosidases with varying specificity for oligosaccharide structures and linkages. Quantitative sialic acid expression pre- and post glycosidase treatment was studied using both lectin plate-binding ELISAs and high pressure liquid chromatography (HPLC) analysis.

4.2.1 Analysis of sialic acid on VWF after treatment with α2-3,6,8,9 neuraminidase

To alter endogenous sialic acid levels on VWF, α2-3,6,8,9 neuraminidase from *Arthobacter ureaifaciens* was used. Neuraminidases catalyse the hydrolysis of non-reducing terminal sialic acids from complex oligosaccharides and glycoproteins (Saito, *et al* 1979, Uchida, *et al* 1979). This particular neuraminidase has a broad specificity as it removes sialic acids attached to the underlying carbohydrate chain in α2-3, α2-6, α2-8 or α2-9 linkages. Digestion with α2-3,6,8,9 neuraminidase resulted in a dose-dependent reduction in VWF sialic acid expression detected by *Sambucus nigra* lectin (which has

specific affinity for α2-6 linked, and to a lesser extent α2-3 linked sialic acid; Figure 4-1). On the basis of initial lectin plate-binding ELISA, incubation with 8mU or 1250mU of α2-3,6,8,9 neuraminidase resulted in ~50% (50% Neu-VWF) and 95% (Neu-VWF) reductions in total *Sambucus nigra* detectable sialic acid. Digestion with PNGase F (PNG-VWF) resulted in loss of 85 ± 2% of total *Sambucus* binding, suggesting that the majority of sialic acid on VWF is expressed on its N-linked carbohydrate chains.

4.2.2 Analysis of O-linked sialic acid on VWF after treatment with α2-3 neuraminidase

Sialic acid is predominantly attached to O-linked glycans in an α2-3 linkage. To reduce α2-3 sialic acid levels, VWF was treated with α2-3 neuraminidase from *Streptococcus pneumoniae.* Using a lectin plate-binding ELISA, incubation with 95mU or 1560mU of α2-3 neuraminidase resulted in a dose-dependent removal of detectable α2-3 linked sialic acid (Figure 4-2). Interestingly, treatment with PNGase F to remove complex N-linked glycan structures significantly enhanced *Maackia amurensis* lectin binding (148 ± 10%; $p< 0.05$). This unexpected increase in *Maackia* binding is presumably due to improved lectin accessibility to O-linked sialic acid due to a deshielding affect following removal of larger and more complex N-glycans, and highlights the inherent limitations of lectin plate-binding ELISAs for studying VWF.

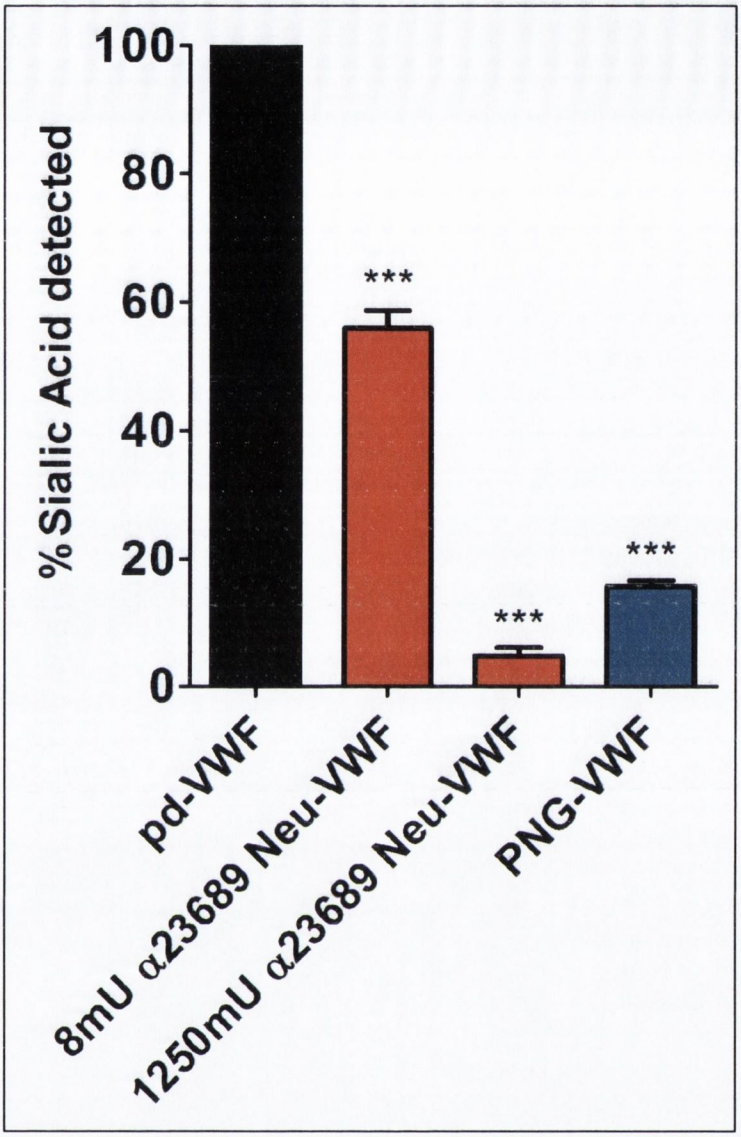


Figure 4-1 Levels of α2-3,6 linked sialic acid on VWF after glycosidase digestion.

Purified plasma-derived human VWF (pd-VWF) was incubated overnight with either α2-3,6,8,9 neuraminidase (Neu-VWF) or PNGase F (PNG-VWF), and residual expression of α2-3,6 linked sialic acid on VWF was analysed by modified *Sambucus nigra* lectin binding ELISA as described in Chapter 2, section 2.7.1. All experiments were performed in triplicate, and results described represent the mean ± SEM (*** $p < 0.001$).

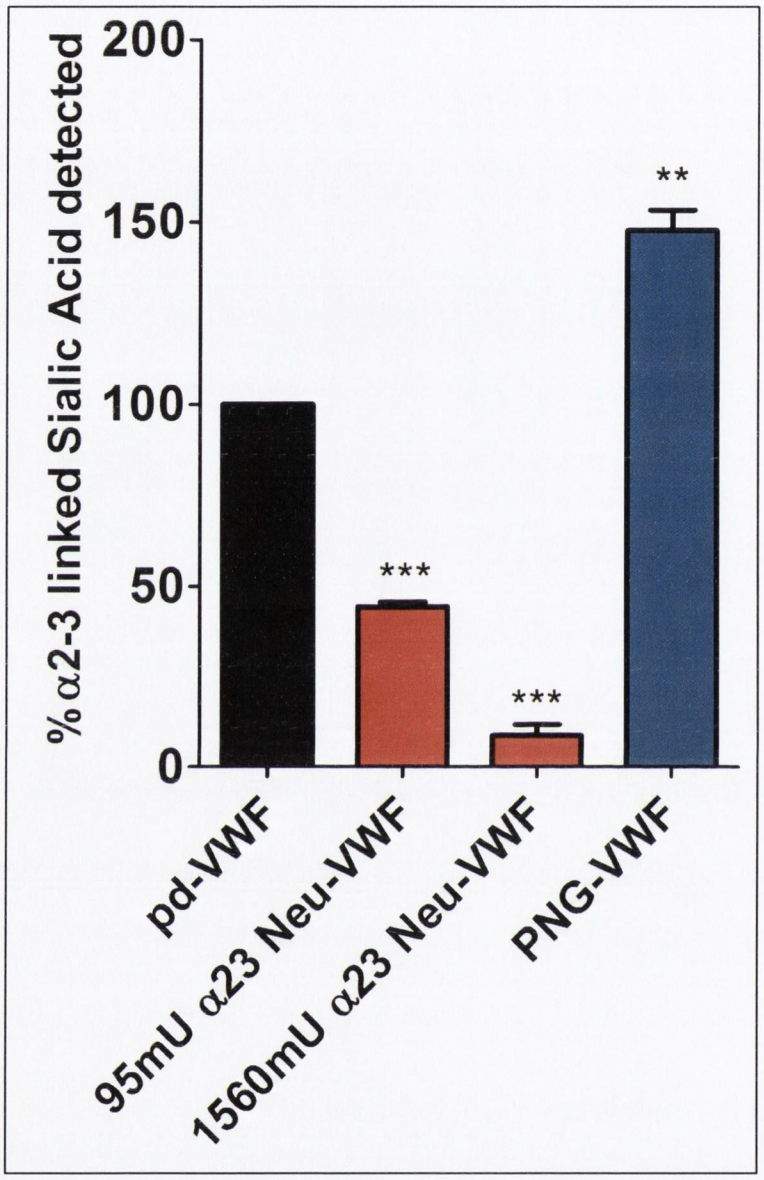


Figure 4-2 Levels of α2-3 linked sialic acid on VWF after glycosidase digestion.

VWF was treated with either α2-3 neuraminidase or PNGase F, and residual expression of α2-3 linked sialic acid on VWF was analysed by modified *Maackia amurensis II* lectin binding ELISA as described in section 2.7.1.

All experiments were performed in triplicate, and results described represent the mean ± SEM (** $p < 0.01$; *** $p < 0.001$).

4.3 Quantification of VWF sialic acid expression using HPLC-based analysis

Lectin plate binding ELISAs have been shown to be limited in their ability to analyse sialic acid content, as VWF is a large multimeric protein that contains inaccessible carbohydrate structures in its native state. Consequently, to further characterise sialic acid expression on VWF, definitive high pressure liquid chromatography (HPLC) was also performed. The analyses reported in the following sections (4.3.1-4.3.3) were carried out in collaboration with Dr. Barry Byrne and Dr. Richard O'Kennedy in the Applied Biochemistry Group and School of Biotechnology, Dublin City University.

4.3.1 Calibration and optimisation of HPLC

Reverse-phase HPLC was used to define quantitative sialic acid expression on plasma VWF. Free sialic acid (Neu5Ac) was labelled with OPD to form a quinoxaline derivative that presented a baseline-resolved peak with retention time of 36.3 minutes (Figure 4-3A). Analysis of acid-hydrolysed VWF (referenced with a calibration curve of free sialic acid; R2=0.99) demonstrated total sialic acid expression was 167 nMol/mg VWF (equivalent to 5.2% of total VWF mass), which is in keeping with previous reports (Gralnick, *et al* 1983).

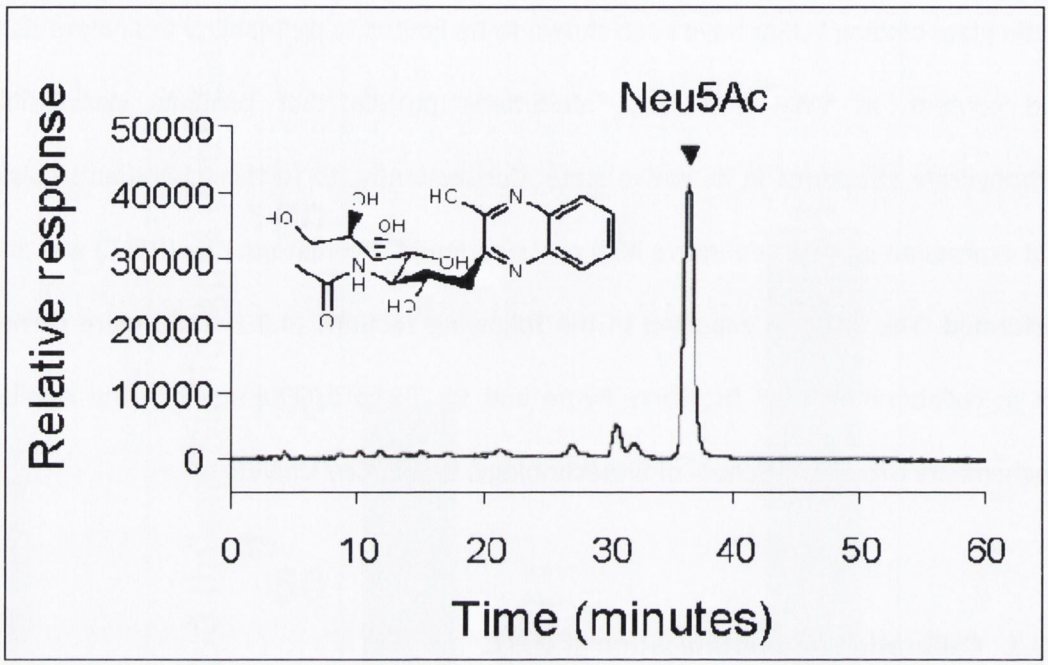


Figure 4-3A HPLC analysis of sialic acid on VWF.

Reverse phase HPLC analysis was used to quantify sialic acid expression on purified pd-VWF. A sample chromatograph of liberated sialic acid from bovine fetuin is represented. Hydrolysed sialic acid (Neu5Ac) was labelled with OPD and a baseline-resolved peak representing Neu5Ac, with a retention time of 36.3 minutes was observed. A structural representation of Neu5Ac derivatised with OPD is shown.

4.3.2 The majority of sialic acid on VWF is N-linked

To determine the relative amount of sialic acid expressed on the N-linked glycans of VWF, pd-VWF was digested with O-glycosidase and liberated oligosaccharide chains were separated from the intact protein as described in Chapter 2, section 2.7.3. Sialic acid moieties from the retained protein (e.g. N-linked) were subsequently liberated by mild acid hydrolysis, derivatised with OPD and quantified (Figure 4-3B). This experiment was also performed in reverse by digesting a separate aliquot of protein at the same concentration with PNGase F and, subsequently, separating intact (O-linked) and FT (N-linked) fractions (Figure 4-3B). N-linked sialic acid expression accounted for 133.4 nMol/mg VWF (80.1% total VWF sialic acid) whilst O-linked sialic acid represented 19.4 nMol/mg (11.6% total VWF sialic acid expression) (Table 4-1 and Figure 4-3B). After combined PNGase F and O-glycosidase digestion, 8% of total sialic acid (removed by acid hydrolysis) remained undigested.

Sample ID	Sample	[SA in µg/ml]	[moles of SA]
1.	Total	1.9	6.15×10^{-11}
2.	Free	0.003	1.2×10^{-12}
3.	O-linked	0.22	7.2×10^{-12}
4.	N-linked (in solution)	1.44	4.65×10^{-11}
5.	N-linked	1.52	4.92×10^{-11}
6.	O-linked (in solution)	0.18	5.7×10^{-12}

Table 4-1 Values obtained for sialic acid (SA) on VWF after glycosidase digestion in µg/ml and moles of SA.

This table corresponds to Figure 4-3B.

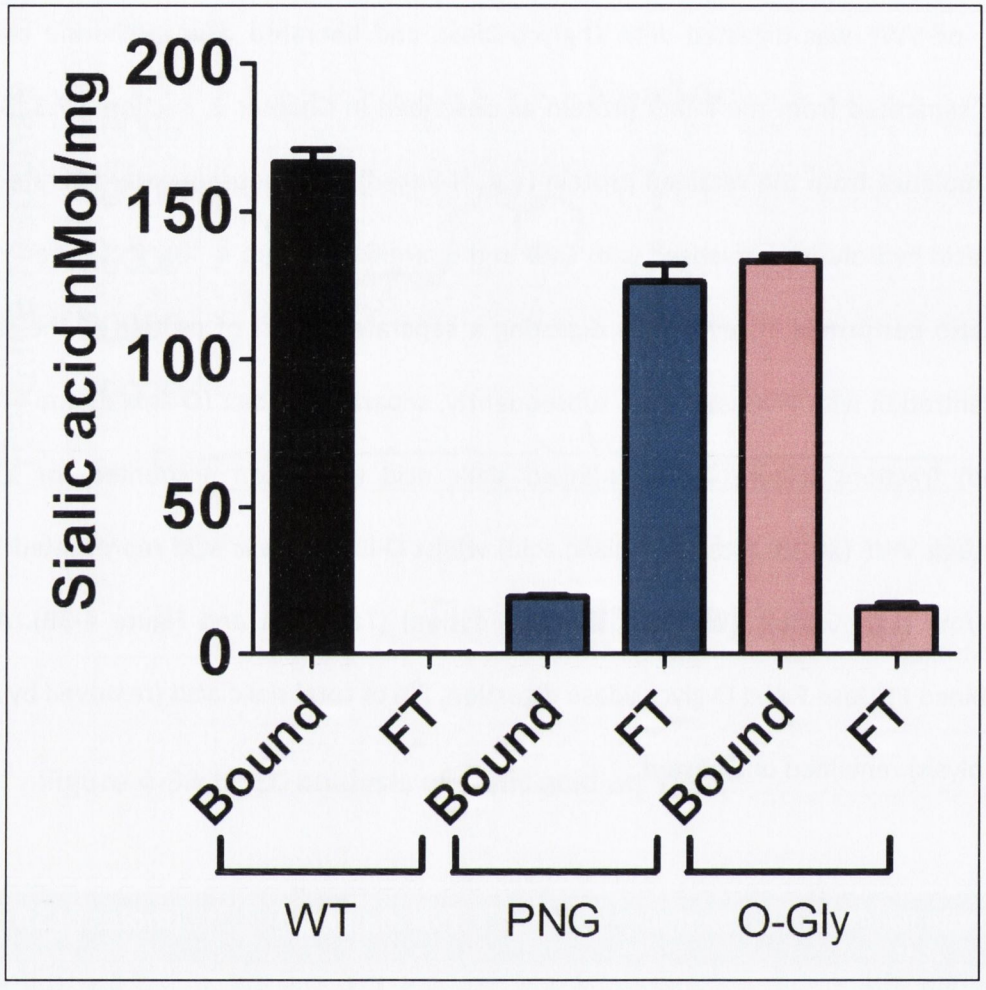


Figure 4-3B Quantification of plasma-derived VWF sialic acid expression using HPLC.

To determine relative quantitative sialic acid expression on the N- and O-linked carbohydrate structures of VWF, HPLC analysis of residual VWF-bound sialic acid (bound) and free (flow through, FT) was performed following digestion with either PNGase F (PNG) or O-glycosidase (O-Gly).

4.3.3 Quantification of sialic acid on VWF after α2-3,6,8,9 neuraminidase digestion by HPLC

Lectin plate-binding ELISAs are strongly influenced by steric hindrance, and preferentially detect glycans structures exposed on the surface of glycoproteins. These limitations are particularly relevant for VWF, given its multimeric composition and complex N-linked glycan structures. Consequently, quantitative sialic acid expression on VWF was also determined by HPLC following α2-3,6,8,9 neuraminidase digestion. Following treatment with 8mU neuraminidase, HPLC analysis demonstrated removal of 50% of total sialic acid expression (Figure 4-3C), which is consistent with results of lectin binding ELISA (Figure 4-1). However, after digestion with 1250mU α2-3,6,8,9 neuraminidase, HPLC analysis demonstrated that 29.2% of total sialic acid remained, despite the fact that the lectin ELISA suggested that > 90% total sialic acid had been removed.

Cumulatively, these HPLC data confirm that plasma VWF is heavily sialylated, and demonstrate for the first time that 80% of this sialic acid expression is present on the N-linked glycan chains, where it is predominantly α2-6 linked to penultimate galactose residues. Moreover, approximately 30% of total sialic acid is not readily accessible to digestion with neuraminidase, and cannot be detected by lectin binding plate assays.

Sample ID	Sample	[moles of SA]	% SA remaining	% SA removed
1.	Total	5.64×10^{-11}	100%	0%
2.	50% Neu-VWF	2.78×10^{-11}	49.40%	50.6%
3.	Neu-VWF	1.65×10^{-11}	29.22%	70.78%

Table 4-2 Sialic acid (SA) expression on VWF after treatment with various concentrations of α2-3,6,8,9 neuraminidase.

The values represented include moles of SA, % SA remaining after digestion and % SA removed. This table corresponds to Figure 4-3D.

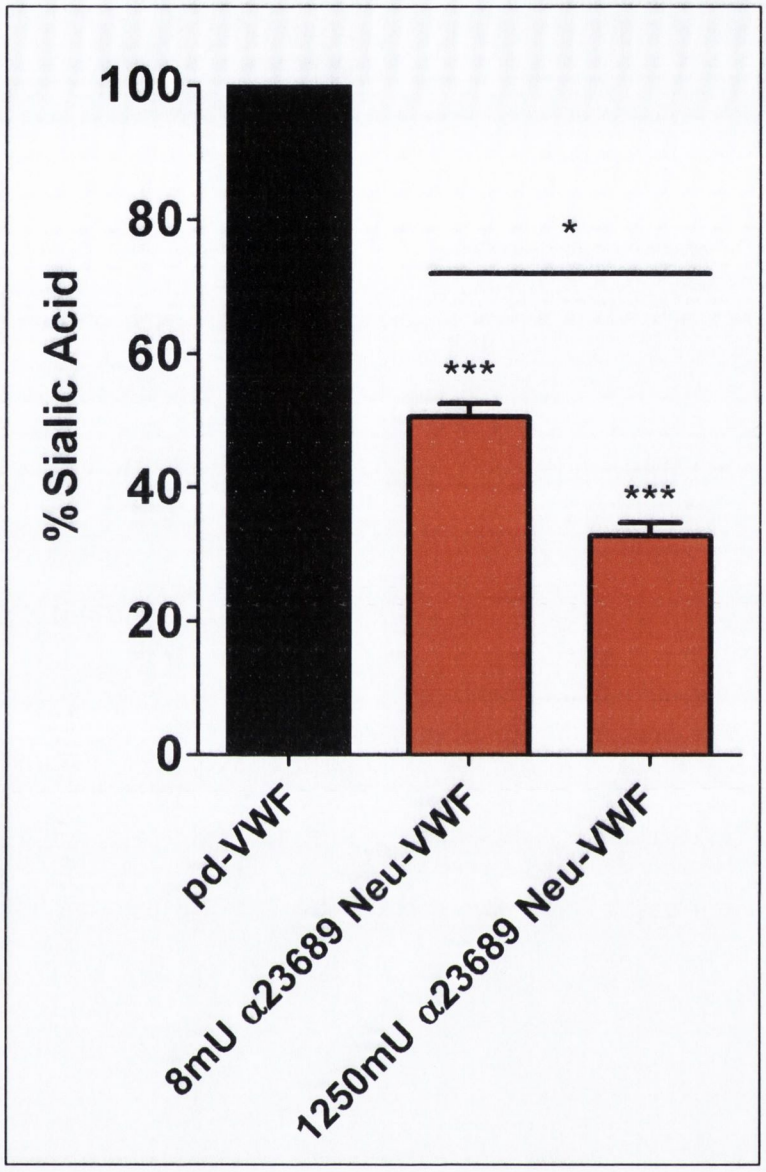


Figure 4-3C Quantification of VWF sialic acid expression after α2-3,6,8,9 neuraminidase digestion using HPLC.

Purified human VWF was incubated overnight with varying concentrations of α2-3,6,8,9 neuraminidase as before, and residual α2-3,6 linked sialic acid expression on VWF analysed by HPLC as described in section 2.7.3 (* $p < 0.05$, *** $p < 0.001$).

4.4 Modification of VWF glycan structures has no effect on collagen binding activity

Previous studies suggested that modification of glycosylation can alter VWF affinity for collagen binding. However, it was subsequently demonstrated that this was an indirect effect based on enhanced susceptibility of glycan modified VWF to proteolysis, which in turn reduces multimer size and therefore affects VWF:CB (Federici, *et al* 1984).

To ensure that VWF:CB can be reliably used as a tool to assess ADAMTS13 cleavage of VWF, I confirmed that modification of VWF glycosylation had no effect on VWF:CB. VWF was digested with various concentrations of α2-3,6,8,9 neuraminidase, α2-3 neuraminidase and PNGase F as described in Chapter 2, section 2.6. Removal of terminal sialic acid or complete N-linked glycan structures had did not alter VWF binding to collagen (data not shown). Similarly, no difference between VWF:CB/VWF:Ag for untreated pd-VWF and glycan modified VWF was observed, which indicates that removal of sialic acid from VWF does not affect multimeric composition (Figures 4-4A, 4-4B and 4-4C).

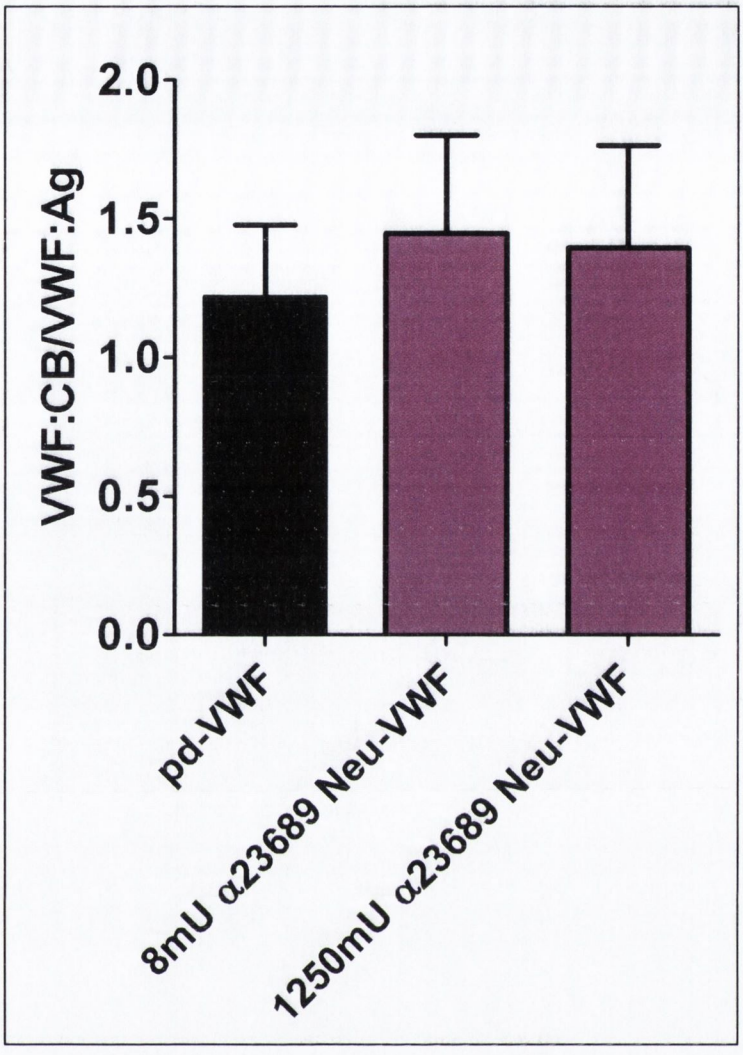


Figure 4-4A Removal of terminal sialic acid has no effect on VWF collagen binding activity.

To investigate whether VWF sialic acid expression influences collagen binding activity, VWF binding to recombinant human collagen III after treatment with various concentrations of α2-3,6,8,9 neuraminidase (Neu-VWF) was assessed. A ratio of VWF:CB/VWF:Ag was used to characterise multimer size of each of the samples. Experiments were performed in triplicate and results are expressed mean ± SEM. Removal of sialic acid has no effect on VWF multimer size or collagen binding activity (p = ns).

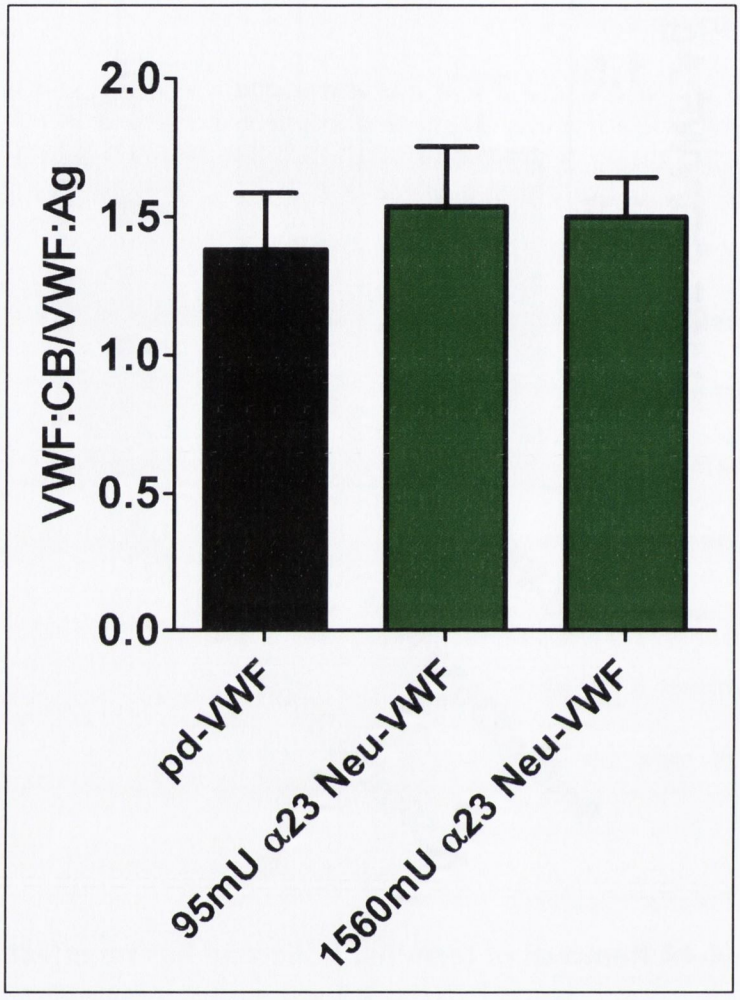


Figure 4-4B Specific removal of terminal O-linked sialic acid has no effect on VWF collagen binding activity.

To examine whether sialic acid expressed on the O-glycans of VWF influences collagen binding activity, VWF binding to recombinant human collagen III after treatment with various concentrations of α2-3 neuraminidase (α23 Neu-VWF) was assessed. A ratio of VWF:CB/VWF:Ag was used to characterise multimer size of each of the samples. Experiments were performed in triplicate and results are expressed mean ± SEM. Removal of O-linked sialic acid has no effect on VWF multimer size or collagen binding activity (p = ns).

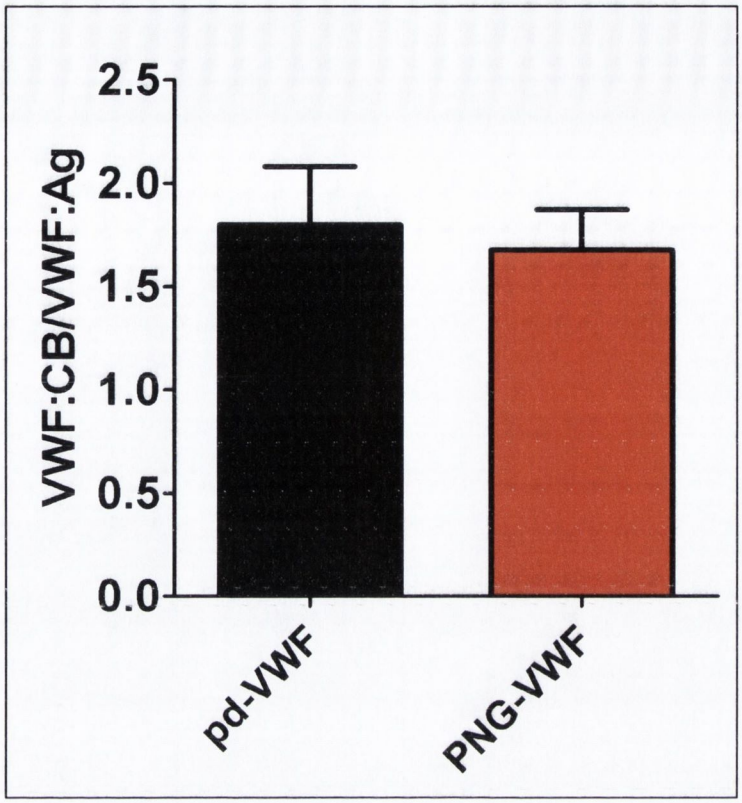


Figure 4-4C Removal of N-glycans does not influence VWF collagen binding activity.

To ascertain whether reduction of VWF N-linked glycan expression after treatment with PNGase F affects VWF:CB, PNG-VWF collagen binding activity was characterised as before. Experiments were performed in triplicate and results are expressed mean ± SEM. Removal of N-linked glycans has no effect on VWF multimer size or collagen binding activity (p = ns).

4.5 Neuraminidase treatment protects VWF from proteolysis by ADAMTS13

A previous study demonstrated that removal of N-linked glycans from VWF, and in particular N1574 which lies next to the ADAMTS13 cleavage site, significantly increased VWF susceptibility to ADAMTS13 proteolysis (McKinnon, *et al* 2008). As both the N- and O-linked glycans of VWF are abundantly sialylated, sialic acid may be playing a role in the regulation of ADAMTS13 cleavage of VWF. To elucidate the significance of N- and O-linked sialic acid expression in modulating VWF proteolysis, glycosidase-treated VWF preparations were incubated with rADAMTS13 and rate of cleavage determined by residual VWF:CB over time. The extent of proteolysis can be established by the reduction in VWF:CB, as larger multimers of VWF bind to collagen with a higher affinity. Therefore, VWF:CB decreases over time corresponding to the degree of ADAMTS13 proteolysis.

4.5.1 Removal of sialic acid from VWF results in increased resistance to ADAMTS13 cleavage

In keeping with recent reports, removal of N-linked glycans by treating VWF with PNGase F (PNG-VWF) significantly *enhanced* the rate of VWF proteolysis by ADAMTS13 (Figure 4-5A). Paradoxically, in contrast to the increased rate of cleavage observed for PNG-VWF, ADAMTS13 proteolysis of Neu-VWF was significantly *inhibited* compared to both untreated pd-VWF and PNG-VWF respectively (Figure 4-5A). At all time points after 60 minutes, Neu-VWF demonstrated significant increased resistance to ADAMTS13 proteolysis ($p < 0.05$). After incubation with ADAMTS13 (3nM for 120 mins), Neu-VWF

collagen binding activity was reduced to 50 ± 14%, compared to 11 ± 7% for pd-VWF ($p <$ 0.01). Furthermore, 50% Neu-VWF also demonstrated partial resistance to ADAMTS13 cleavage. The partial resistance was most apparent at 60 mins, where residual VWF:CB values for pd-VWF, 50% Neu-VWF and Neu-VWF were 27± 6%; 36 ± 7% and 63 ± 15% respectively (p = 0.08; Figure 4-5B). This shows that sialic acid on VWF specifically enhances the rate of cleavage by ADAMTS13.

4.5.2 Removal of α2-3 linked sialic acid from VWF has no effect on susceptibility to ADAMTS13

Despite the ADAMTS13-resistant phenotype of Neu-VWF compared to pd-VWF, digestion of VWF with α2-3 neuraminidase to specifically remove only α2-3 linked sialic acid expression (predominantly O-linked) did not influence ADAMTS13 proteolysis (Figure 4-5C). Both pd-VWF and α23-Neu-VWF were cleaved at a similar rate and to the same extent by ADAMTS13. This indicates that terminal α2-3 linked sialic acid, the majority of which is O-linked, does not directly mediate VWF susceptibility to ADAMTS13 cleavage. Thus, the data suggests a novel role for terminal α2-6 linked sialic acid expression on VWF in modulating proteolysis by ADAMTS13.

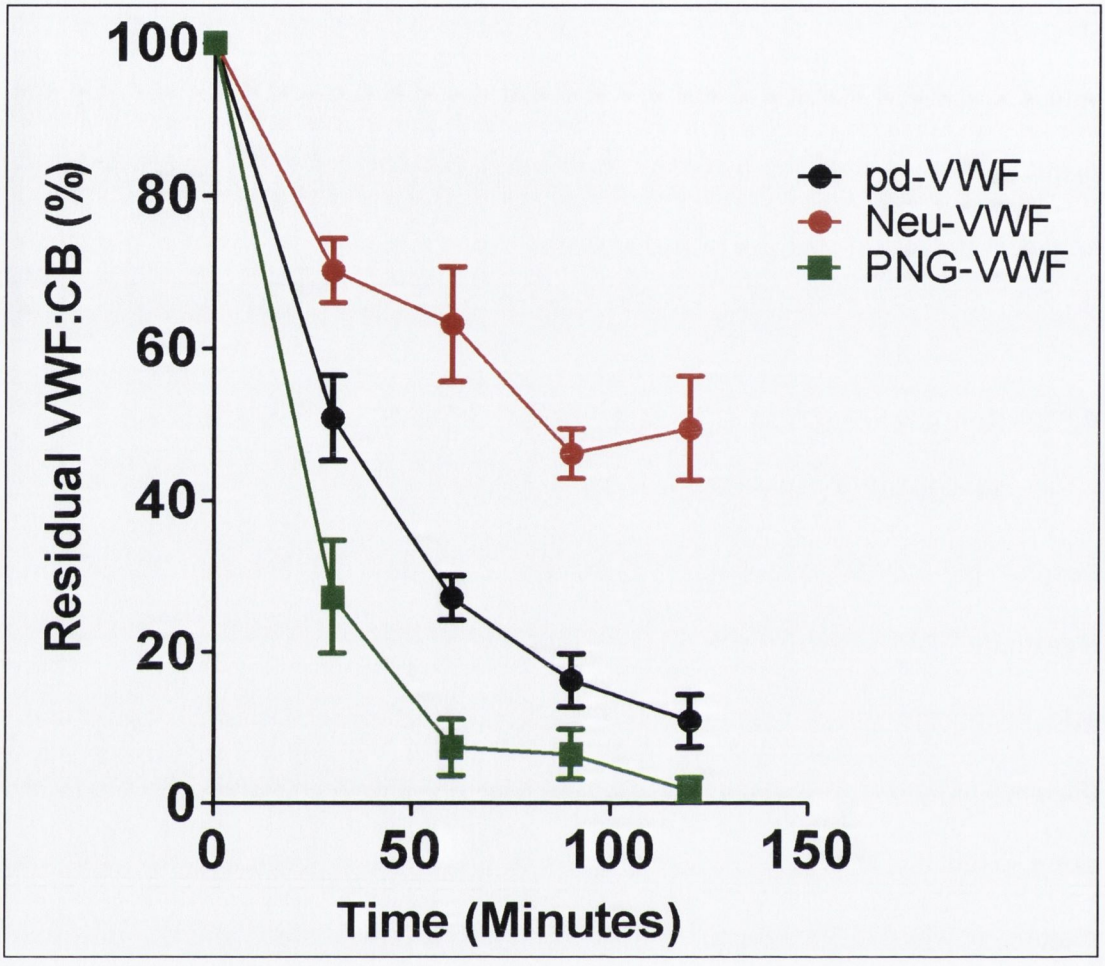


Figure 4-5A Terminal sialic acid expression on VWF promotes proteolysis by ADAMTS13.

To investigate whether sialic acid expression on VWF influences susceptibility to ADAMTS13 proteolysis, α2-3,6,8,9 neuraminidase treated VWF (Neu-VWF) and PNGase treated VWF (PNG-VWF) preparations were incubated with 3nM human rADAMTS13 in the presence of 1.5M urea. Rate of VWF cleavage was assessed by determining the rate of VWF:CB decrease over time. Results are expressed as residual VWF:CB (mean ± SEM). (pd-VWF, ●; Neu-VWF, ●; PNG-VWF ■). Removal of sialic acid impairs ADAMTS13 proteolysis of VWF ($p < 0.05$).

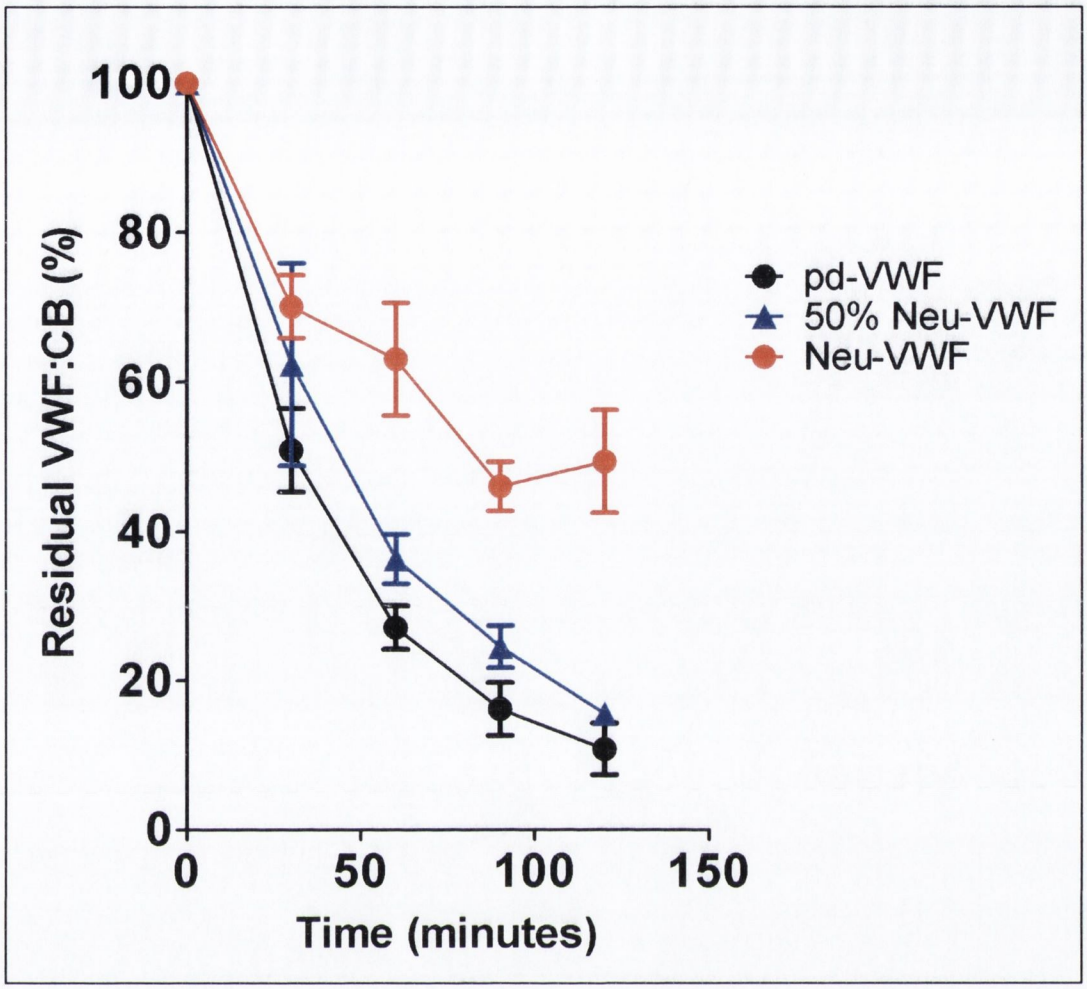


Figure 4-5B Sialic acid expression on VWF modulates ADAMTS13 proteolysis in a quantitative manner.

VWF was digested with 8mU and 1250mU of α2-3,6,8,9 neuraminidase to remove ~50% and total accessible sialic acid respectively. 50% Neu-VWF demonstrated partial resistance to ADAMTS13 proteolysis as determined by residual VWF:CB over time. This difference in susceptibility to cleavage was most marked at 60 mins (p = 0.08). pd-VWF (●); Neu-VWF (●); and 50% Neu-VWF (▲).

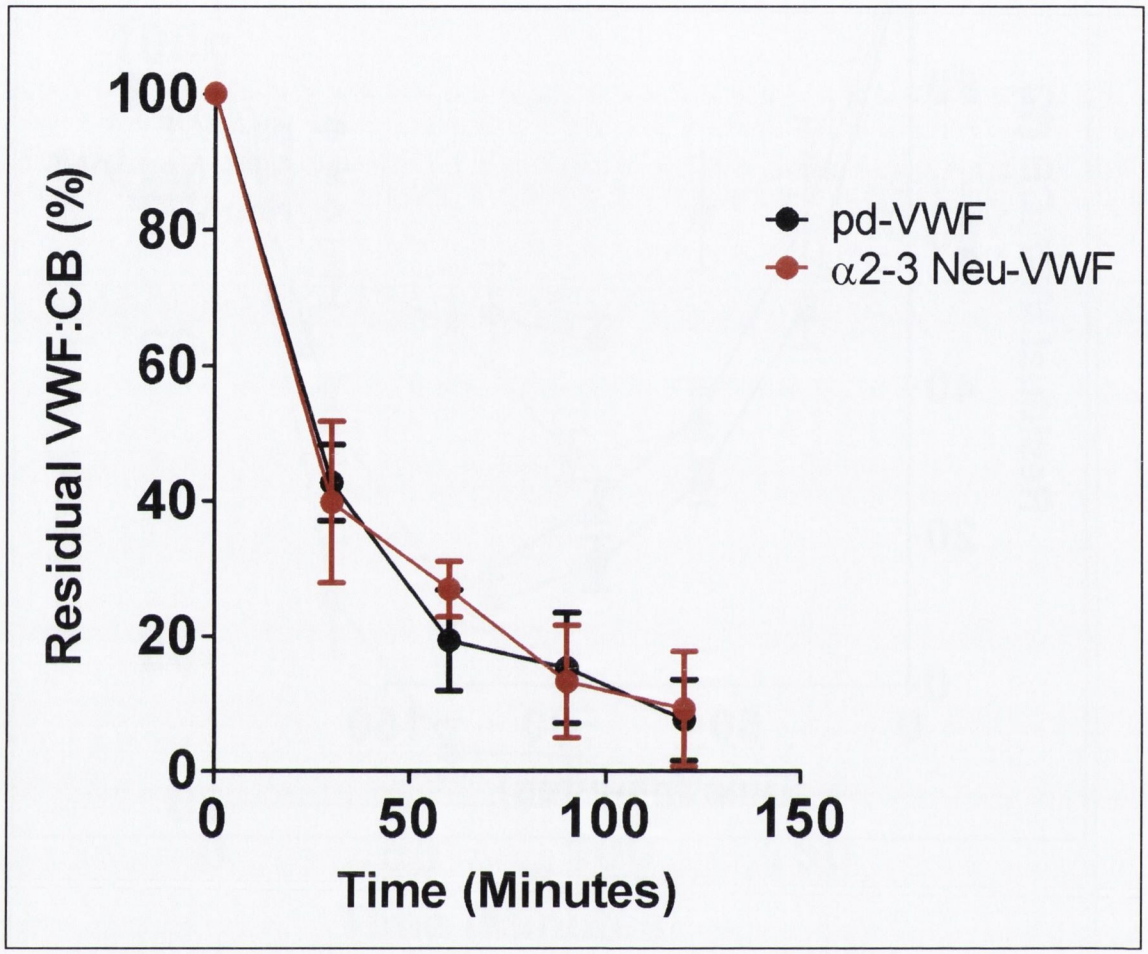


Figure 4-5C Removal of α2-3 linked sialic acid has no effect on VWF susceptibility to ADAMTS13 cleavage.

To determine whether α2-3 linked sialic acid on VWF influences ADAMTS13 proteolysis, α2-3 neuraminidase treated VWF was incubated with recombinant ADAMTS13 in the presence of 1.5M urea as before. Rate of proteolysis for α2-3Neu-VWF (●) was compared to pd-VWF (●) by determining residual collagen binding activity. In contrast to the marked resistance to ADAMTS13 cleavage observed following treatment with α2-3,6,8,9 neuraminidase, α2-3Neu-VWF was proteolysed at a rate indistinguishable from that of pd-VWF.

Discussion

To maintain normal physiologic haemostasis, VWF proteolysis by ADAMTS13 requires tight regulation (Levy, *et al* 2005). In patients with type 2A von Willebrand disease (VWD), increased proteolysis results in loss of high molecular weight multimer and confers a significant bleeding phenotype. Conversely, in thrombotic thrombocytopenic purpura, inherited or acquired ADAMTS13 deficiency causes accumulation of ultra large VWF multimers in plasma and thereby contributes to the development of pathologic platelet-rich thrombi in the microvasculature (Levy, *et al* 2001). In addition, multivariate analyses suggest that reduced plasma ADAMTS13 levels are associated with increased risk of myocardial infarction (Chion, *et al* 2007, Crawley, *et al* 2008). In this context, understanding the molecular basis through which VWF proteolysis by ADAMTS13 is regulated in vivo is of direct translational importance.

Recent reports have shown that carbohydrate structures expressed on VWF play an important role in regulating ADAMTS13 proteolysis. In this study, I demonstrate a novel role for sialic acid expression in mediating this effect, such that removal of terminal sialic acid significantly inhibits VWF cleavage by ADAMTS13 in a dose-dependent manner. Although impaired ADAMTS13 proteolysis was apparent after α2-3,6,8,9 neuraminidase treatment, α2-3 neuraminidase had no significant effect. This highlights the role for N-linked sialic acid alone in modulation of the rate of ADAMTS13 proteolysis, whereas O-linked sialic acid is not participating directly.

Previous studies that used different methods have consistently shown that most sialic acid present on the N-linked chains of VWF is α2-6 linked to sub-terminal underlying

galactose residues (Debeire, *et al* 1983, Matsui, *et al* 1992, Samor, *et al* 1982), whereas O-linked sialic acid can be either α2-3 or α2-6 linked (Samor, *et al* 1989). Neither α2-8 nor α2-9 linked sialic acid have been identified on the N- or O-linked glycans of human pd-VWF. Consequently, it seems highly probable that loss of α2-6 linked sialic acid (on either N- or O-linked chains) is responsible for mediating the ADAMTS13-resistant phenotype observed after α2-3,6,8,9 neuraminidase treatment. It is well established that sialic acid expression on other circulating glycoproteins influences susceptibility to proteolysis (Garner, *et al* 2001, Rudd, *et al* 1999, Varki 1993). However, removal of sialic acid has always previously been reported to result in significantly enhanced proteolysis rates. In contrast to this accepted paradigm, the data in this chapter show that α2-6 linked sialic acid on VWF exerts different effects on susceptibility to proteolysis, depending on the protease involved.

Herein I report the novel observation that α2-6 linked sialic acid expression on VWF specifically enhances proteolysis by ADAMTS13 in a dose-dependent manner. I further demonstrate that this α2-6 linked sialic acid is predominantly expressed on the N-linked glycans of VWF, as determined by HPLC analysis. Lectin plate binding ELISAs are not suitable for quantitative analysis of VWF glycosylation, due to the multimeric nature of the protein, and the abundance of glycan structures, some of which are inaccessible unless VWF is fully denatured. Alteration of VWF glycan pattern does not affect collagen binding, making this assay suitable for the assessment of ADAMTS13 cleavage in vitro. Further experiments will be carried out to determine the molecular mechanism responsible for Neu-VWF resistance to ADAMTS13 proteolysis.

CHAPTER 5:

DETERMINATION OF THE MOLECULAR MECHANISMS RESPONSIBLE FOR SIALIC ACID MEDIATED VWF RESISTANCE TO ADAMTS13 PROTEOLYSIS

5.1 Introduction

As demonstrated in the previous chapter, alteration of sialic acid expression levels on plasma-derived human VWF affects susceptibility to ADAMTS13 proteolysis. More specifically, removal of terminal α2-6 linked sialic acid, which is predominantly found on the N-linked carbohydrate chains of VWF, impairs ADAMTS13 cleavage.

Removal of sialic acid from glycoproteins usually renders them more susceptible to proteolytic degradation. The finding that VWF desialylation protects from regulatory cleavage by ADAMTS13 was therefore surprising. To further understand and determine the molecular mechanisms responsible for this phenomenon, several approaches were taken. Previous studies have demonstrated that variation of the denaturing conditions used in a static ADAMTS13 cleavage assay can help to identify the role of VWF conformation in the modulation of proteolysis (McKinnon, *et al* 2008, O'Donnell, *et al* 2005). Similarly, sequential digestion of sugar structures present on N- and O-linked glycan chains can pinpoint the exact carbohydrate moiety and/or linkage responsible for exerting a particular effect on glycoprotein function. Moreover, other aspects that may affect sialic acid mediated resistance to proteolysis, or the VWF-ADAMTS13 interaction

need to be addressed including the negative charge on sialic acid, the role of FVIII and/or the role of ABO blood group antigenic structures.

In summary, the questions I hoped to answer with this section of experiments were:

A) Does removal of sialic acid protect VWF from proteolysis by other enzymes?

B) Does removal of sialic acid mediate resistance to ADAMTS13 through a conformation based mechanism, via removal of the negative charge?

C) Is exposure of sub-terminal underlying galactose residues important in relation to mediating resistance to ADAMTS13?

D) Does factor VIII play a part in mediating Neu-VWF resistance to ADAMTS13?

E) How do terminal ABO(H) blood group antigens and terminal sialic acid interact to modulate ADAMTS13 proteolysis?

5.2 Sialic acid specifically enhances VWF proteolysis by ADAMTS13

Previous research has shown that several plasma proteins (e.g. erythropoietin) are particularly sensitive to cleavage after sialic acid expression levels have been reduced (Goldwasser, *et al* 1974). Moreover, studies carried out in the 1980's demonstrated that desialylation dramatically increases the rate of VWF proteolysis by various serine proteases. Consequently, sialic acid on VWF is essential for maintaining VWF multimeric structure and protecting the protein from degradation in plasma (Berkowitz and Federici 1988, Federici, *et al* 1984).

In view of the inhibitory effect of α2-3,6,8,9 neuraminidase treatment on VWF cleavage by ADAMTS13, susceptibility of Neu-VWF to a series of other serine and cysteine

proteases was investigated. In contrast to its resistance to ADAMTS13, Neu-VWF demonstrated significantly *enhanced* cleavage by both chymotrypsin (final concentration 60U/mg VWF) and cathepsin B (final concentration 4U/mg VWF) after 90 minutes compared to untreated pd-VWF ($p < 0.001$ and $p < 0.05$ respectively; Figure 5-1). Consequently, although loss of sialic acid expression *specifically* protects VWF against ADAMTS13 proteolysis, it renders VWF more susceptible to cleavage by other proteases.

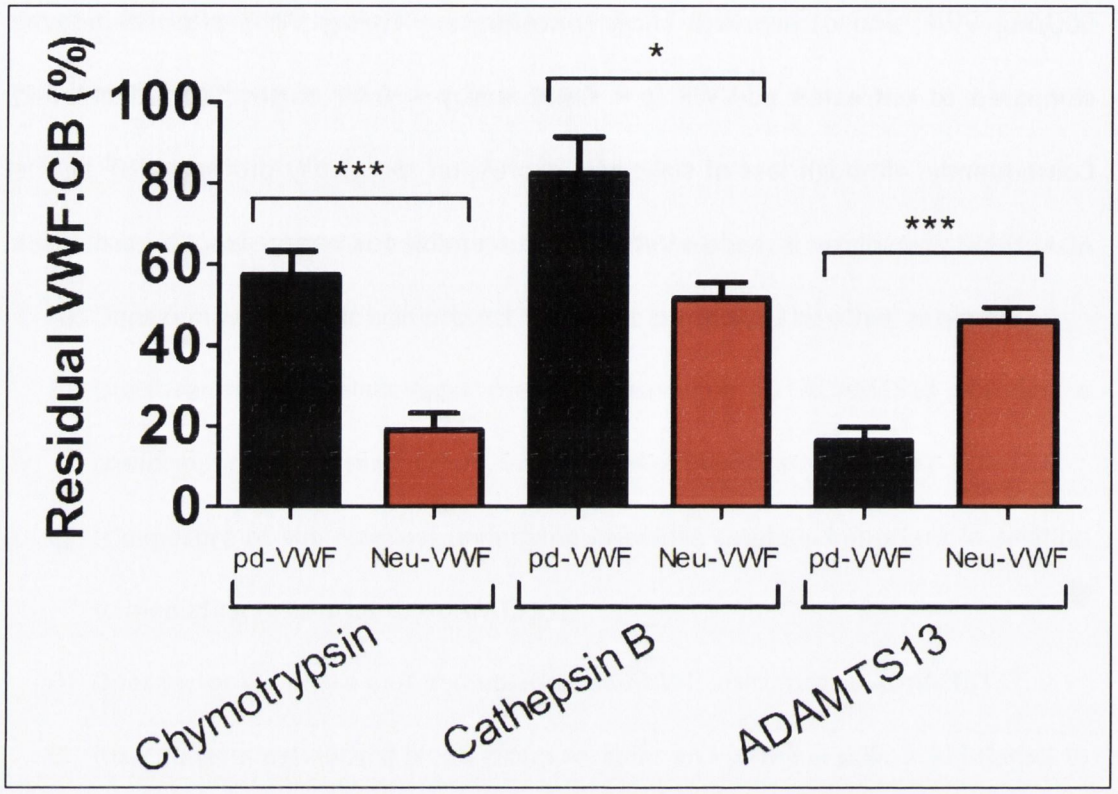


Figure 5-1 Sialic acid specifically modulates VWF proteolysis by ADAMTS13.

To establish the effect of desialylation of VWF on susceptibility to non specific proteolysis, VWF was treated with α2-3,6,8,9 neuraminidase and subsequently subjected to cleavage by chymotrypsin (60U/mg VWF), cathepsin B (4U/mg VWF) and ADAMTS13 (500nM/mg VWF) at 37°C for 90 minutes. Results are shown as residual collagen binding activity at 90 minutes ± SEM for each of the samples. (* $p < 0.05$, *** $p < 0.001$).

5.3 α2-6 linked sialic acid increases VWF proteolysis by ADAMTS13 through a conformational mechanism

Previous studies have demonstrated that changes in glycan structures can influence glycoprotein conformation. Oligosaccharide structures are vital to the maintenance of structure, stability and conformation of certain glycoproteins, and alteration of endogenous glycan patterns not only affects conformation, but can also have resultant effects on half-life, function and binding (Elbein 1991, Varki 1993). Moreover, modification of both N- and O-linked carbohydrate chains via chemical or enzymatic modification can directly influence protein conformation by altering conformational freedom of the local peptide backbone; via charge mediated or steric effects (Keutmann, *et al* 1985, Mitra, *et al* 2006, Narhi, *et al* 1991, Wyss, *et al* 1995).

5.3.1 Further denaturation of VWF by increasing the urea concentration ablates Neu-VWF resistance to ADAMTS13 cleavage

To investigate whether α2-6 linked sialic acid expression enhances VWF proteolysis by altering its tertiary conformation, the effect of VWF desialylation on ADAMTS13 cleavage at different urea concentrations was studied. In keeping with previous reports (O'Donnell, *et al* 2005), the rate of VWF proteolysis increased significantly as urea concentration was increased from 1M to 2M to progressively denature VWF (Figure 5-2A and 5-2B). However, the ability of α2-6 linked sialic acid to enhance VWF cleavage by ADAMTS13 was less evident at higher urea levels (Figure 5-2B). These data suggest that sialic acid may directly influence the conformation of pd-VWF. Alternatively, sialic acid

may rely upon normal VWF conformation in order to be able to affect specific interactions between VWF and ADAMTS13.

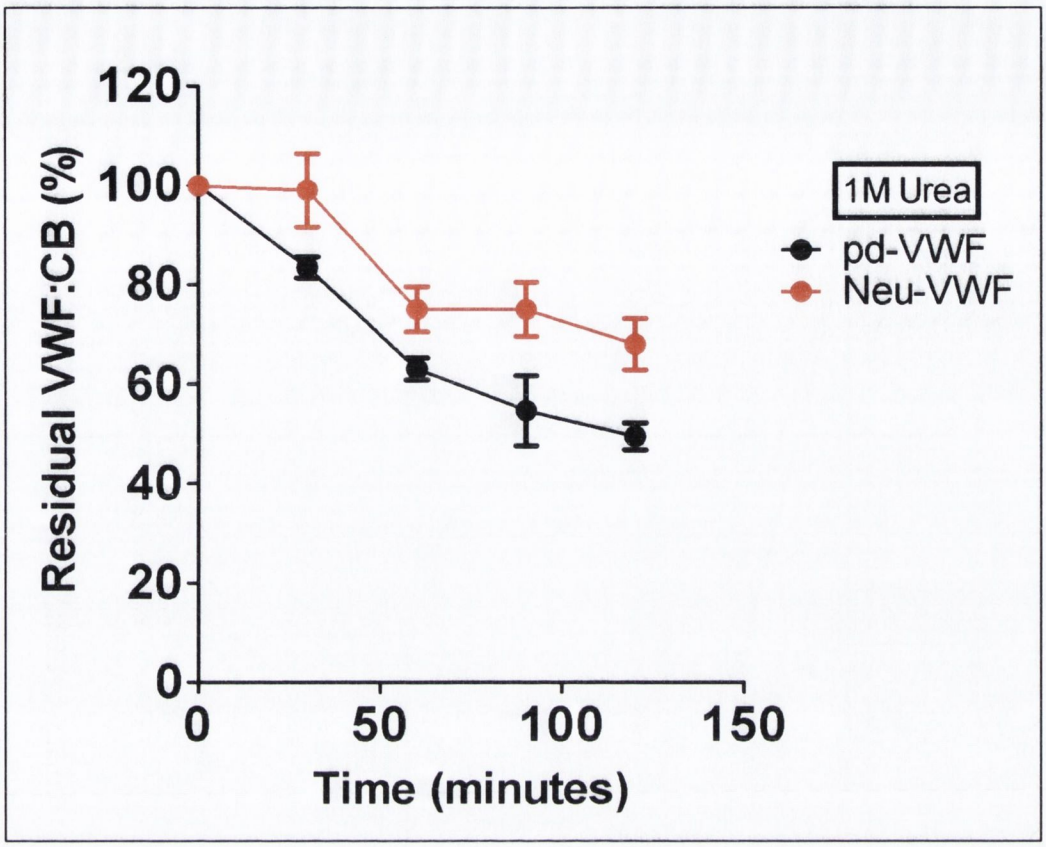


Figure 5-2A Effect of variation of urea concentration on ADAMTS13 proteolysis of Neu-VWF.

The effect of α2-3,6,8,9 neuraminidase treatment in regulating VWF susceptibility to ADAMTS13 proteolysis was investigated at different urea concentrations. At 1M urea, the rate of VWF proteolysis was significantly decreased for both pd-VWF (●) and Neu-VWF (●), compared to 1.5M urea (Figure 4-5A).

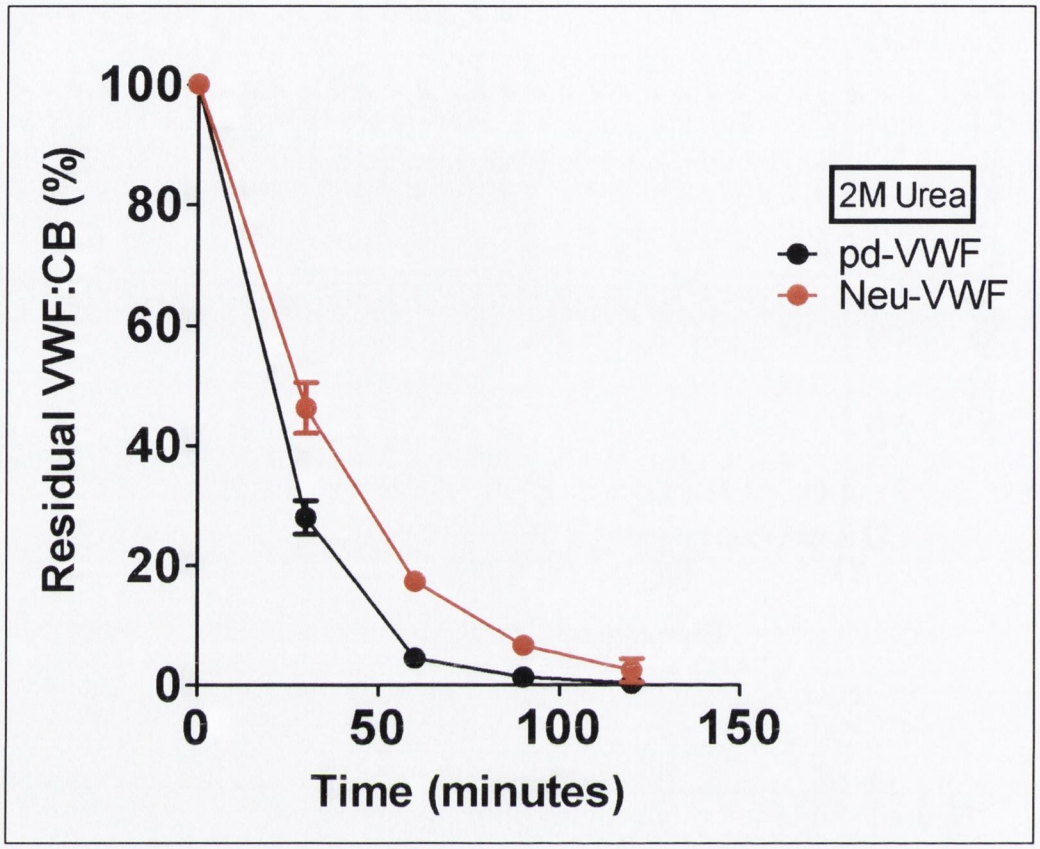


Figure 5-2B Effect of variation of urea concentration on ADAMTS13 proteolysis of Neu- VWF.

The effects of α2-3,6,8,9 neuraminidase treatment in regulating VWF susceptibility to ADAMTS13 proteolysis were investigated at different urea concentrations. At 2M urea, the rate of VWF proteolysis was significantly increased for both pd-VWF (●) and Neu-VWF (●), compared to 1.5M urea (Figure 4-5A). This suggests that sialic acid mediated resistance of VWF to ADAMTS13 cleavage is dependent on VWF conformation.

5.3.2 Negative charge on sialic acid does not directly influence ADAMTS13 proteolysis of VWF

Sialic acid moieties possess a net negative charge due to an electron-withdrawing carboxyl group linked to the anomeric carbon (Chen and Varki 2010, Schauer 2009). To determine whether loss of sialic acid negative charge was important in mediating VWF resistance to ADAMTS13 proteolysis, sialic acid on VWF was oxidised by treatment with sodium *meta*-periodate ($NaIO_4$). Sodium *meta*-periodate cleaves bonds between two neighbouring carbon atoms that contain hydroxyl groups, leading to the formation of two aldehyde groups (Van Lenten and Ashwell 1971). Selective oxidation of sialic acid residues can be achieved by incubating glycoproteins with a low concentration of sodium *meta*-periodate (1-10mM) on ice for a short period of time (30-60 min). Oxidation of VWF sialic acid had no effect on the rate or extent of proteolysis by ADAMTS13 (Figure 5-3), suggesting that the negative charge carried on terminal sialic acid residues does not play a critical role in mediating the ADAMTS13-VWF interaction.

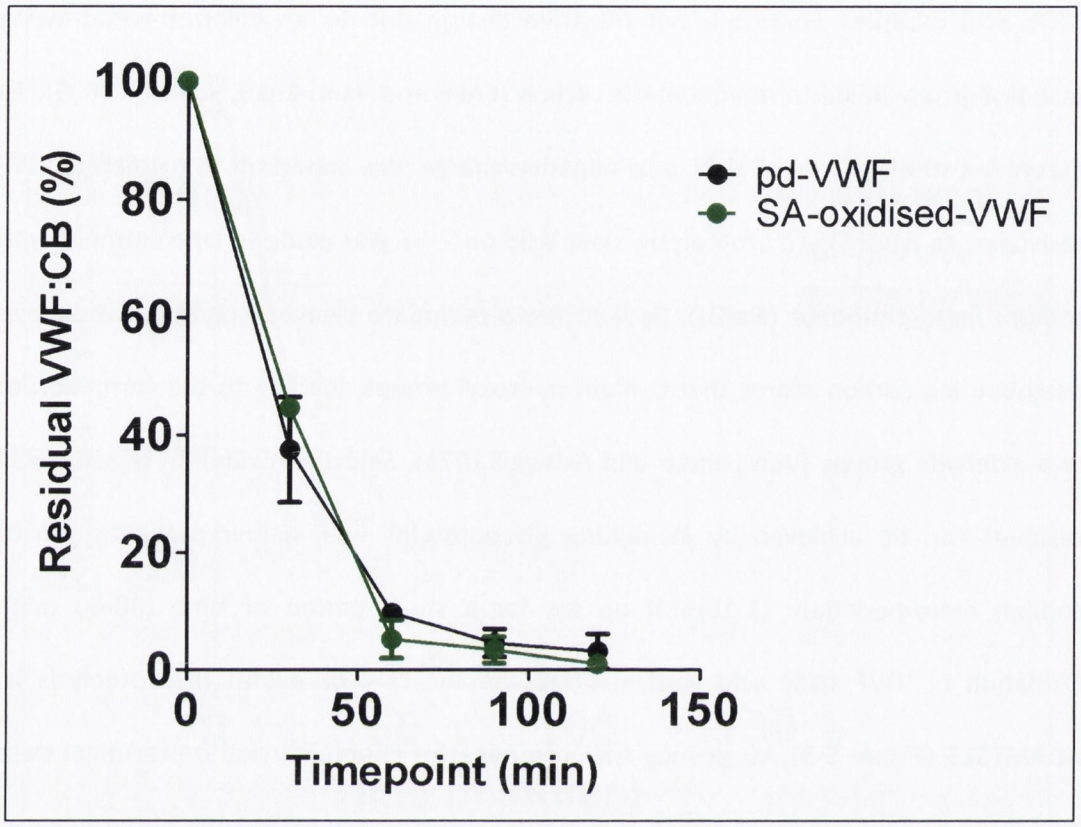


Figure 5-3 Loss of negative charge on sialic acid does not affect VWF susceptibility to ADAMTS13 proteolysis.

Sialic acid negative charge can directly mediate protein-protein interactions. To determine whether loss of negative charge was involved in inhibiting VWF proteolysis by ADAMTS13, sialic acid on VWF was oxidised by treatment with 5mM sodium metaperiodate, and ADAMTS13 proteolysis was investigated as before. pd-VWF (●) and sialic acid (SA) oxidised-VWF (●) were cleaved in an identical fashion.

5.4 Sialic acid on ADAMTS13 is not required for proteolysis of VWF

Due to their negative charge and terminal location on oligosaccharide chains, sialic acid residues have also been shown to directly influence enzyme-substrate interactions. Interestingly ADAMTS13 also circulates as a sialoglycoprotein, with putative N- and O-linked glycosylation sites. Moreover, Ricketts *et al* demonstrated that O-fucosylation is required for ADAMTS13 secretion (Ricketts, *et al* 2007). Furthermore, Zhou *et al* recently showed that ADAMTS13 N-linked glycosylation is necessary for efficient secretion, while after secretion, ADAMTS13 does not require N-glycans for its VWF cleaving activity (Zhou and Tsai 2009).

To ascertain whether sialic acid on ADAMTS13 plays a role in regulation of VWF cleaving activity, ADAMTS13 was treated with α2-3,6,8,9 neuraminidase (Neu-A13) as described in Chapter 2, section 2.6. Following reduction of ADAMTS13 sialic acid expression levels, proteolysis of VWF was carried out as before. Desialylation of ADAMTS13 has no effect on VWF cleaving ability. Neu-A13 cleaved both pd- and Neu-VWF in an identical fashion (Figure 5-4). Neu-VWF still demonstrated resistance to ADAMTS13 in the absence of ADAMTS13 sialic acid. Overall, this data indicates that terminal sialic acid expression on ADAMTS13 is not required for cleavage of full length VWF. In addition, sialic acid on VWF alone modulates susceptibility to ADAMTS13 cleavage, with ADAMTS13 glycosylation exerting no effect.

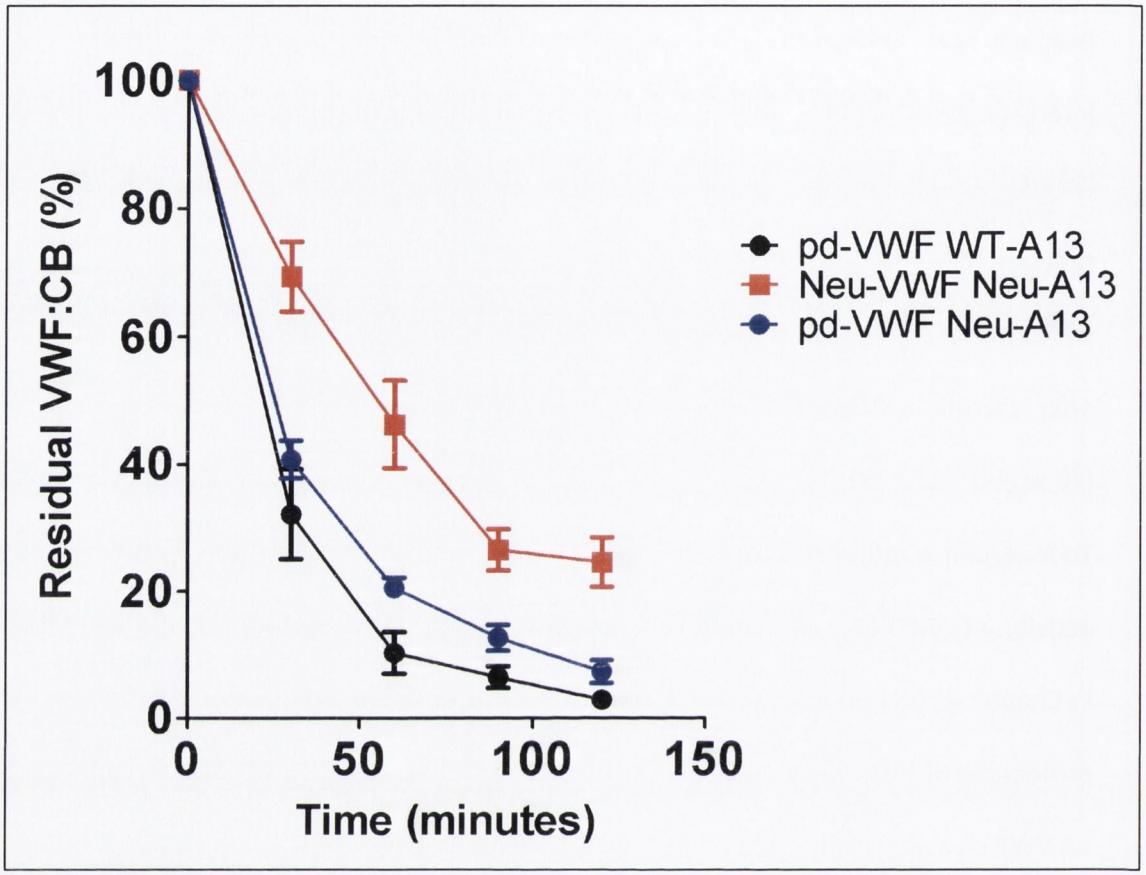


Figure 5-4 Desialylation of ADAMTS13 has no effect on proteolytic activity.

To establish whether sialic acid on ADAMTS13 is important for VWF proteolysis, WT-ADAMTS13 was desialylated by treatment with α2-3,6,8,9 neuraminidase as described in section 2.6. Untreated ADAMTS13 (WT-ADAMTS13) and α2-3,6,8,9 neuraminidase treated ADAMTS13 (Neu-ADAMTS13) cleaved pd-VWF and Neu-VWF in an identical manner. pd-VWF, WT-ADAMTS13 (●); Neu-VWF, Neu-ADAMTS13 (■); WT-VWF, Neu-ADAMTS13 (●).

5.5 Subsequent removal of penultimate galactose residues from VWF glycan chains after desialylation ablates resistance to ADAMTS13 cleavage

Removal of α2-6 linked sialic acid from the N-linked glycans of VWF results in exposure of additional underlying sub-terminal D-galactose residues (Gralnick 1978, Matsui, *et al* 1992). To determine whether these exposed galactose residues may play a role in mediating the ADAMTS13-resistant phenotype of Neu-VWF, further treatment of Neu-VWF with β1-3,4-galactosidase was carried out. Galactose expression pre- and post galactosidase treatment was determined using a lectin binding assay utilising *Ricinus communis* agglutinin (RCA), which specifically binds to β-D-galactosyl residues (Ellies, *et al* 2002).

β-galactosidase treatment of pd-VWF alone resulted in removal of ~60% of exposed galactose (Figure 5-5A, $p < 0.001$), but did not influence susceptibility to ADAMTS13 cleavage (Figure 5-5B). Galactosidase treatment of sialoglycoproteins, however, does not fully remove all galactose residues, as some are masked by overlying sialic acid moieties. Therefore, VWF was digested with a combination of neuraminidase and galactosidase, as described in chapter 3, section 3.2. Galactosidase treatment of Neu-VWF resulted in the removal of 92 ± 3 % exposed galactose (Figure 5-6A, $p < 0.001$). Interestingly, upon galactose removal, Neu-Gal-VWF no longer demonstrated significant resistance to ADAMTS13 proteolysis and was cleaved at a rate identical to wild type VWF (Figure 5-6C). Consequently, the results imply that the balance of sialic acid to galactose expression at the termini of VWF glycans significantly influences susceptibility of pd-VWF to ADAMTS13 proteolysis.

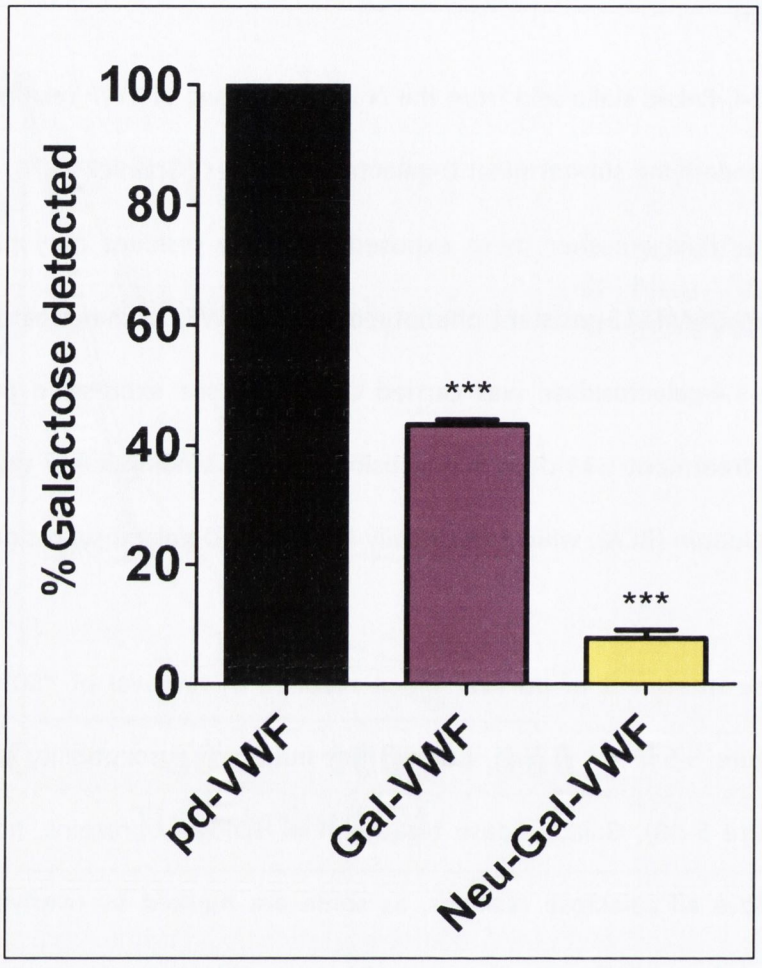


Figure 5-5A Levels of β-galactose on VWF after glycosidase digestion.

Purified plasma-derived human VWF (pdVWF) was incubated overnight with either α2-3,6,8,9 neuraminidase, β1-3,4 galactosidase or a combination of both glycosidases. Residual expression of β1-3,4 linked galactose on VWF was analysed by modified *Ricinus Communis* agluttinin (lectin) binding ELISA as described in Chapter 2, section 2.7.1.
All experiments were performed in triplicate, and results described represent the mean ± SEM (*** $p < 0.001$).

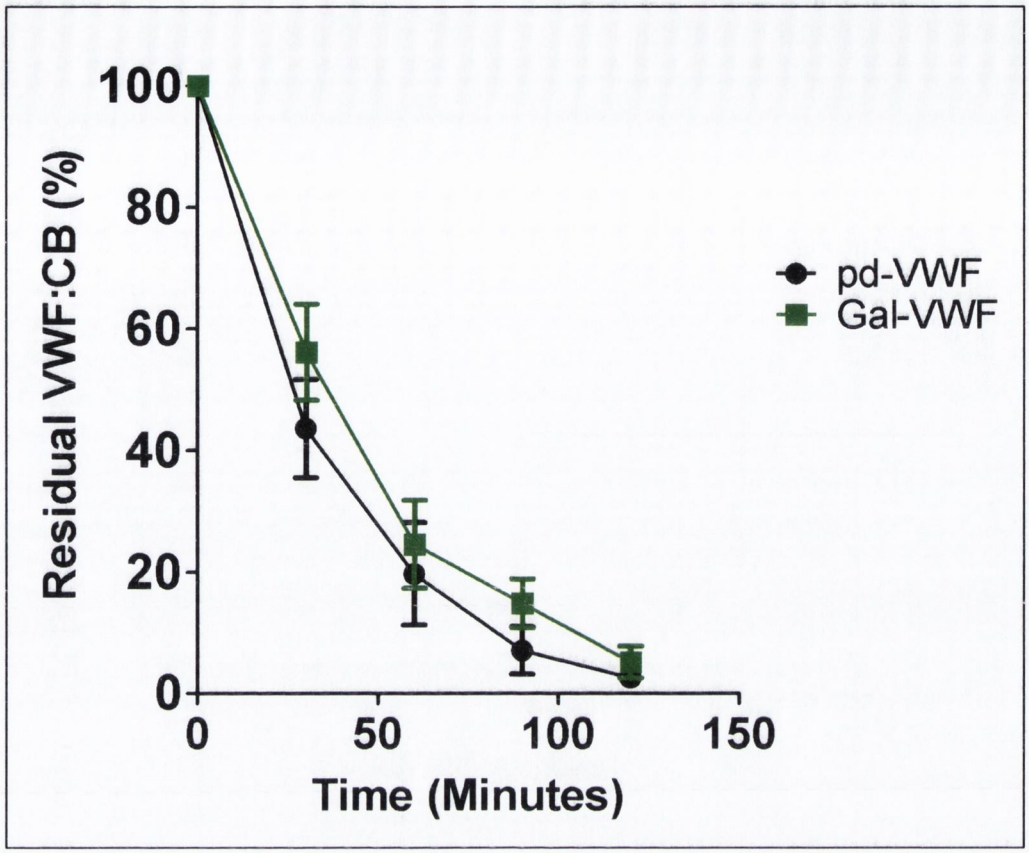


Figure 5-5B Removal of sub-terminal galactose carbohydrate structures from VWF has no effect on rate of ADAMTS13 proteolysis.

VWF was digested with β1,3-4 galactosidase and subjected to ADAMTS13 proteolysis as before. (pd-VWF, ●; Gal-VWF, ■). Removal of galactose residues has no effect on VWF susceptibility to ADAMTS13 cleavage, as pd-VWF and Gal-VWF were proteolysed at a similar rate.

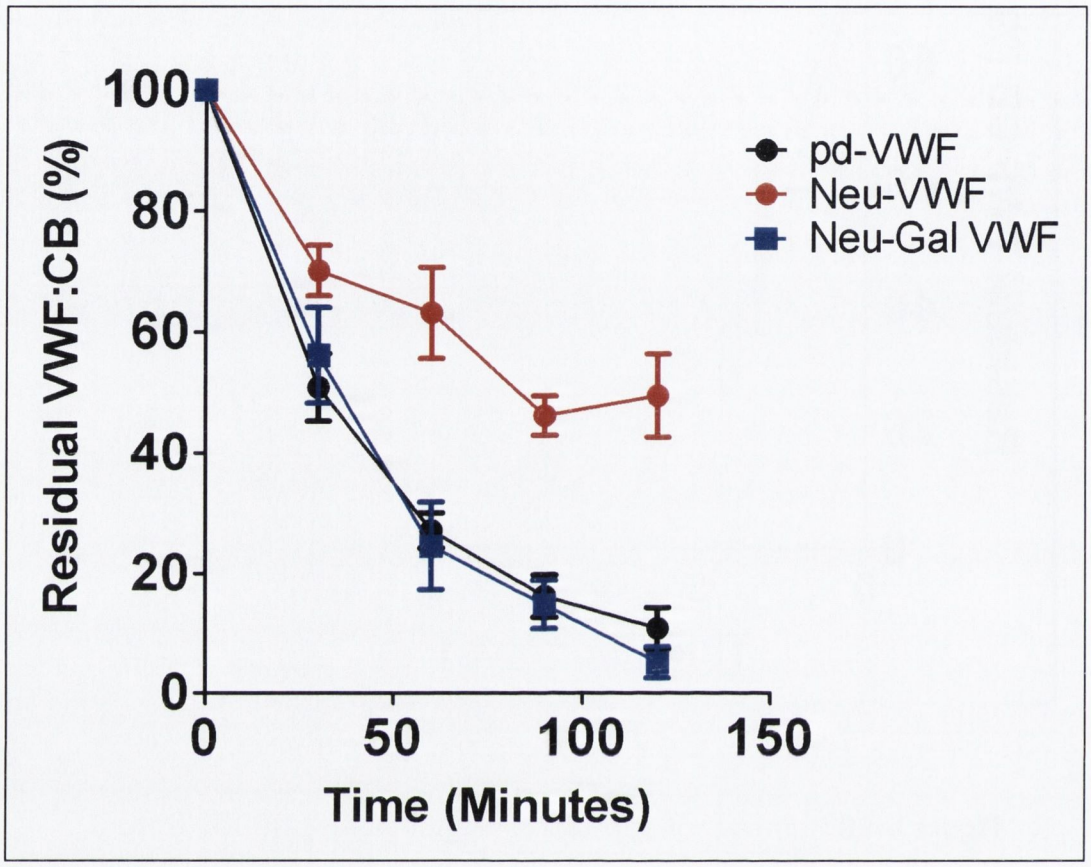


Figure 5-5C Loss of sialic acid alone mediates VWF resistance to ADAMTS13 cleavage.

Removal of capping α2-6 linked sialic acid from the N-linked glycans of VWF results in exposure of penultimate D-galactose residues. To determine whether the protective effect of α2-3,6,8,9 neuraminidase treatment was mediated through loss of sialic acid, or was attributable to the subsequent increased galactose exposure, we studied the effects of combined α2-3,6,8,9 neuraminidase and β-galactosidase digestion (pd-VWF, ●; Neu-VWF, ●; Neu-Gal-VWF, ■). Following combined glycosidase digestion, the protective effect of loss of α2-6 linked sialic acid only was no longer observed.

5.6 FVIII does not influence Neu-VWF resistance to ADAMTS13 proteolysis

Recent studies have shown that FVIII enhances the rate of VWF proteolysis by ADAMTS13 under shear stress (Cao, *et al* 2008). To study the effect of FVIII on Neu-VWF resistance to ADAMTS13, commercial FVIII-free plasma-derived human VWF was used (Haematologic Technologies Inc. VT, USA). FVIII-free VWF contained < 1% FVIII activity compared to the pd-VWF, as determined by a one stage FVIII:C assay.

Neu-VWF resistance to ADAMTS13 cleavage was evident in both the presence (≤20nM FVIII) or absence of FVIII:C (Figure 5-6). These results demonstrate that FVIII does not influence the rate of ADAMTS13 cleavage of Neu-VWF.

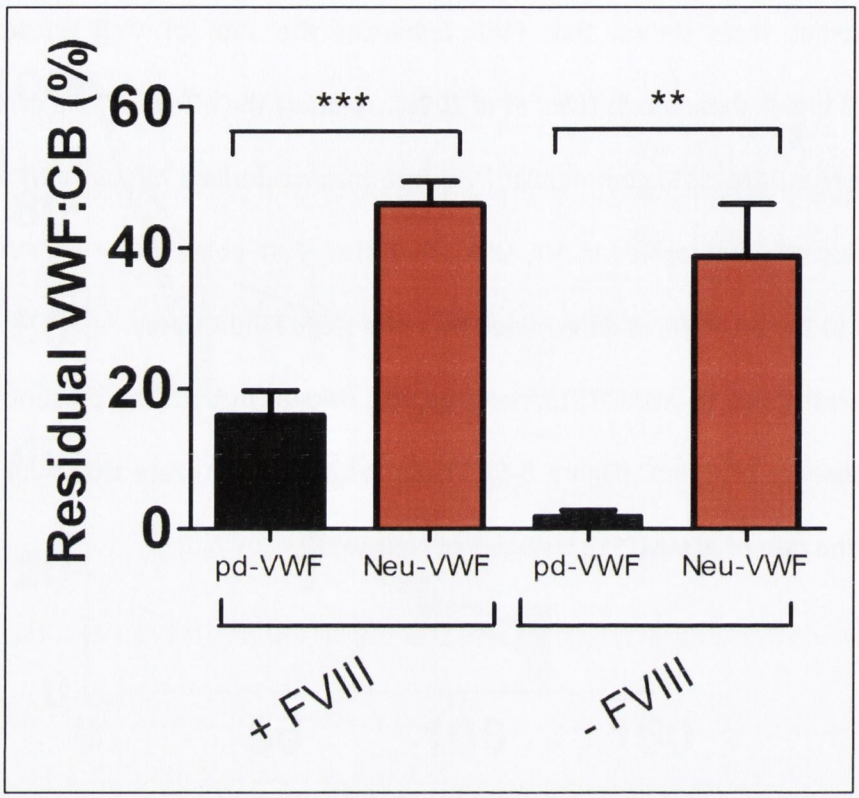


Figure 5-6 Absence of FVIII has no effect on sialic acid mediated VWF resistance to ADAMTS13 cleavage.

To determine whether the protective effect of α2-3,6,8,9 neuraminidase treatment was mediated through modification of sialic acid expression on FVIII, or was attributable to removal of sialic acid from VWF alone, pd-VWF and FVIII-free VWF were digested with α2-3,6,8,9 neuraminidase as before. Results are shown as residual collagen binding activity at 90 minutes ± SEM for each of the samples. (** $p < 0.01$, *** $p < 0.001$).

5.7 N-linked sialic acid expression regulates the effect of ABO blood group on VWF proteolysis by ADAMTS13

It has previously been shown that VWF isolated from plasma of different ABO blood groups exhibits differential susceptibility to cleavage by ADAMTS13 (O ≥ B > A ≥ AB) (Bowen 2003, O'Donnell, *et al* 2005). Although this effect was attributed to ABH determinant expression on VWF glycan structures, recent evidence suggests that the presence of ABH antigens can also modulate interactions involving α2-6 linked sialic acid expressed on adjacent glycans (Cohen, *et al* 2009). Since α2-6 linked sialic acid and ABO(H) determinants are both expressed as terminal antigens on the both the N- and O-linked glycans of VWF (Canis, *et al* 2010, Matsui, *et al* 1992), I further investigated the relationship between ABO(H) and sialic acid expression in modulating susceptibility to ADAMTS13 cleavage.

5.7.1 Blood group O VWF is more susceptible to ADAMTS13 proteolysis than blood group AB VWF

Blood group AB and blood group O VWF were isolated and purified from plasma using a combination of cryo-precipitation and gel filtration (Chapter 2, section 2.3). As determined by the reduction in percentage residual VWF:CB over time, blood group O VWF is cleaved at a faster rate and to a greater extent by ADAMTS13 than blood group AB VWF (Figure 5-7A).

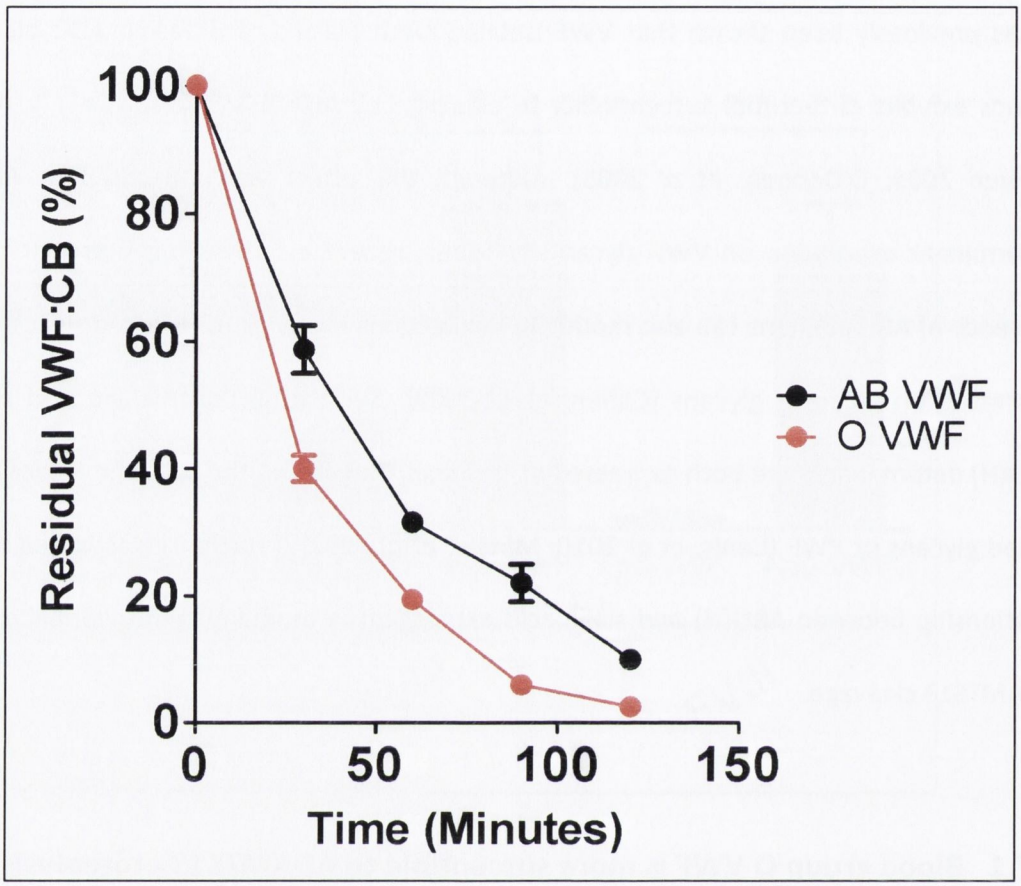


Figure 5-7A Blood group modulates VWF susceptibility to ADAMTS13.

Purified group O and AB VWF were subjected to ADAMTS13 proteolysis in the presence of 1.5M urea. As has previously been shown, blood group O specific VWF (●) is cleaved at a faster rate and to a greater extent by ADAMTS13 than blood group AB VWF (●) ($p < 0.05$). At some points error bars cannot be seen due to small size.

5.7.2 Removal of terminal A antigen from blood group A VWF has no effect on ADAMTS13 cleavage

To further define the role of ABH determinants in mediating the effect of ABO blood group on VWF proteolysis, purified group A VWF was digested with A-Zyme (N-acetyl-galactosaminidase treatment) and the rate of proteolysis by ADAMTS13 was studied as before. Although A-Zyme removed all detectable A antigen expression on VWF (Figure 5-7B), rate of cleavage by ADAMTS13 was not significantly different from untreated group A VWF (Figure 5-7C). Furthermore, A-Zyme-treated group A VWF was still cleaved significantly more slowly than group O VWF ($p < 0.05$). This suggests that blood group carbohydrate structures themselves do not modulate ADAMTS13 proteolysis of VWF.

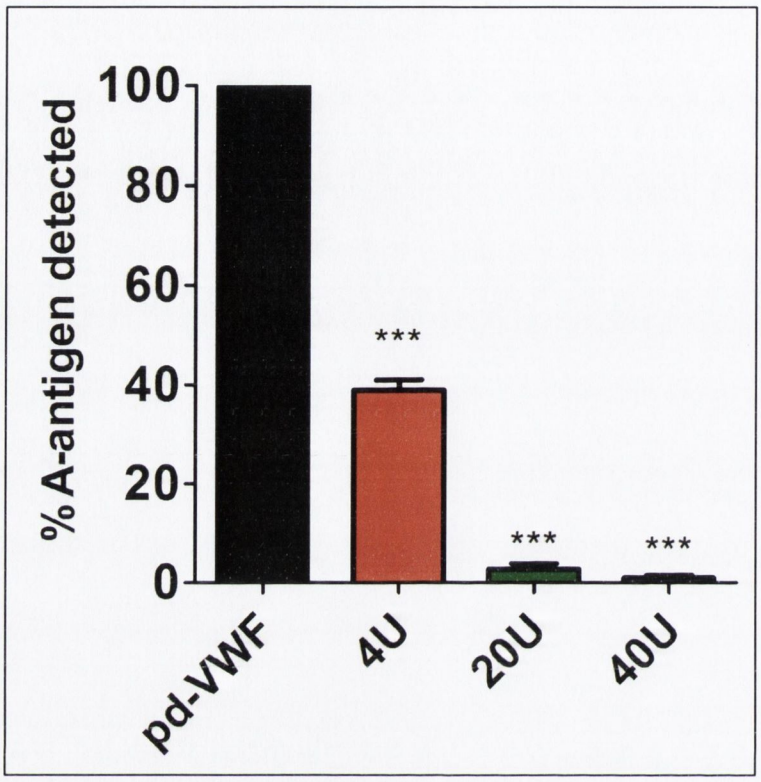


Figure 5-7B Dose-dependent reduction in levels of A antigen detected on VWF after digestion with A-Zyme.

Blood group A VWF (A-VWF) was digested with increasing units of A-Zyme (N-acetyl-galactosaminidase) and residual expression of A antigenic carbohydrate structures were quantified by modified A-Antigen ELISA as described in Chapter 2, section 2.7.2. All experiments were performed in triplicate, and the results shown represent the mean ± SEM (*** $p <$ 0.001).

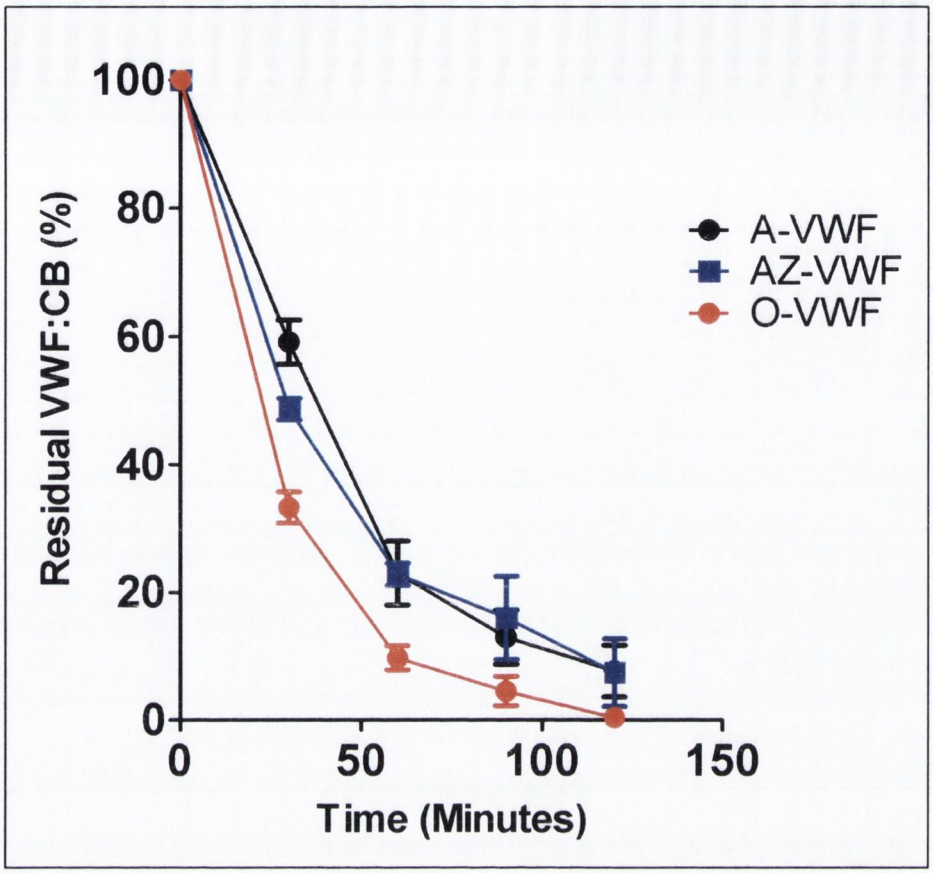


Figure 5-7C Loss of A antigen from blood group A VWF has no effect on susceptibility to ADAMTS13 proteolysis.

To determine whether loss of the A antigenic carbohydrate structure from blood group A VWF (A-VWF) affects susceptibility to ADAMTS13 proteolysis, A-VWF was digested with A-Zyme (N-acetyl-galactosaminidase) and ADAMTS13 cleavage assay was carried out as before. A-VWF (●) and A-Zyme treated VWF (AZ-VWF) (■) were cleaved at the same rate by ADAMTS13, and both were cleaved significantly more slowly than O-VWF (●) ($p < 0.05$).

5.8 Discussion

It is well established that sialic acid expression on other circulating glycoproteins influences susceptibility to proteolysis (Nishiyama, *et al* 2000). However, removal of sialic acid has always previously been reported to result in significantly *enhanced* proteolysis rates. In contrast to this accepted paradigm, I have demonstrated that α2-6 linked sialic acid on VWF exerts different effects on susceptibility to proteolysis dependent upon the protease involved. Thus, as shown in Figure 5-1, α2-6 linked sialic acid protects VWF against proteolysis by a variety of different serine and cysteine proteases, namely chymotrypsin and cathepsin B. In contrast to this, terminal sialic acid on VWF also serves to specifically *promote* cleavage by ADAMTS13 (Figure 4-8).

Previous studies have demonstrated that glycan structures can directly mediate protein-protein interactions through either conformational and/or charge-mediated mechanisms (Kimura, *et al* 1998, Mitra, *et al* 2006, Narhi, *et al* 1991, Wyss, *et al* 1995). Subtle variations in glycan structures directly influence glycoprotein conformation, by altering conformational freedom of the local peptide backbone (Gralnick, *et al* 1983). I found that the ability of sialic acid to enhance ADAMTS13 cleavage became less marked at progressively higher urea concentrations, suggesting that sialic acid may influence the conformation of VWF, and that removal of N-linked sialic acid results in the A2 domain adopting a conformation that is less permissive for ADAMTS13 cleavage. A putative role for α2-6 linked sialic acid in determining VWF conformation is supported by previous studies demonstrating that neuraminidase-treated VWF induced spontaneous platelet aggregation in the absence of ristocetin (Federici, *et al* 1988). Interestingly, Federici *et al* also reported that combined treatment of VWF with neuraminidase and galactosidase

(to remove terminal sialic acid and penultimate galactose residues) significantly attenuated the rate of spontaneous aggregation compared to that following VWF digestion with neuraminidase alone. These data are in keeping with the finding that treatment with galactosidase after α2-3,6,8,9 neuraminidase ablated the ADAMTS13 resistance observed upon neuraminidase digestion (Figure 5-6C). Cumulatively, these results support the hypothesis that the N-linked carbohydrate structures of VWF play a critical role in regulating the conformation of both the A1 and A2 domains of VWF. Further studies will be required to determine whether this effect is mediated by specific glycosylation site(s) within these domains.

ABO(H) blood group sugar determinants are expressed on the N-linked carbohydrate chains of human VWF and influence susceptibility to ADAMTS13 (Bowen 2003, O'Donnell, *et al* 2005). The significant effect of blood group on VWF proteolysis is surprising, given that ABO(H) determinants differ with respect to a single terminal sugar residue, and are expressed on only 13% of N-linked glycan chains, and on < 1% of O-glycans (Canis, *et al* 2010, Matsui, *et al* 1992). As shown in Chapter 4, Section 4.3, results arising from this study have demonstrated by HPLC analysis that VWF is heavily sialylated, and that the majority (80%) of this sialic acid is expressed on N-linked carbohydrate chains. Consequently, the ability of VWF glycans to modulate ADAMTS13 cleavage is not simply determined by the length of the N-linked glycan chains as previously proposed (McKinnon, *et al* 2008, O'Donnell, *et al* 2002). Rather, specific individual monosaccharides residues expressed at the terminal end of the N-linked glycans of VWF directly influence susceptibility to ADAMTS13 proteolysis. Intriguingly, I also observed that even following removal of A antigenic determinants, blood group A

VWF remained significantly more resistant to ADAMTS13 proteolysis than group O VWF. These findings are interesting given that previous studies reported variation in quantitative sialic acid expression on erythrocyte membrane glycoproteins according to ABO blood group (Federici, *et al* 1988, Petrescu, *et al* 2004). Furthermore, an elegant study by Cohen et al has clearly demonstrated that ABO blood group can also modulate qualitative sialic acid presentation on erythrocyte surfaces (Cohen, *et al* 2009). Further studies will be required in order to define whether quantitative and/or qualitative variations in sialic acid expression on VWF are important in mediating the effects of ABO blood group on VWF proteolysis and/or clearance.

Quantitative sialic acid and ABO(H) expression on the N-linked glycans of plasma VWF varies widely in the normal population (Ellies, *et al* 2002, O'Donnell, *et al* 2002). In addition, sialyltransferase expression levels vary between different tissues, and in different pathological states (Byrne, *et al* 2007, Lopes, *et al* 2000). The physiological and pathological significance of quantitative variation in VWF sialic acid expression remain unclear. Unsurprisingly however, loss of sialic acid significantly reduced the half-life of VWF in animal studies, presumably due to enhanced clearance via the asialoglycoprotein receptor (Sodetz, *et al* 1977). Moreover, a murine knockout of a specific sialyltransferase (ST3GalIV) also resulted in VWF deficiency due to increased clearance (Ellies, *et al* 2002). Given its dual effects in regulating VWF proteolysis (both by ADAMTS13 and other plasma proteases) and VWF clearance, it is also significant that reduced VWF sialic acid expression has been observed in a cohort of patients with type I VWD (Ellies, *et al* 2002, Gralnick, *et al* 1976). Furthermore, van Schooten *et al* recently reported an inverse correlation between O-linked sialylated T antigen expression and plasma VWF levels

(van Schooten, *et al* 2007). It is well established that up to 40% of families with Type I VWD fail to demonstrate linkage to the VWF gene locus on chromosome 12 (Eikenboom, *et al* 2006). Further clinical studies will be required to determine the potential role of mutations and/or polymorphisms influencing quantitative α2-6 linked sialic acid expression in this group of patients.

On the basis of the data shown in this chapter, and also in chapter 4, we propose that VWF synthesized within endothelial cells is sialylated in the Golgi before constitutive or regulated secretion. This sialic acid expression specifically enhances ADAMTS13 cleavage at the endothelial surface after initial secretion, while also protecting VWF against nonspecific proteolysis by other plasma proteases. The sialic acid is progressively lost with VWF aging in the plasma, precipitating eventual clearance by the asialoglycoprotein receptor.

CHAPTER 6:

PLATELET-DERIVED VWF EXHIBITS SPECIFIC RESISTANCE TO ADAMTS13 PROTEOLYSIS THROUGH A GLYCAN-DEPENDENT MECHANISM

6.1 Introduction

Synthesis of VWF occurs exclusively in endothelial cells and megakaryocytes (platelet precursors); see Chapter 1, Section 1.6. Platelet-derived VWF is stored in platelet α-granules and ultra large platelet-VWF multimers are secreted at sites of vascular injury in high concentrations, following platelet activation in vivo (Fernandez, *et al* 1982, Koutts, *et al* 1978). Platelet-VWF constitutes ~20% of the circulating levels of VWF, and has been found to be important in the maintenance of primary haemostasis in several animal models (Bowie, *et al* 1986, Nichols, *et al* 1993, Nichols, *et al* 1990).

Previous studies have suggested that post-translational modification of VWF synthesised within megakaryocytes varies significantly to that of VWF produced in endothelial cells. Specifically, the glycosylation of platelet-derived VWF has been shown to differ greatly from endothelial-derived plasma-VWF. Preliminary analysis of platelet-VWF using SDS-PAGE demonstrated a slight difference in molecular weight in comparison to plasma-VWF; this variation was attributed to differences in carbohydrate processing (Sporn, *et al* 1985). Moreover, both the sialic acid and galactose content of platelet-VWF are

reduced ~50% compared to plasma-VWF, and plasma-VWF N-linked glycans are composed of more complex and heterogeneous oligosaccharide chains (Kagami, *et al* 2000, Williams, *et al* 1994). Furthermore, platelet-VWF glycans do not express blood group antigenic carbohydrate structures (Brown, *et al* 2002, Matsui, *et al* 1999).

In light of the role of that VWF oligosaccharide structures play in modulation of ADAMTS13 cleavage, and the differences between plasma- and platelet-VWF glycosylation, I studied the susceptibility of platelet-VWF to proteolysis by ADAMTS13 as before (Chapters 4 and 5). Platelet-derived VWF was isolated from lysed platelets (see Chapter 3, section 3.3) and the role of platelet-VWF glycosylation in mediating susceptibility to ADAMTS13 cleavage was investigated. Glycosidase treatment of platelet-VWF to modify the endogenous carbohydrate structures was carried out, and the subsequent effect on the rate ADAMTS13 proteolysis evaluated. Finally, sialic acid expression levels on platelet-VWF were definitively quantified by HPLC analysis.

6.2 Platelet-VWF preparations

6.2.1 Preliminary analysis of VWF in platelet lysate and releasate

As discussed in Chapter 3, section 3.3, several approaches were taken to lyse platelets, with the aim of isolating a high antigen level of platelet-derived VWF (VWF:Ag), while maintaining elevated functional activity (collagen binding). The optimal conditions for platelet lysis were found to be snap freeze-thawing (rapid movement from -200°C in liquid nitrogen to 37°C in H_2O), in the presence of a protease inhibitor cocktail, followed by dialysis of platelet proteins into a physiological buffer (50mM Tris-HCL, pH 7.4). Platelet releasate containing secreted proteins after platelet activation was also used as a source of VWF. Platelet releasate was obtained as detailed in Chapter 2, section 2.2.4.

The protein profiles of platelet lysate and platelet releasate were assessed using SDS-PAGE followed by coomassie® blue staining (Figure 6-1, Image A). The gel shows that VWF present in both platelet lysate and releasate is impure, with many contaminating protein bands observed. Interestingly, different expression levels of platelet proteins are present in lysate versus releasate. Platelet releasate expresses fewer proteins overall, but some are present at a higher concentration in comparison to platelet lysate (Figure 6-1, Image A). Both lysate and releasate contained significant amounts of VWF as determined by VWF:Ag ELISA, however platelet-releasate derived VWF could not be used for further assays, as the VWF:CB/VWF:Ag value was < 0.2 (data not shown). This highlights the absence of functionally active high or intermediate molecular weight multimers of VWF, suggesting that platelet lysate is a better source of VWF for ensuing purification and additional experiments.

6.2.2 Purification of VWF by immunoaffinity chromatography

To further study platelet-VWF resistance to ADAMTS13 proteolysis, purification of VWF from platelet lysate was carried out in collaboration with the Department of Plasma Proteins, Sanquin Research, as detailed in Chapter 2, section 2.2.5. Plasma-derived VWF was purified in parallel to serve as a control. Following immunoaffinity chromatography, purity of VWF was assessed by SDS-PAGE and silver staining (Figure 6-1, Image B). Double bands representing both plasma- and platelet-VWF at ~250kDa are visible. Other protein bands are not detected, which indicates the samples are of very high purity.

6.2.3 Functional assessment of purified VWF

To determine if VWF obtained from the purification process was suitable for use in further functional assays, multimer gel analysis was carried out. The multimeric structure of VWF was assessed by agarose gel electrophoresis in 1.8% gels under non-reducing conditions. Similar multimeric profiles were observed for plasma- versus platelet-VWF (Figure 6-2). The triplet structure, characteristic of VWF that has been cleaved by ADAMTS13 (Schneppenheim, *et al* 2003), is present for plasma-derived VWF, but not platelet-VWF. This is expected, as PL-VWF is exposed to ADAMTS13 proteolysis in plasma, whereas PT-VWF is not. ADAMTS13 stored in platelets may be released into platelet lysate, however addition of protease inhibitors prevents cleavage of VWF.

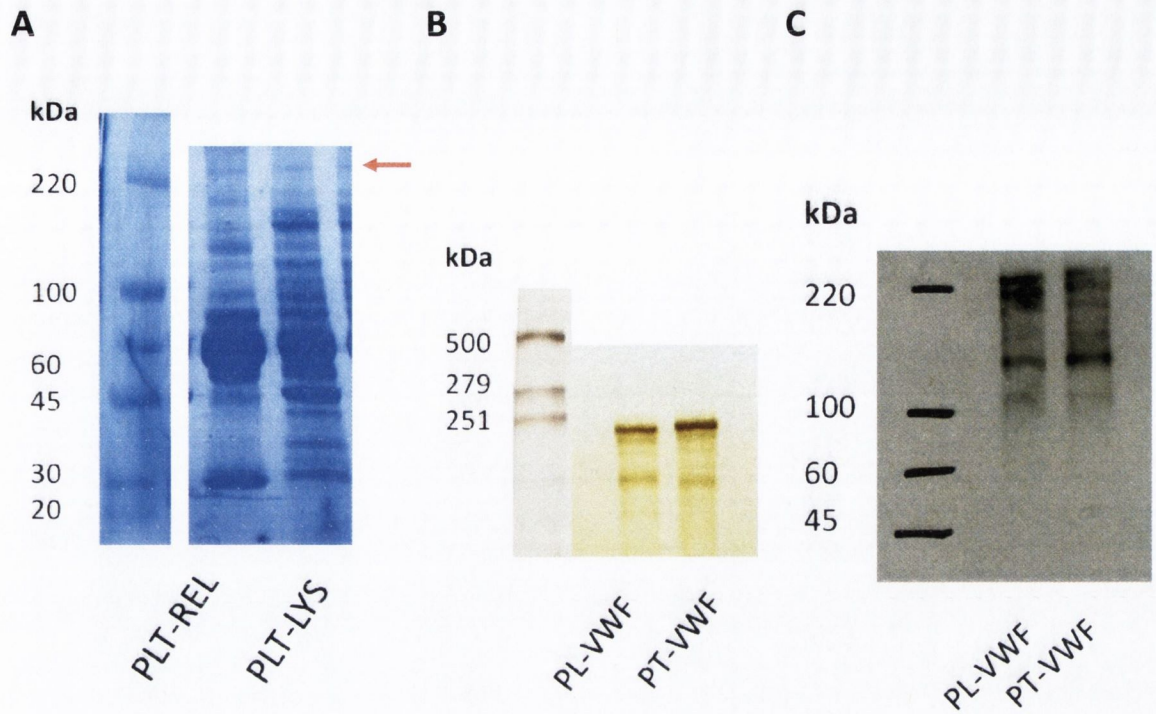


Figure 6-1 Analysis of platelet-VWF by SDS-PAGE.

Image A: The protein profiles of platelet lysate (PLT-LYS) and platelet releasate (PLT-REL) were studied by SDS-PAGE and coomassie® blue staining. Both platelet lysate and releasate contain many contaminating bands, indicating impurity of the samples. VWF is visible in both lanes at ~250kDa (shown by red arrow).

Image B: Purity of plasma- and platelet-VWF after immunoaffinity chromatography was assessed by SDS-PAGE and silver staining. Double bands present at ~250kDa highlight the presence of VWF.

Image C: Other bands present on the silver stain in Image B are degradation products of VWF, as demonstrated by western blotting.

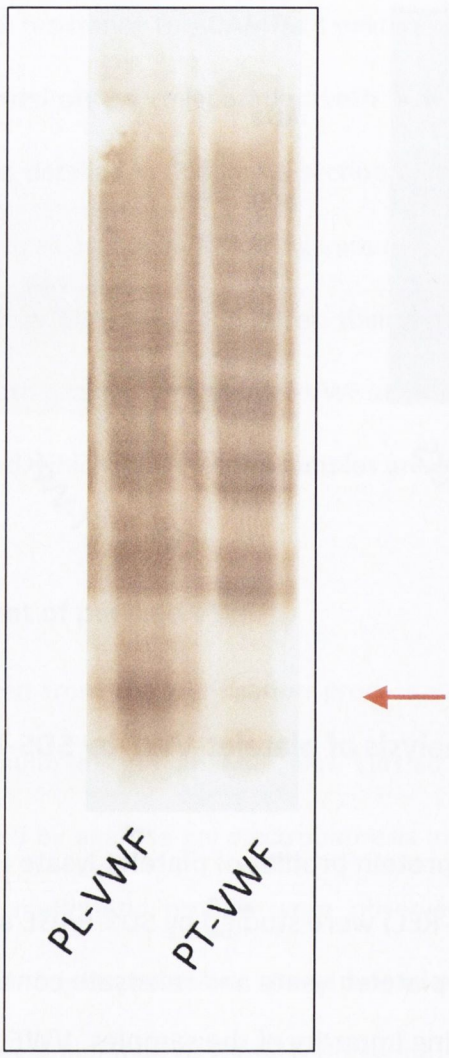


Figure 6-2 Multimer analysis of plasma- and platelet-VWF.

The multimeric structure of VWF was assessed by agarose gel electrophoresis in 1.8% gels under non-reducing conditions. Both plasma- and platelet-VWF show a similar multimeric profile, indicating that these samples are suitable for use in further comparative functional assays. The characteristic triplet structure observed after VWF proteolysis by ADAMTS13 is seen for PL-VWF but not for PT-VWF (indicated by red arrow), as platelet-VWF has not been exposed to ADAMTS13.

6.3 Platelet- and plasma-derived VWF bind to collagen III with similar affinity

In preliminary experiments, endothelial-derived plasma VWF (PL-VWF) and platelet lysate-derived VWF (PT-VWF) collagen binding activity was assessed. Soluble recombinant human collagen type III was coated onto a 96-well plate at a set concentration of 5μg/ml, subsequently a range of concentrations of VWF were added (1-22.5nM). Both PL-VWF and PT-VWF bound to collagen III with a similar affinity, with K_d apparent values of 2.8 ± 0.6nM and 3.8 ± 1.2nM observed respectively (Figure 6-3). This is in keeping with previous reports (Williams, *et al* 1994) and highlights the suitability of the collagen binding assay for the assessment of VWF proteolysis in vitro.

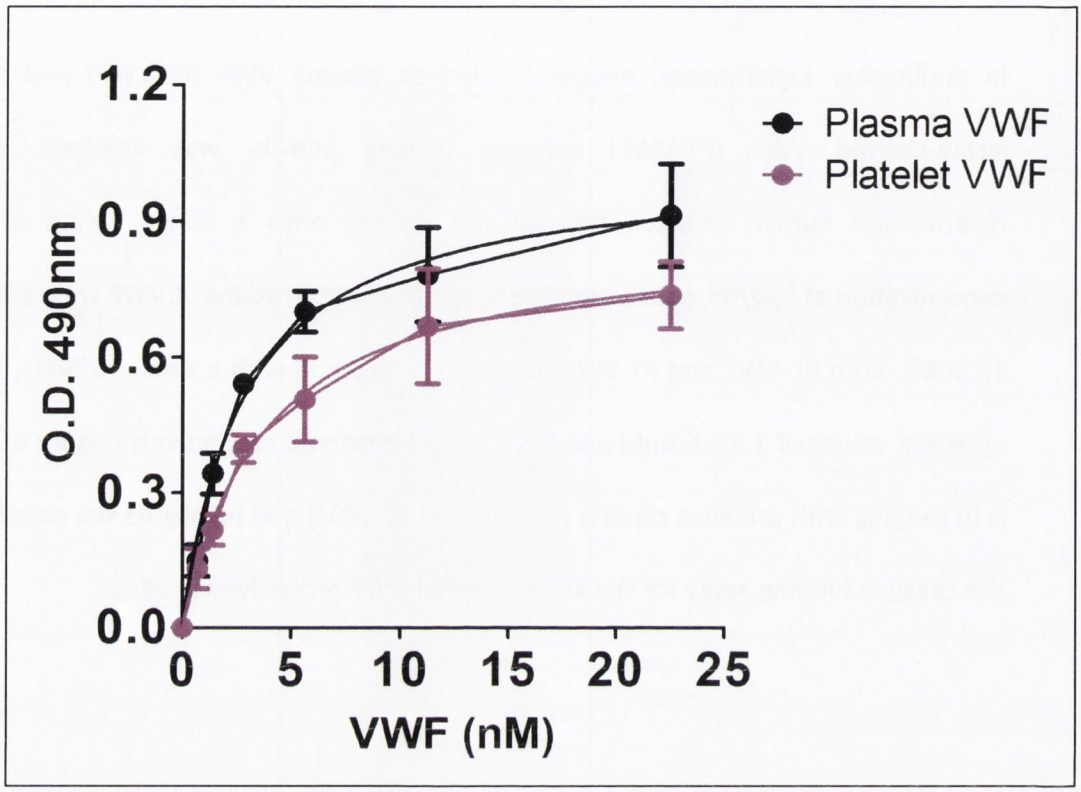


Figure 6-3 Plasma- and platelet-derived VWF binding to human collagen III.

No difference in binding kinetics was observed for PL-VWF (●) vs. PT-VWF (●) binding to collagen III, with apparent K_d values of 2.8 ± 0.6nM and 3.8 ± 1.2nM observed respectively. Experiments were performed in triplicate and mean ± SEM is shown.

6.4 Platelet-derived VWF in platelet lysate is resistant to ADAMTS13 cleavage

Regulation of VWF multimer composition and functional activity in normal plasma occurs through the action of ADAMTS13, which specifically cleaves VWF at the Tyr1605-Met1606 bond within the VWF A2 domain (Zheng, *et al* 2001). However, the role that ADAMTS13 proteolysis plays in the modulation of platelet-VWF functional activity is currently unknown. Platelet-derived VWF susceptibility to ADAMTS13 proteolysis, in comparison to plasma-derived VWF, was evaluated. The rate and extent of ADAMTS13 cleavage was determined by the reduction in residual VWF collagen binding activity (VWF:CB) over time.

Incubation of PL-VWF with 1.5nM ADAMTS13 resulted in a drop of VWF:CB to 8 ± 1% after 120 minutes (Figure 6-4A). Interestingly, PT-VWF exhibited a significant resistance to ADAMTS13 cleavage in comparison to PL-VWF, such that residual VWF:CB fell to only 31 ± 1% after 120 minutes (Figure 6-4A, $p < 0.01$). A more marked impairment of cleavage was apparent at 0.75nM ADAMTS13, where VWF:CB for PT-VWF fell to 57 ± 1% compared to 18 ± 3% for PL-VWF, after 120 minutes (Figure 6-4B, $p < 0.001$).

This result is consistent with our previous findings that subtle differences in the glycosylation profile of VWF can modulate its functional properties, including susceptibility to ADAMTS13 proteolysis. Interestingly, variations in platelet- versus plasma-VWF function (GPIIb/IIIa, GPIbα and heparin binding) have also previously been reported. These differences have also been attributed in part to differences in VWF glycosylation (Williams, *et al* 1994).

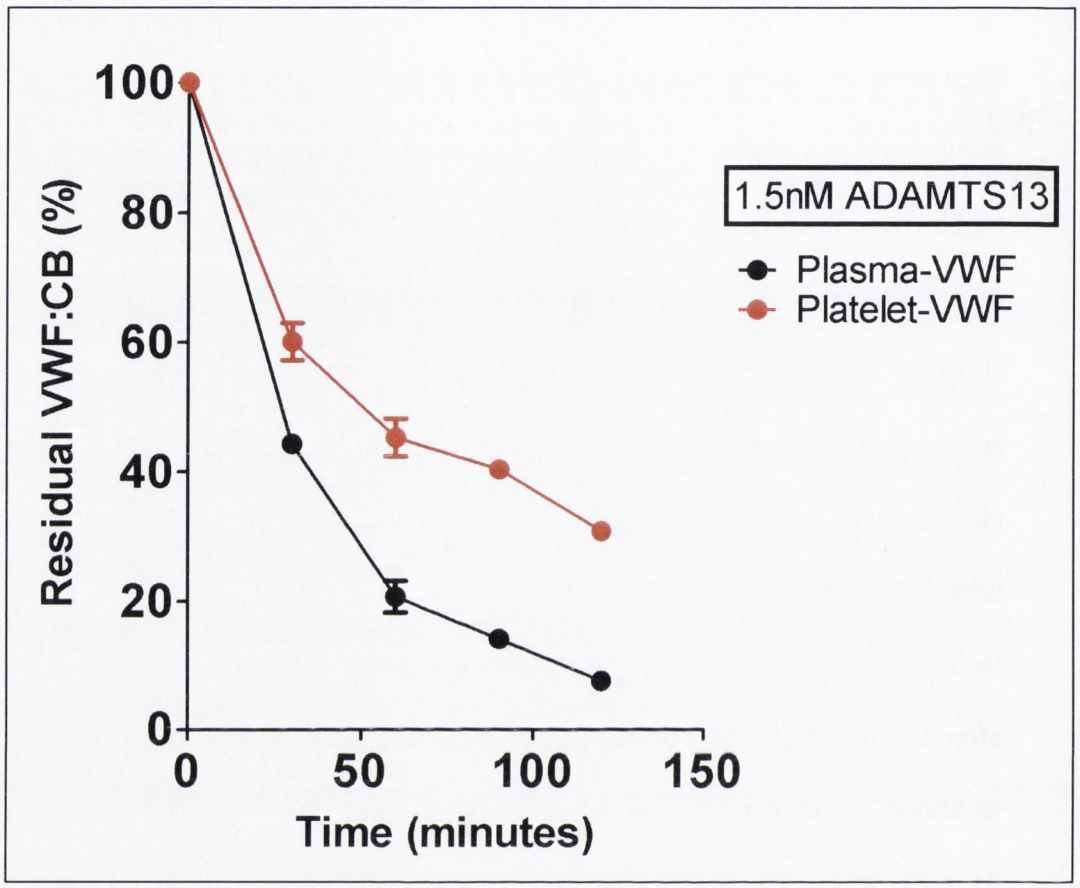


Figure 6-4A Platelet-VWF is resistant to ADAMTS13 proteolysis.

To investigate platelet-VWF susceptibility to ADAMTS13 proteolysis, both plasma- and platelet-VWF were incubated with 1.5nM ADAMTS13 in the presence of 1.5M urea. Rate of VWF cleavage was assessed by determining the percentage decrease in residual VWF:CB over time. Results are expressed as mean ± SEM, and experiments were performed in triplicate. At certain time points, error bars cannot be seen due to their small size. Platelet-VWF (●) was cleaved at a slower rate and to a lesser extent than plasma-VWF (●; ** $p < 0.01$).

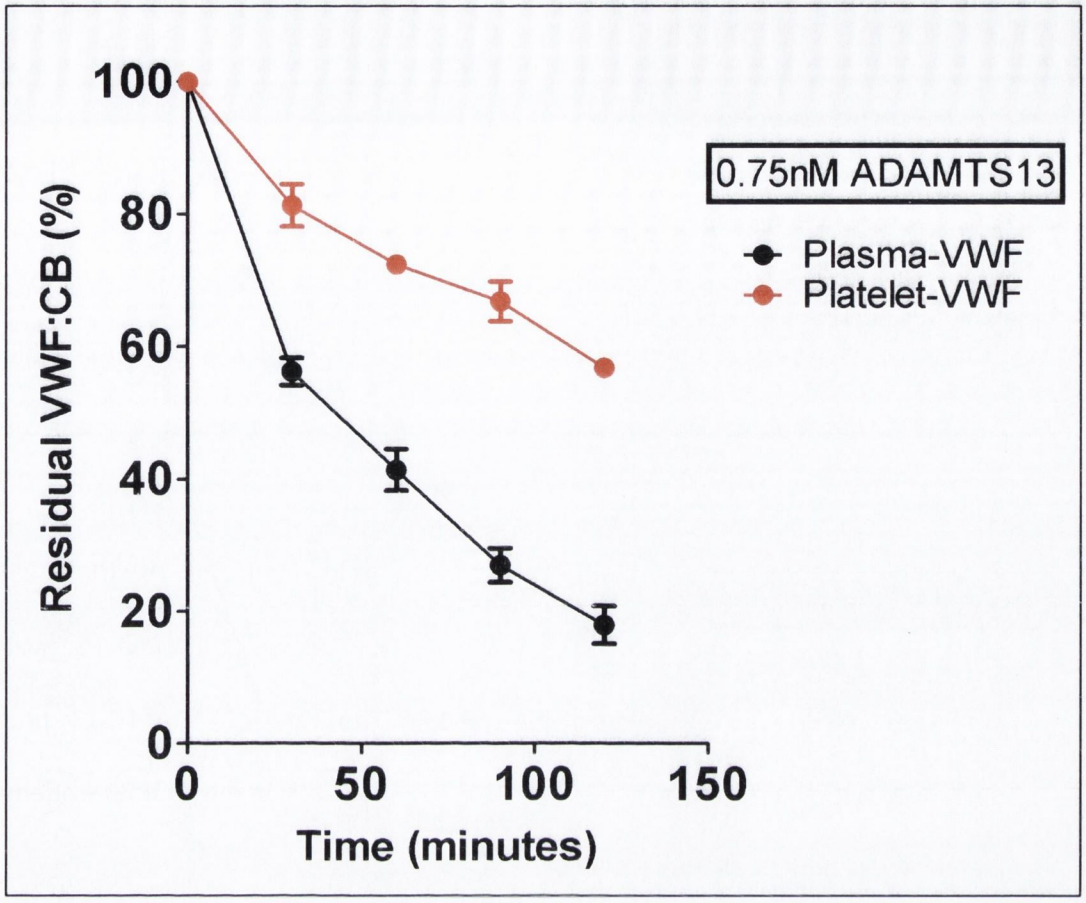


Figure 6-4B Platelet-VWF is resistant to ADAMTS13 proteolysis.

To further investigate platelet-derived VWF susceptibility to ADAMTS13 proteolysis, both plasma- and platelet-VWF were incubated with 0.75nM ADAMTS13 in the presence of 1.5M urea. Residual VWF:CB for platelet-VWF (●) fell to 57 ± 1% compared to 18 ± 3% for plasma-VWF (●), after 120 minutes (*** $p < 0.001$). Error bars cannot be seen at certain time points, due to their small size.

6.5 Inhibitor of ADAMTS13 activity was not detected in platelet lysate

As VWF in platelet lysate is impure, we hypothesized that there may be a factor present that inhibits ADAMTS13 enzymatic. To investigate whether a potential inhibitor of ADAMTS13 proteolytic activity was present in the PT-VWF preparation, classical mixing studies were carried out.

PL-VWF and PT-VWF dilutions were mixed in the following ratios: 0%:100%, 25%:75%, 50%:50%, 75%:25% and 100%:0%, respectively. Following immediate mixing, no inhibitory effect was observed (Figure 6-5A). The rate of ADAMTS13 proteolysis decreased with increasing amounts of PT-VWF in the reaction mix, but no evidence of an inhibitor was detected (Figure 6-5A).

To further investigate if an inhibitor of ADAMTS13 was present in the platelet lysate, rADAMTS13 (20nM) was incubated with a 1:1 (v/v) mix of platelet lysate and ADAMTS13 activity was assessed using the FRETS-VWF73 assay (as described in Chapter 2, section 2.8.3). Incubation of ADAMTS13 with platelet lysate had no effect on enzymatic activity (Figure 6-5B).

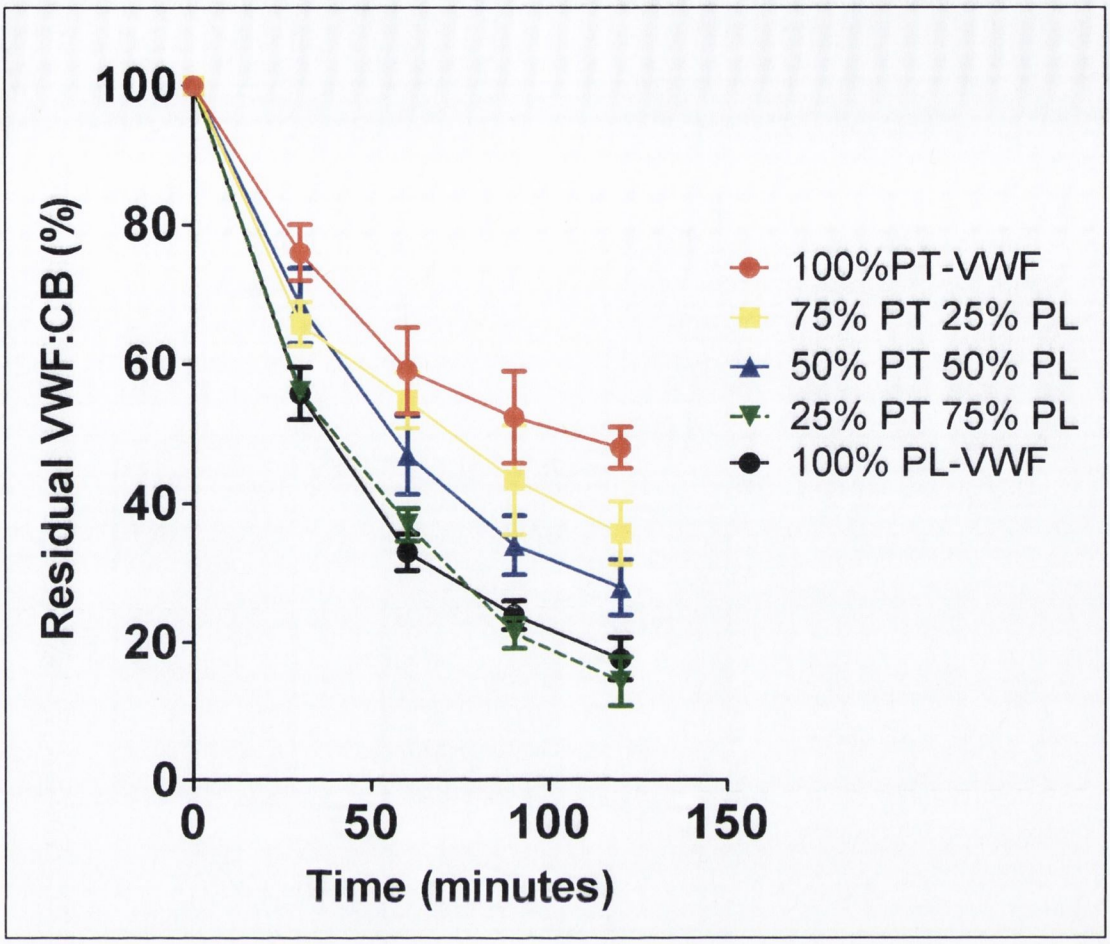


Figure 6-5A Platelet lysate does not contain an inhibitor of ADAMTS13 proteolytic activity.

Mixing studies of PL- and PT-VWF were carried out to ascertain whether an inhibitor of ADAMTS13 is present in platelet lysate. PL- and PT-VWF were mixed in the following ratios: 100%:0% (●); 25%:75% (▼); 50%:50% (▲); 75%:25% (■); and 0%:100% (●), respectively.

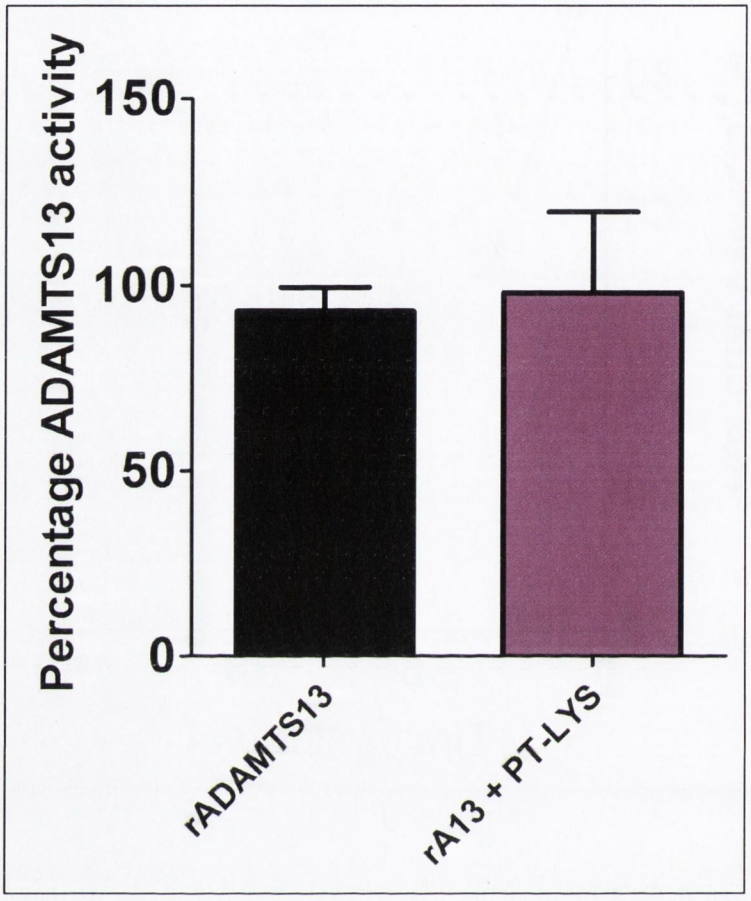


Figure 6-5B Platelet lysate does not inhibit ADAMTS13 activity.

Prior to assessment of enzymatic activity using the FRETS-VWF73 assay, rADAMTS13 was incubated with platelet lysate for 30 min @ 37°C. Incubation with platelet lysate had no effect on ADAMTS13 activity.

6.6 Immunoaffinity purified PT-VWF is resistant to ADAMTS13 cleavage

To further investigate the resistance of VWF in platelet-lysate to ADAMTS13, purified PT-VWF was also subjected to ADAMTS13 proteolysis. Immunoaffinity purified PL-VWF was used in parallel as a control.

Optimisation of the most suitable parameters to be used in the static cleavage assay showed that 3nM ADAMTS13 digested 10μg/ml VWF at a urea concentration of 1.5M. A steady fall off in residual VWF:CB was observed when these conditions were used, and the protein concentrations employed are within the physiological range. Residual VWF:CB for PL-VWF fell to 28 ± 3% after incubation with ADAMTS13 for 2 hours (Figure 6-6). Again, a significant impairment of cleavage was observed for PT-VWF, as VWF:CB remained at 59 ± 8% after 120 min (Figure 6-6; $p < 0.05$).

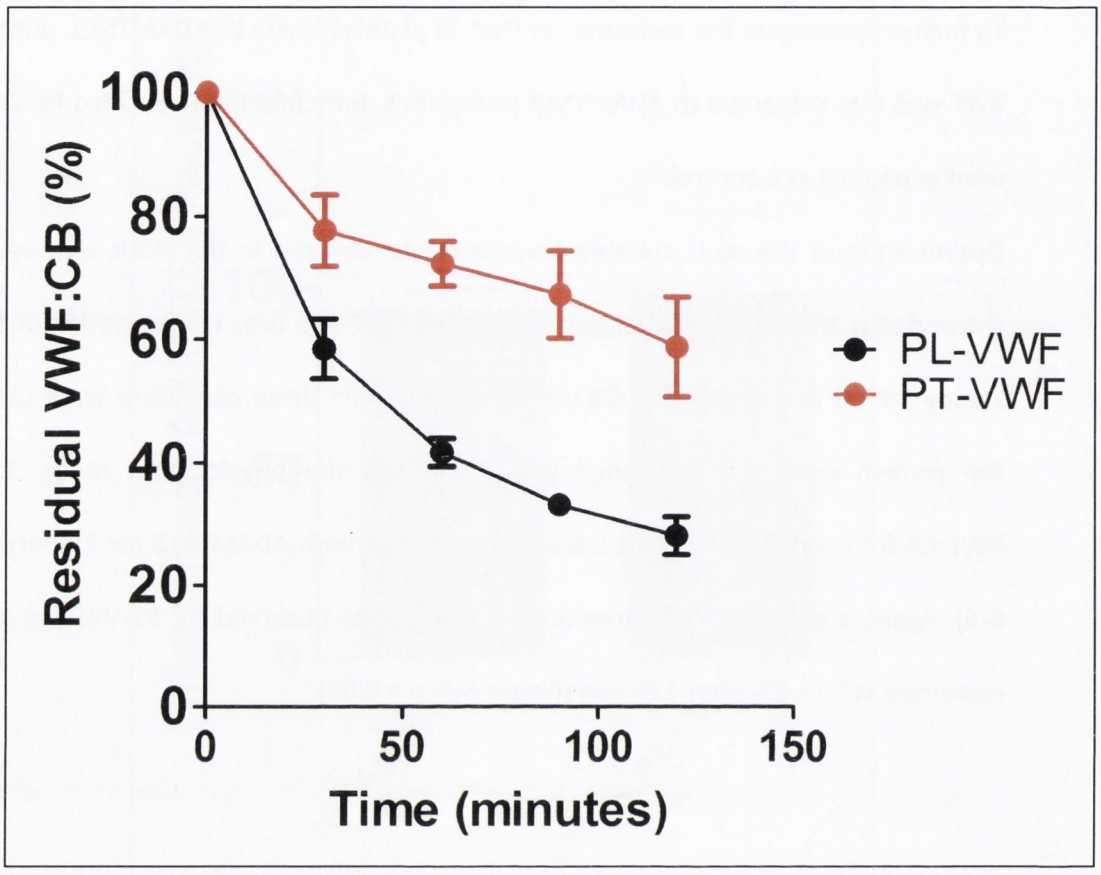


Figure 6-6 Immunoaffinity purified platelet-derived VWF is resistant to ADAMTS13 proteolysis.

After purification by immunoaffinity chromatography, PL- and PT-VWF were subjected to ADAMTS13 proteolysis in a static assay as before. PT-VWF (●) exhibits significant resistance to ADAMTS13 and is cleaved at a significantly slower rate in comparison to PL-VWF (●; * $p < 0.05$). Error bars are not visible at certain time points, due to their small size.

6.7 Platelet-VWF is specifically resistant to proteolysis by ADAMTS13

To further investigate the properties of platelet-VWF, general proteolysis by other serine proteases chymotrypsin and carboxypeptidase Y was studied. No significant difference between the rate and extent of PL-VWF versus PT-VWF cleavage was observed (Figure 6-7; Chymotrypsin: PL-VWF:CB 6 ± 3% vs. PT-VWF:CB 2 ± 1%, p = ns; Carboxypeptidase Y: PL-VWF:CB 62 ± 3% vs. PT-VWF:CB 63 ± 6%, p = ns), indicating PT-VWF resistance to proteolysis is ADAMTS13 specific. In contrast to this, a significant impairment of proteolysis by ADAMTS13 is again apparent at 90 minutes (Figure 6-7, 31 ± 10% vs. 67 ± 5% for PL- and PT-VWF respectively, $p < 0.01$).

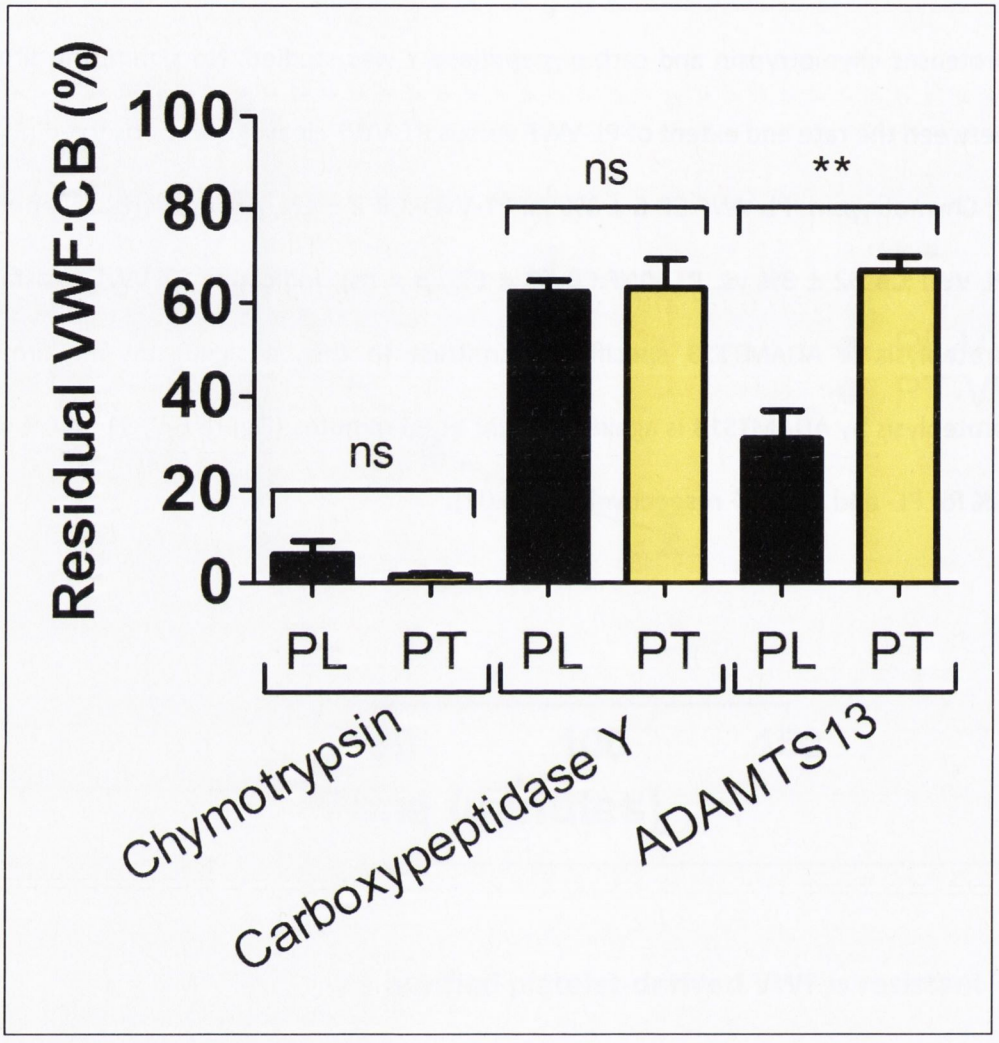


Figure 6-7 Platelet-derived VWF is specifically resistant to ADAMTS13 cleavage.

To establish whether impairment of PT-VWF proteolysis is ADAMTS13 specific, both PL- and PT-VWF were subjected to cleavage by chymotrypsin (60U/mg VWF), carboxypeptidase Y (19U/mg VWF) and ADAMTS13 (500nM/mg VWF) at 37°C for 90 minutes. Results are shown as residual collagen binding activity at 90 minutes ± SEM for each of the samples. (ns, p value is non-significant; ** $p < 0.01$).

6.8 Characterisation of plasma- and platelet-VWF binding to ADAMTS13

Modification of VWF conformation via truncation studies has shown that conformational changes in VWF can dramatically affect interactions with ADAMTS13 (Feys, *et al* 2009, Zanardelli, *et al* 2009). Moreover, alteration of the endogenous VWF glycosylation pattern affects the VWF-ADAMTS13 interaction, as demonstrated by binding experiments in previous studies (McKinnon, *et al* 2008).

Using a plate binding assay (Chapter 2, section 2.11) a two-fold difference in binding kinetics was observed for plasma- and platelet-VWF as half-maximal binding values of 3.7 ± 0.4nM and 7.5 ± 0.6nM were obtained respectively (Figure 6-8).

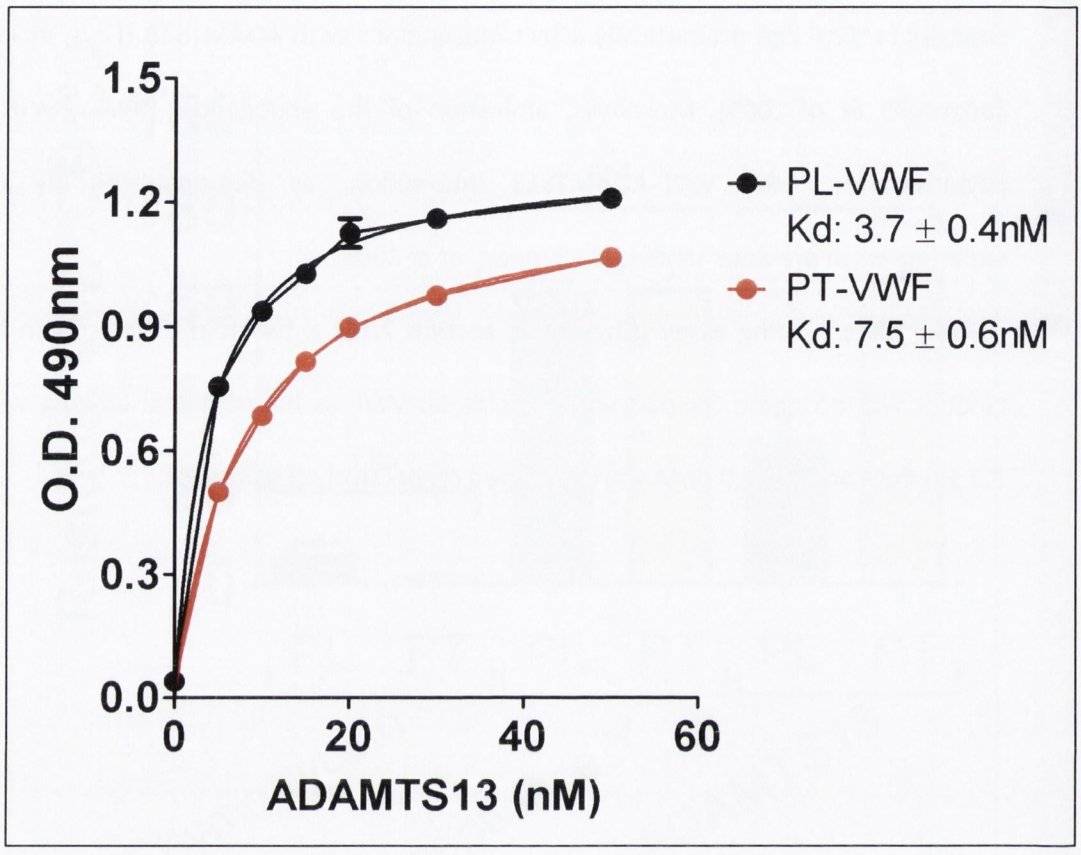


Figure 6-8 Platelet-VWF binds to ADAMTS13 with less affinity than plasma-VWF

Plasma- and platelet-VWF binding to ADAMTS13 was assessed using a plate binding assay. Plasma-VWF (●) bound to ADAMTS13 with two-fold higher affinity than platelet-VWF (●), as determined by K_d apparent values obtained by one-site specific binding analysis (GraphPad Prism, Version 5.0). Results are expressed as mean ± SEM and experiments were performed in triplicate. At certain timepoints, error bars are not visible due to their small size.

6.9 Blood group antigen H is expressed on platelet-derived VWF

Endothelial-derived plasma-VWF (PL-VWF) expresses ABO (H) antigens on terminal ends of both N- and O-glycan chains. Previous studies have demonstrated that platelet-derived VWF does not express blood group A and B antigenic structures (Brown, *et al* 2002, Matsui, *et al* 1999). However, the presence or absence of the H antigen structure (α-L-fucose linked to underlying D-galactose) has not been addressed.

I further investigated platelet-VWF glycosylation by determining whether the H antigenic structure is expressed using a modified lectin ELISA utilising *Ulex europaeus* lectin.

This lectin ELISA shows that platelet-VWF does express the H antigenic carbohydrate structure. Furthermore, it is present in relatively the same amount as on plasma VWF (Figure 6-9). This is an interesting finding, as platelet-VWF contains ~50% less glycans than plasma-VWF, and suggests a higher level of H antigen expression per oligosaccharide unit on platelet-VWF. Alternatively, more H antigen may be detectable on platelet-derived VWF as capping blood group AB antigenic structures are not present, which could block underlying H antigen detection on plasma-VWF.

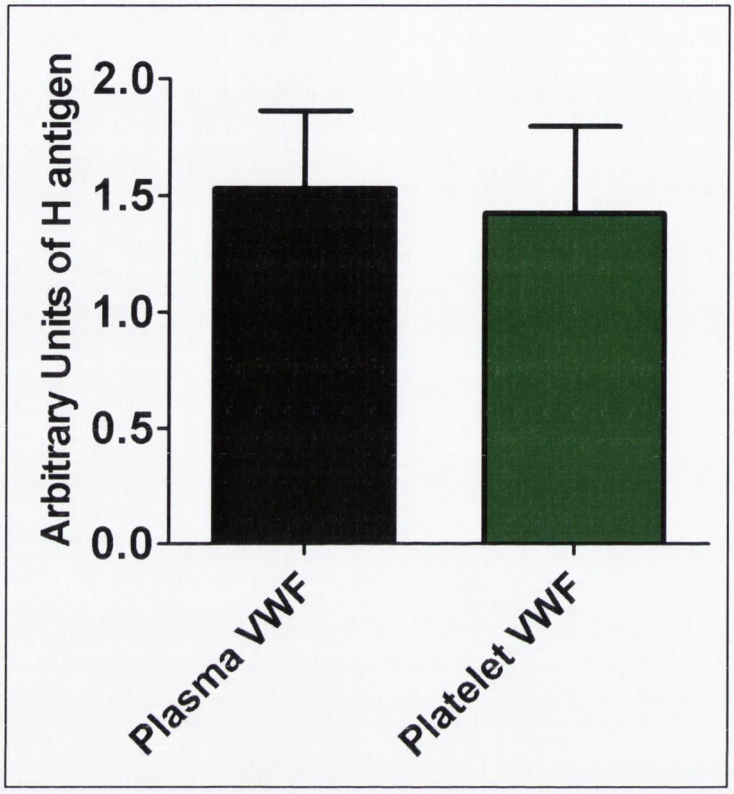


Figure 6- 9 Blood group H antigen is expressed on platelet VWF glycans.

The H antigen blood group carbohydrate structure was detected on both on plasma- and platelet-VWF by *Ulex* lectin ELISA. Arbitrary units of H antigen indicate levels of H antigen detected per μg of VWF. All experiments were performed in triplicate, and results described represent the mean ± SEM.

6.10 N- and O-linked glycosylation of platelet-VWF modulates proteolysis by ADAMTS13

Platelet-derived VWF exhibits significant differences in glycan composition compared to plasma-derived VWF. Several studies have recently confirmed that VWF glycosylation is a major modulator of ADAMTS13 proteolysis (Bowen 2003, McGrath, *et al* 2009, McKinnon, *et al* 2008, O'Donnell, *et al* 2005). To ascertain the role of platelet-VWF glycans in the modulation of ADAMTS13 cleavage, both PL- and PT-VWF were treated with PNGase F and O-glycosidase to remove total N-linked glycans and O-linked glycan chains respectively. The consequent effect on ADAMTS13 cleavage was assessed.

Treatment of PT-VWF with PNGase F (PNG-PT-VWF) to remove N-linked glycan structures ablated the observed resistance to ADAMTS13 cleavage, as PNG-PT-VWF was cleaved in a similar fashion to wild-type untreated PL-VWF (Figure 6-10A; PNG-PT-VWF:CB 13 ± 0.5% vs. WT-PL-VWF:CB 11 ± 3% at 120 min, p = ns). However, N-linked deglycosylation of PL-VWF increases susceptibility to ADAMTS13 proteolysis, such that PNG-PL-VWF is proteolysed to a greater extent than both WT-PL-VWF and PNG-PT-VWF ($p < 0.001$; Figure 6-10A).

Similarly, removal of O-linked glycans enhanced ADAMTS13 proteolysis of PT-VWF (Figure 6-10B; WT-PT-VWF:CB 38 ± 2% vs. O-Gly-PT-VWF:CB 17 ± 4% at 120 min; $p <$ 0.01), whereas O-linked deglycosylation had no effect on PL-VWF cleavage (WT-PL-VWF:CB 20 ± 1% vs. O-Gly-PL-VWF:CB 18 ± 7% at 120 min, p = ns; Figure 6-11B).

Taken together, these results indicate that both the N- and O-linked glycans on platelet-derived VWF cooperatively modulate ADAMTS13 proteolysis, whereas N-linked glycan structures alone determine plasma-derived VWF susceptibility to cleavage by ADAMTS13.

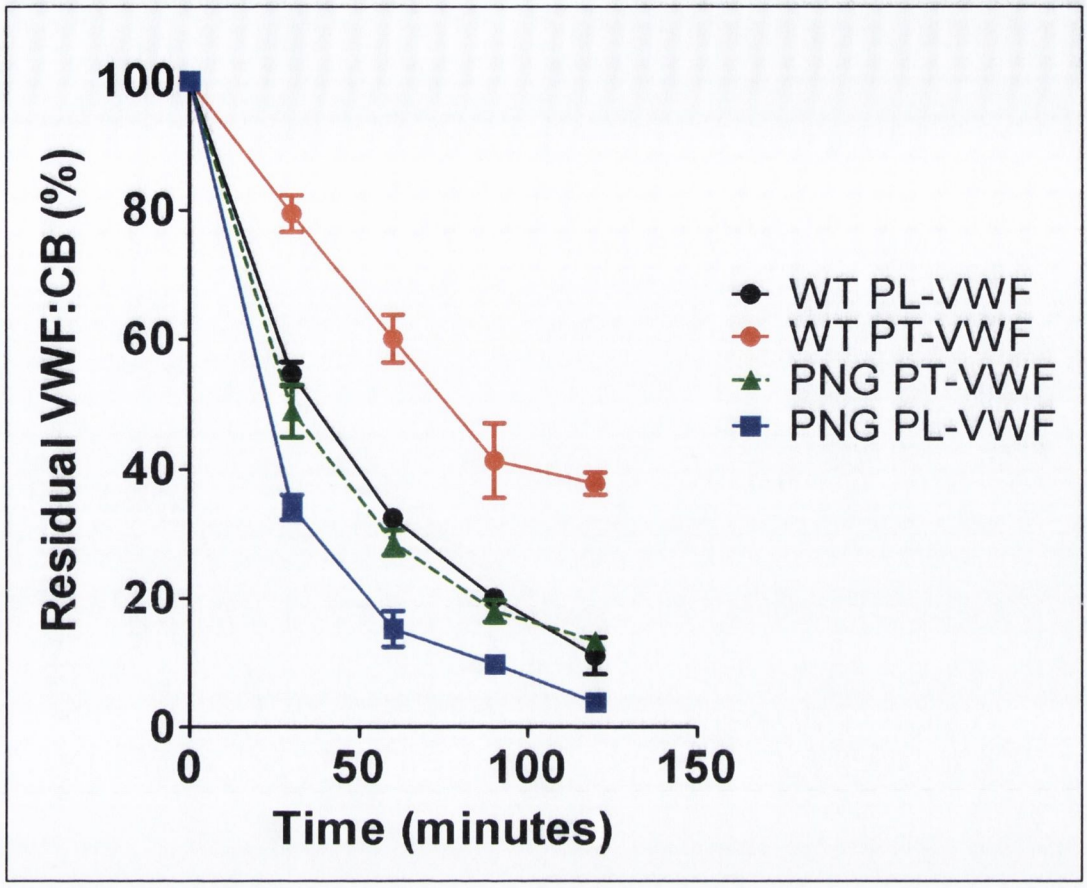


Figure 6-10A N-Glycans modulate platelet-VWF susceptibility to ADAMTS13 proteolysis

Following N-linked deglycosylation, PL- and PT-VWF were subjected to ADAMTS13 proteolysis in a static cleavage assay. Removal of N-linked glycans increased PL-VWF susceptibility to ADAMTS13 cleavage (PL-VWF (●) vs. PNG-PL-VWF (■); *** $p < 0.001$). Treatment with PNGase F ablated PT-VWF resistance to ADAMTS13 (PT-VWF (●) vs. PNG-PT-VWF (▲); *** $p < 0.001$), suggesting that N-linked glycans on both plasma- and platelet-derived VWF modulate susceptibility to ADAMTS13.

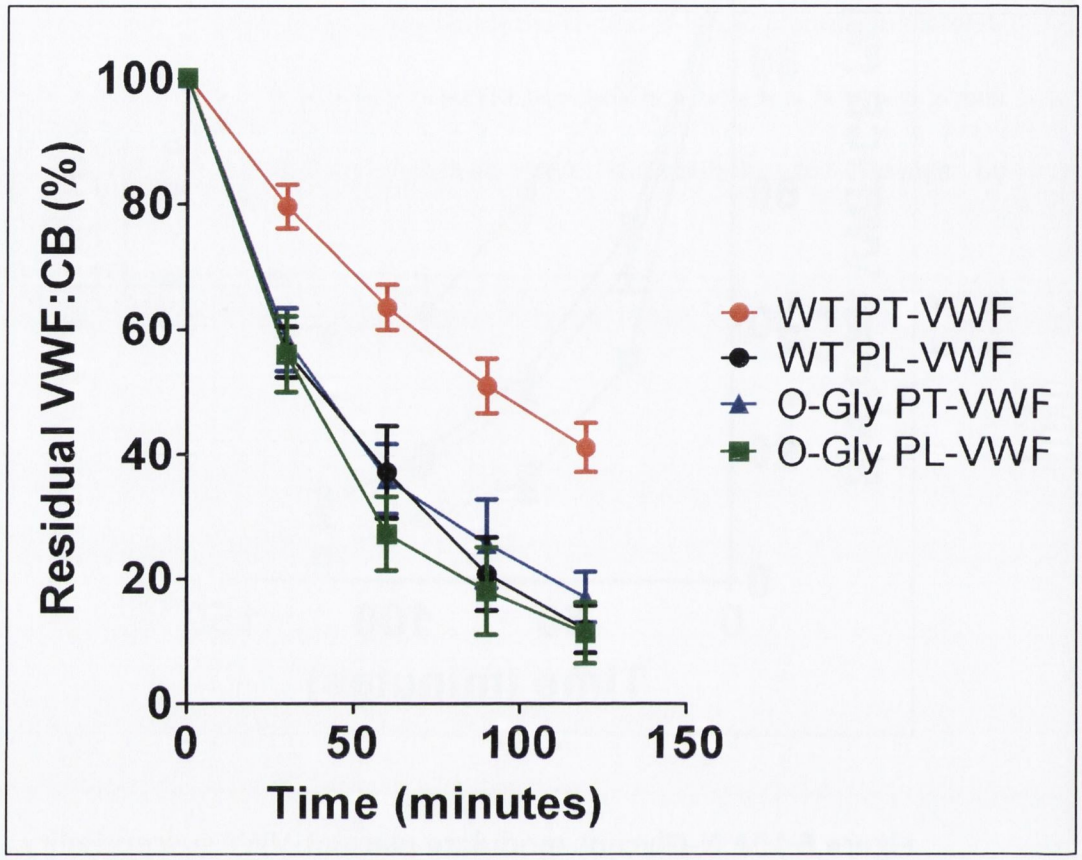


Figure 6-10B O-Glycans modulate platelet-VWF susceptibility to ADAMTS13 proteolysis

The effect of removal of O-linked glycans from VWF on susceptibility to ADAMTS13 cleavage was studied. O-linked deglycosylation had no effect on the rate of ADAMTS13 cleavage of PL-VWF (PL-VWF (●) vs. O-Gly-PL-VWF (■); p = ns). Treatment with O-glycosidase ablated PT-VWF resistance to ADAMTS13 (PT-VWF (●) vs. O-Gly-PT-VWF (▲); ** $p < 0.01$), suggesting that both N- and O-linked glycans platelet-derived VWF regulate ADAMTS13 proteolysis.

6.11 Quantification of terminal sialic acid levels on plasma- versus platelet-derived VWF by HPLC

The data presented in Chapters 4 and 5 highlight the importance of sialic acid in modulating VWF susceptibility to ADAMTS13 proteolysis. Previously, terminal sialic acid expression levels on platelet-VWF have been quantified using several approaches, including a resorcinol-based colorimetry assay, and Fluorophore-Assisted-Carbohydrate Electrophoresis (FACE) in conjunction with isoelectric focusing (Kagami, *et al* 2000, Williams, *et al* 1994). To further quantify and characterise platelet-VWF sialic acid levels, definitive HPLC analysis was used as described in Chapter 4, section 4.3.

Free sialic acid (Neu5Ac) labelled with OPD to form a quinoxaline derivative that, when analysed by reverse-phase HPLC, presented a baseline-resolved peak with a retention time of 33.5 minutes. A calibration curve of free sialic acid (R^2=0.99), consisting of five different sialic acid standards ranging from $1.29x10^{-9}$ Moles to $5.05x10^{-12}$ Moles per injection (10μl) was subsequently implemented for quantitative determination. As shown in Chapter 4, section 4.3.3, the total sialic acid present in plasma-derived VWF is 166.57nMol of sialic acid per mg VWF protein, an observation consistent with the findings of Sodetz and colleagues (Sodetz, *et al* 1977) who detected 154±15nMol/mg of protein. Similar observations were again seen for plasma-derived VWF during this analysis (data not shown).

Notably, comparative quantitative analysis of the sialic acid content on both plasma- and platelet-derived VWF demonstrated that the latter contained 45.7% of the total sialic

acid content of its co-analysed sample (Figure 6-11, $p < 0.001$), an observation that correlates well with the findings of Williams et al, who showed that platelet-derived VWF contained approximately half of the amount of sialic acid as its plasma-based counterpart (Williams, *et al* 1994).

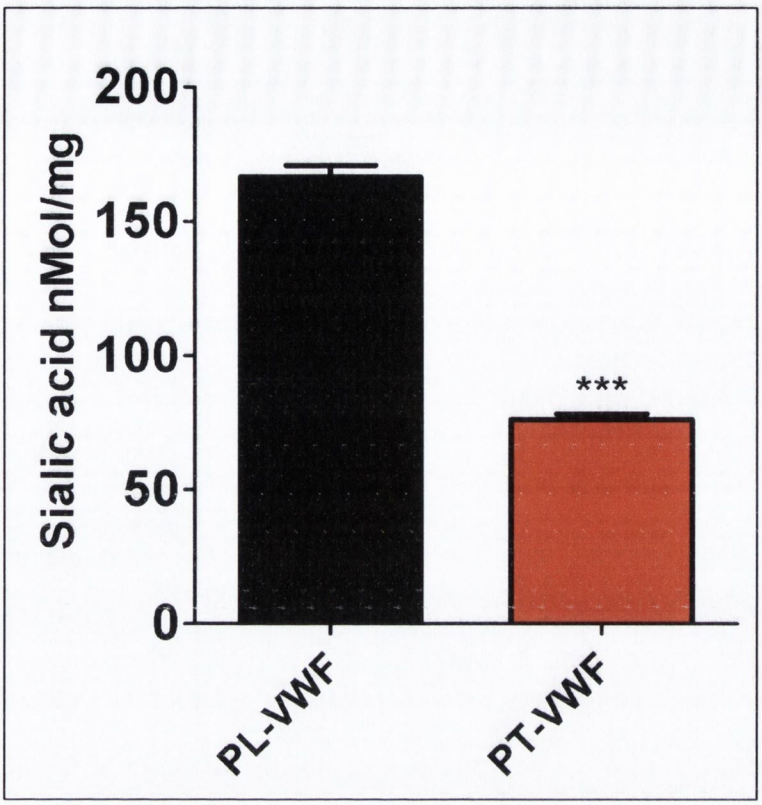


Figure 6-11 Quantification of VWF sialic acid expression on plasma- vs. platelet-VWF using HPLC.

Purified human plasma-derived VWF (PL-VWF) and platelet-derived VWF (PT-VWF) α2-3,6 linked sialic acid expression was analysed by HPLC as described in Section 4.3. Total sialic acid expression on PL-VWF was 167nMol/mg vs. 76nMol/mg for PT-VWF (*** $p < 0.001$). This demonstrates that there is ~54% less sialic acid on PT-VWF in comparison to PL-VWF.

6.12 Sialic acid protects platelet-VWF from ADAMTS13 proteolysis

To further understand the role by which carbohydrate structures mediate the interaction between platelet-derived VWF and ADAMTS13, the effect of sialic acid modification on ADAMTS13 proteolysis was also investigated. In agreement with my previous findings (Chapter 4, section 4.5.1), enzymatic removal of α2-6 linked sialic acid from plasma VWF confers resistance to ADAMTS13 proteolysis, as Neu-PL-VWF:CB remained at 62 ± 4% compared to a reduction to 11 ± 3% for WT PL-VWF (Figure 6-12; $p < 0.001$). However, in contrast to the observed resistance for PL-VWF, desialylation of PT-VWF results in a slight increase in susceptibility to ADAMTS13 cleavage, as residual VWF:CB for Neu-PT-VWF fell to 28 ± 4% after 120 minutes compared to 38 ± 2% for WT PT-VWF (Figure 6-12; $p < 0.05$). This suggests that sialic acid on plasma- vs. platelet-VWF has different functions in mediating the rate of ADAMTS13 cleavage.

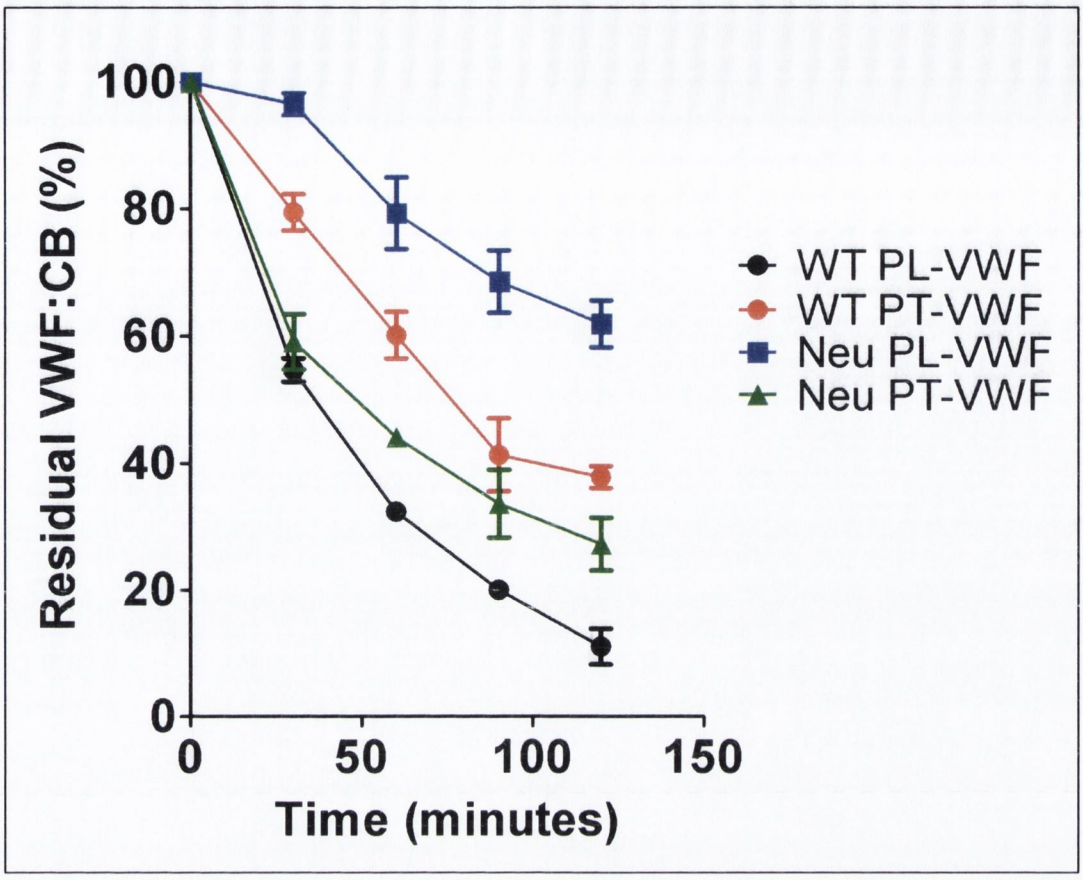


Figure 6-12 Sialic acid protects platelet-VWF from ADAMTS13 cleavage

Removal of sialic acid from platelet-VWF by digestion with α2-3,6,8,9 neuraminidase increases susceptibility to ADAMTS13 proteolysis (WT-PT-VWF (●) vs. Neu-PT-VWF (▲); * $p < 0.05$). This is in contract to the role of sialic acid on plasma-VWF N-linked glycans, which selectively promotes cleavage by ADAMTS13 (WT-PL-VWF (●) vs. Neu-PL-VWF (■); *** $p < 0.001$). Experiments were performed a minimum of 3 times and results are expressed as mean ± SEM at each time point.

Source of VWF	Glycosidase treatment	Glycan modification	Result - Susceptibility to ADAMTS13
Plasma	PNGase F	Removal of N-Glycans	Increase
	O-Glycosidase	Removal of O-Glycans	No change
	α2-3,6,8,9 neuraminidase	Removal of sialic acid	Decrease
Platelet	PNGase F	Removal of N-Glycans	Increase
	O-Glycosidase	Removal of O-Glycans	Increase
	α2-3,6,8,9 neuraminidase	Removal of sialic acid	Slight increase

Table 6-1 Glycan modification of plasma- and platelet-derived VWF and the subsequent effect on rate of ADAMTS13 proteolysis in vitro.

6.13 Discussion

The susceptibility of platelet-derived VWF to regulatory proteolysis by ADAMTS13 was investigated in this chapter. Impure platelet-VWF present in platelet lysate and purified platelet-VWF demonstrated significant resistance to ADAMTS13 cleavage, in comparison to endothelial derived plasma-VWF. This resistance was found to be ADAMTS13 specific, as platelet-VWF was cleaved in a similar fashion to plasma-VWF by several serine and cysteine proteases. Alteration of platelet-VWF glycosylation ablated the observed impairment of ADAMTS13 proteolysis, with roles for both N- and O-linked glycans, and also terminal sialic acid expression being exhibited. Table 6-1 provides an overview of the effect of modification of both plasma- and platelet-VWf glycsoylation on susceptibility to cleavage by ADAMTS13. These results underline the importance of VWF carbohydrate expression levels in the regulation of VWF functional activity.

Earlier studies have suggested that platelet-VWF function differs greatly to that of plasma-VWF, including decreased GPIbα binding and enhanced binding to both GPIIb/IIIa and unfractionated heparin. These functional dissimilarities have been in part based on variations in plasma- vs. platelet-VWF glycosylation pattern. As the glycan pattern of VWF can greatly affect functional abilities such as those listed above, it is conceivable that platelet-VWF oligosaccharide expression also modulates ADAMTS13 proteolysis. The mechanism by which glycosylation modulates VWF proteolysis by ADAMTS13 remains unclear. However, previous studies have demonstrated that N- and O-linked glycans, and even single monosaccharides, can directly influence glycoprotein folding (Kimura, *et al* 1998, Petrescu, *et al* 2004). This in turn may affect ADAMTS13 accessibility to the cleavage site in the platelet-VWF A2 domain. Variation in the rates of

ADAMTS13 proteolysis may be due to the initial difference in levels and/or distribution of sialic acid on wild-type PL-VWF and PT-VWF glycan chains. Quantification of sialic acid levels by HPLC analysis showed that platelet-VWF glycans express less than half of the sialic acid found on plasma-VWF, which is in keeping with previous reports (Kagami, *et al* 2000, Williams, *et al* 1994). As I have demonstrated in Chapters 4 and 5, reduction of the sialylation expression pattern of plasma-derived VWF renders the protein less susceptible to ADAMTS13. Platelet-VWF may naturally be present in a conformation that is less prone to proteolysis by ADAMTS13, based on sialic acid distribution and/or expression, just like desialylated plasma-VWF. Removal of sialic acid by treatment with α2-3,6,8,9 neuraminidase increases platelet-VWF susceptibility to ADAMTS13 cleavage, suggesting endogenous sialic acid on platelet-VWF has a protective function, in contrast to promoting ADAMTS13 proteolysis of plasma-VWF (McGrath, *et al* 2009). Furthermore, the absence of blood group antigen structures on platelet-VWF glycans may also have an effect on sialic acid composition, as AB blood group antigens have recently been shown to modulate the conformation and distribution of sialic acid on red cells (Cohen, *et al* 2009). Moreover, Platelet-VWF is not bound to factor VIII (Shi, *et al* 2006, Yarovoi, *et al* 2003), which could have an effect on the rate of ADAMTS13 proteolysis, as FVIII has previously been shown to promote VWF cleavage (Cao, *et al* 2008).

Impairment of ADAMTS13 cleavage through a glycan mediated mechanism could represent an intrinsic protective role of platelet-VWF carbohydrate chains, in maintaining critical UL-VWF function at sites of platelet activation and thrombus formation. Interestingly, ADAMTS13 is also expressed in platelets, which may indicate a

role for regulated proteolysis of platelet-VWF upon secretion of platelet granule contents (Liu, *et al* 2005, Suzuki, *et al* 2004). Furthermore, several studies have reported that platelet-VWF function is vital under high levels of shear stress, in mediating adherence of platelets to collagen under conditions of flow (d'Alessio, *et al* 1990, Fressinaud, *et al* 1987, Fressinaud, *et al* 1994). This could partially explain why endogenous platelet-VWF is less likely to be proteolysed by ADAMTS13, as VWF function is crucial to platelet accumulation and aggregation to ensure the arrest of bleeding (Ikeda, *et al* 1991). Studies to determine the role of platelet-VWF susceptibility to ADAMTS13 under various conditions of shear stress and to further characterise the role of platelet-VWF glycans in the modulation of ADAMTS13 proteolysis under flow are currently being carried out by our group.

Several subsets of type I von Willebrand disease (VWD) have been described based upon low levels of platelet-VWF antigen and/or activity (Mannucci, *et al* 1985, Weiss, *et al* 1983). Similarly, platelet-VWF levels have previously been shown to correlate better with bleeding time than plasma-VWF levels (Gralnick, *et al* 1986, Rodeghiero, *et al* 1992). Furthermore, in some cases of severe type III VWD, infusion of platelets improved patient outcome, when administered in plasma or concentrate (Castillo, *et al* 1997, Castillo, *et al* 1991, Mannucci 1995). This highlights the translational significance and importance of platelet-VWF function in vivo, and therefore the elucidation of platelet-VWF regulation by ADAMTS13 is of patho-physiological consequence.

In conclusion, my findings demonstrate that platelet-derived VWF exhibits significant resistance to ADAMTS13 proteolysis via a glycan mediated mechanism. This resistance is

specific to ADAMTS13 cleavage, and is dependent on both N- and O-linked glycan chains and also on the sialylation pattern of platelet-VWF. These results provide another example of how endogenous glycan patterns can regulate protein function, and may also explain why plasma- and platelet-derived VWF exhibit different carbohydrate compositions in vivo. Further studies are required to determine the exact molecular mechanisms responsible, as well as the physiological significance of these findings in the context of von Willebrand disease, and the overall regulation of platelet-VWF activity following platelet activation at sites of vascular injury.

CHAPTER 7:

CONCLUSIONS AND FUTURE STUDIES

Deficiency or loss of function of von Willebrand factor (VWF) leads to the most common hereditary bleeding disorder in humans, von Willebrand disease (VWD). Type I VWD is characterised as a partial deficiency of VWF, whereas type II VWF is a qualitative loss of functional VWF activity, in terms of platelet- or FVIII-binding. The rarest form of VWD; type III, is an inherited recessive trait which leads to undetectable VWF levels in plasma and a spontaneous mucocutaneous bleeding tendency (Lillicrap 2009). The frequency of VWD can be up to ~1 individual per 100 in the population (Sadler, *et al* 2006) and the more severe type II and type III cases of VWD are occasionally difficult to treat (Rodeghiero, *et al* 2009).

Several factors have been shown to contribute to the pathogenesis of VWD including defects in VWF synthesis, secretion, proteolysis and clearance. Furthermore, in approximately 30% of VWF cases, no direct link to the *VWF* gene has been confirmed (Goodeve 2007), indicating that other factors, including VWF glycosylation, may be having an effect.

Conversely, reduced activity or deficiency of ADAMTS13 can contribute to the development of several disease states, including malaria, thrombotic thrombocytopenic purpura (TTP) and sepsis (Claus, *et al* 2010). Recent studies have shown that loss of

ADAMTS13 activity itself does not participate in the progress of these diseases; it is the consequential increase in the size and frequency of pro-thrombotic circulating ULVWF that mediates a direct effect.

The mechanisms governing ADAMTS13 proteolysis of VWF in plasma are therefore, of direct translational importance. The molecular mechanisms responsible for VWD have not been elucidated in all cases, and the regulation of ADAMTS13 proteolysis of VWF in plasma has not been fully addressed either. The work presented in this thesis highlights the crucial role that VWF glycosylation plays in the modulation of susceptibility to regulatory proteolysis by ADAMTS13. I investigated the role of plasma-derived VWF sialic acid expression levels in mediating the rate of cleavage by ADAMTS13. I also examined platelet-VWF susceptibility to proteolysis by ADAMTS13, and further determined the role that endogenous platelet-VWF oligosaccharide structures play in the modulation of this process.

7.1 Lectin plate binding assays are not suitable for quantification of VWF carbohydrate structures

To determine the relative reduction in VWF glycan levels after digestion with specific exoglycosidases, lectin plate binding assays were used. Levels of oligosaccharide moieties on VWF were quantified before and after glycosidase digestion. As evidenced by definitive HPLC analysis, removal of > 90% detectable sialic acid by digestion with α2-3,6,8,9 neuraminidase actually only corresponds to a reduction of ~70% of the total

amount of sialic acid expression on VWF. HPLC analysis further demonstrated that the majority of sialic acid present on VWF is N-linked.

This highlights the limitations of lectin plate binding assays for the quantification of glycans on VWF, as presumably, VWF present in a globular confirmation displays oligosaccharide structures that are inaccessible to lectins. Denaturation of VWF is therefore required to access partially buried or hidden oligosaccharide structures. Lectin assays may be used for qualitative characterisation of glycan levels on VWF; however a more sensitive assay is needed to determine the exact levels of individual carbohydrate moieties.

7.2 Sialic acid on plasma-VWF specifically promotes proteolysis by ADAMTS13

Removal of sialic acid from VWF renders the protein less susceptible to ADAMTS13 cleavage. This effect is specific, as desialylation increases the rate of VWF proteolysis by non-specific serine and cysteine proteases. Moreover, removal of O-linked sialic acid had no effect on ADAMTS13 cleavage, whereas combined N- and O-linked desialylation has a significant protective effect, suggesting that N-linked sialic acid expression on VWF is a critical modulator of susceptibility to ADAMTS13. Removal of subterminal galactose residues after desialylation ablates the observed resistance for asialo-VWF, indicating that loss of sialic acid alone is responsible for impairment of ADAMTS13 cleavage.

7.3 Desialylation renders VWF less susceptible to ADAMTS13 cleavage via a conformational mechanism

Alteration of VWF sialic acid levels had no effect on ADAMTS13 proteolysis when harsher denaturing conditions were used; suggesting that sialylation of VWF modulates VWF susceptibility to cleavage via a conformational mechanism. Moreover, oxidation of the negative charge present on sialic acid structures has no effect on ADAMTS13 proteolysis, showing that loss of negative charge is not sufficient to promote VWF resistance to ADAMTS13. Loss of sialic acid expression appears to facilitate VWF adopting a conformation that is less permissive to ADAMTS13 proteolysis.

7.4 Removal of blood group antigenic structures does not affect the rate of VWF proteolysis by ADAMTS13

Blood group specific VWF demonstrates differential susceptibility to ADAMTS13 cleavage; with blood group O VWF being cleaved at a faster rate than non-O VWF. To determine the mechanisms responsible for this phenomenon, blood group A VWF was treated with A-zyme to remove terminal blood group A antigenic structures, and was subsequently digested with ADAMTS13. Reduction in A antigen expression on blood group specific VWF has no effect on the rate of ADAMTS13 proteolysis, implying that blood group carbohydrate structures themselves to not modulate susceptibility to ADAMTS13 cleavage.

7.5 Platelet-derived VWF is endogenously resistant to ADAMTS13 cleavage

Platelet-derived VWF was isolated from platelet lysate and purified using immunoaffinity chromatography (Chapter 6, section x). Following purification, the rate of ADAMTS13 cleavage of platelet-VWF was determined. Platelet-VWF displays reduced susceptibility to ADAMTS13 proteolysis in comparison to plasma-derived VWF. Moreover, this resistance is ADAMTS13 specific, as both plasma- and platelet-VWf were cleaved at the same rate and to the same extent by the serine proteases chymotrypsin and carboxypeptidase Y.

7.6 Platelet-VWF expresses ~55% less sialic acid than plasma-VWF; as determined by HPLC analysis

Previous studies have characterised platelet-VWF carbohydrate expression using a variety of techniques (Kagami, *et al* 2000, Williams, *et al* 1994). However, to definitively quantify sialic levels on platelet-VWF using a more sensitive assay, HPLC analysis was carried out. Platelet-VWF expresses ~55% less sialic acid than plasma-derived VWF. This may be of physiological significance, as sialic acid levels modulate plasma-VWF proteolysis by ADAMTS13. Furthermore, a naturally expressed reduction in platelet-VWF sialic acid expression may render the protein less susceptible to ADAMTS13 cleavage.

7.7 Platelet-VWF glycosylation modulates susceptibility to ADAMTS13 proteolysis

As VWF glycosylation modulates plasma- VWF proteolysis by ADAMTS13, we hypothesized that differences in platelet-VWF carbohydrate expression levels may also have an effect on the rate of ADAMTS13 cleavage. Accordingly, removal of N- or O-linked glycans or terminal sialic acid increases platelet-VWF susceptibility to ADAMTS13. This indicates that both N- and O-linked carbohydrate chains on VWF have a functional role in regulating platelet-VWF proteolysis in plasma.

7.8 Overall conclusions

Glycosylation of VWF is a vital mediator of the ADAMTS13 interaction, and thus has a direct role in the regulation of VWF functional activity in plasma. The novel data presented in this thesis demonstrate that VWF derived from different cellular sources exhibits large variation in susceptibility to regulatory proteolysis by ADAMTS13. Due to differences in post-translational modifications, plasma- and platelet-VWF display different glycosylation profiles. In turn, this results in diverse susceptibility to ADAMTS13 proteolysis. Moreover, as outlined in Table 6-1, alteration of endogenous plasma- and platelet-VWF carbohydrate expression levels has dissimilar effects on the rate of ADAMTS13 cleavage. N-linked glycans and in particular, N-linked sialic acid are crucial modulators of the plasma-VWF - ADAMTS13 interaction, whereas roles for both the N- and O-linked oligosaccharide chains (including sialic acid residues) on platelet-VWF appear to have a regulatory role in this process. Levels of sialic acid on VWF may vary in several disease states, and also in cases of VWD. Furthermore, we hypothesize that

newly secreted VWF is hypersialylated, which would facilitate regulatory proteolysis and prevent ultra-large prothrombotic VWF multimers from entering the bloodstream. Subsequently, levels of sialic acid on VWF diminish over time, thus facilitating clearance via the asialoglycoprotein receptor, among others. Clearly, glycosylation represents a key regulator of VWF multimeric composition, and as such, is likely to be of patho-physiological significance.

7.9 Future directions

1. Optimisation of a flow-based ADAMTS13 cleavage assay.

2. Analysis of plasma- and platelet-derived VWF susceptibility to ADAMTS13 proteolysis under various levels of shear stress (25-120 dyne/cm^2) as measured by residual platelet binding using the VenaFluxAssay System (Cellix Ltd., Dublin, Ireland).

3. Analysis of glycan modified plasma- and platelet-VWF susceptibility to ADAMTS13 cleavage under flow.

4. Characterisation of quantitative expression of sialic acid on platelet-VWF; digestion with glycosidase enzymes and analysis by HPLC to ascertain whether sialic acid is predominantly N- or O-linked.

5. Further glycan modification of platelet-VWF and subsequent determination of the effect on rate of ADAMTS13 proteolysis.

6. Analysis of the role of FVIII in ADAMTS13 cleavage of platelet-VWF.

PUBLICATIONS ARISING FROM THIS WORK

Papers published.

- ***Expression of terminal α2-6-linked sialic acid on von Willebrand factor specifically enhances proteolysis by ADAMTS13.***

  Rachel T. McGrath, Thomas A. J. McKinnon, Barry Byrne, Richard O'Kennedy, Virginie Terraube, Emily McRae, Roger J. S. Preston, Mike A. Laffan, and James S. O'Donnell.

  Blood, 1 April 2010, Vol. 115, No. 13, pp. 2666-2673.

- ***Platelet von Willebrand factor-structure, function and biological importance.***

  Rachel T. McGrath, Emily McRae, Owen P. Smith, and James S. O'Donnell.

  British Journal of Haematology, 2010 Mar;148(6):834-43.

Abstracts leading to oral presentations.

- ***Role of Glycan Structures in Mediating von Willebrand Factor Function and Proteolysis.***

  Rachel T. McGrath, Emily McRae, Virginie Terraube, Roger J. S. Preston and James S. O'Donnell.

  Royal Academy of Medicine in Ireland; Section of Biomedical Sciences. Annual Meeting 2008

  Winner of the 2nd prize in The Donegan Medal Competition.

- ***Sialic Acid Expression on von Willebrand Factor - A Novel Critical Regulator of ADAMTS13 Proteolysis and Functional Activity.***

  Rachel T. McGrath, Thomas A. J. McKinnon, Barry Byrne, Richard O'Kennedy, Virginie Terraube, Emily McRae, Roger J. S. Preston, Mike A. Laffan, and James S. O'Donnell.

  Haematology Association Ireland, Annual Meeting 2009

  Winner of the Best Presentation - Postgraduate Student Prize.

- ***Terminal α2-6 Linked Sialic Acid Expression On VWF Specifically Enhances Proteolysis by ADAMTS13.***

  Rachel T. McGrath, Thomas A. J. McKinnon, Barry Byrne, Richard O'Kennedy, Virginie Terraube, Emily McRae, Roger J. S. Preston, Mike A. Laffan, and James S. O'Donnell.

  51st American Society of Haematology Meeting and Exposition, 2009

  Blood (ASH Annual Meeting Abstracts), Nov 2009; 114: 30.

  Selected for inclusion in the Highlights of ASH 2009.

Abstracts leading to poster publications.

- ***Characterisation and modification of VWF Glycosylation.***

  Rachel T. McGrath, Virginie Terraube, Emily McRae, Roger J. S. Preston and James S. O'Donnell.

  GlycoScience Ireland Inaugural Meeting, 2008.

- ***Terminal α2-6 Linked Sialic Acid Modulates VWF Cleavage by ADAMTS13.***

  Rachel T. McGrath, Virginie Terraube, Emily McRae, Deirdre Larkin, Barry White, Roger J. S. Preston and James S. O'Donnell.

  11th Annual Meeting of the Institute of Molecular Medicine, Trinity College Dublin, 2008.

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