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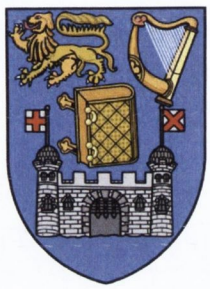
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The role of glycosylation and sequence variation in the regulation of von Willebrand Factor plasma levels.



A thesis submitted to the University of Dublin, Trinity College,
for the Degree of Doctor of Philosophy in the School of Medicine.

Orla Rawley

Submitted March 2014

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Summary

Von Willebrand Factor (VWF) is a multimeric plasma sialoglycoprotein that plays an important role in primary haemostasis by mediating platelet capture at sites of vascular injury. In the normal population, VWF plasma concentrations vary over a wide range (5-20μg/mL). Importantly, VWF glycosylation, and single amino acid substitutions in the VWF molecule, have both been shown to contribute to variation in plasma VWF levels by regulating VWF clearance. However the biological mechanism(s) involved have not been defined.

To date, over 100 single amino acid substitutions have been reported to cause partial quantitative deficiencies of plasma VWF. Moreover, at least 20 of these have been associated with enhanced VWF clearance. The prototypic mutation is the R1205H substitution in the VWF D3 domain which is responsible for the so-called VWD Vicenza phenotype. Although it is well established that patients with VWD Vicenza have significantly reduced plasma VWF levels due to a marked reduction in VWF half-life, the receptors and cell types responsible for the enhanced clearance phenotype remain unknown.

Therefore, the work herein aims to elucidate the underlying molecular mechanisms. Consequently, clearance of a number of recombinant VWF variants as well as a series of VWF domain truncations was studied in $VWF^{-/-}$ mice. Furthermore, hepatic and splenic macrophages were depleted to assess their contribution to pathogenic VWF clearance in VWD Vicenza. Additionally, *in vitro* macrophage binding studies were also carried out to identify the

receptors involved. Briefly, R1205 was identified as a critical regulator of VWF clearance. Moreover, enhanced macrophage-mediated VWF clearance was identified as the major pathogenic mechanism in VWD Vicenza with specific roles for macrophage LRP1 and $\alpha_M\beta_2$ integrins.

To assess the clinical impact of enhanced VWF clearance, VWF plasma levels were determined in a VWD Vicenza cohort following DDAVP administration. Phenotypic responses were characterized, and VWF and FVIII plasma half-lives were determined for each patient. Furthermore, enhanced VWF clearance was also investigated as a potential pathogenic mechanism in a specific patient cohort with intermediate reductions in VWF plasma levels. Interestingly, analysis of these clinical data suggests involvement of several pathogenic mechanisms in modulating variation in VWF plasma levels in these patients.

VWF is extensively glycosylated with significant N-linked and O-linked glycosylation. Moreover, these complex glycan structures critically influence VWF activity and clearance *in vivo*. Although the molecular mechanisms through which VWF glycans regulate VWF biology remain unknown, recent studies have shown that these glycan structures can module interaction between VWF and various lectins in normal plasma. In particular, VWF has been shown to bind to a particular member of the galectin family, galectin-3. Furthermore, galectin 3 binding significantly influences VWF activity *in vivo*. To define the nature of this novel interaction, VWF glycosylation was modified via exoglycosidase digestion and VWF/galectin-3 binding was assessed. Interestingly, both the N- and O-linked VWF glycan determinants were shown to modulate this interaction and terminal sialic acid and ABO blood group

antigen expression were of particular importance. Furthermore, an additional role for specific N-linked glycans was identified.

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Finally, this thesis is dedicated to my family; my Mom and Dad, my brother Colm, and my Grandmother Kitty. Thank you all for your unconditional support and encouragement along the way.

Abstracts leading to oral/poster presentations

- **Elucidating the role of carbohydrate determinants in regulating haemostasis – insights and opportunities.**

  James S. O'Donnell, Orla Rawley.

  7th Annual Congress of the European Association for Haemophilia and Allied Disorders, 2014.

- **Identification of galectin-1 and galectin-3 as novel binding partners for factor FVIII.**

  Jamie O' Sullivan, Orla Rawley, P. Vince Jenkins, Alain Chion, Teresa Brophy and James S. O' Donnell.

  55th American Society of Haematology Annual Meeting, 2010.

  Oral presentation.

- **Defining the molecular basis underlying the physiological interaction between von Willebrand factor and galectins in normal plasma.**

  Orla Rawley*, Jamie O'Sullivan*, Alain Chion, Niamh O'Regan, P. Vince Jenkins, Teresa Brophy and James S. O'Donnell.

  XXIV Congress of the International Society on Thrombosis and Haemostasis, 2013.

  Oral poster presentation.

- **Defining the pathological mechanisms underlying enhanced clearance in VWD type Vicenza.**

  Orla Rawley, Jamie M. O'Sullivan, Alain Chan, Teresa M. Brophy, James S. O'Donnell.

  Haematology Association Ireland, Annual Meeting 2013.

  Oral presentation.

- **Defining the molecular basis underlying the physiological interaction between VWF and galectin-3 in normal plasma.**

  Orla Rawley, Jamie M. O'Sullivan, Alain Chan, Teresa M. Brophy, P. Vince Jenkins, James S. O'Donnell.

  Haematology Association Ireland, Annual Meeting 2012.

  Oral presentation, President's Symposium.

  Winner: President's Prize, Best Overall Abstract.

- **Platelet-derived von Willebrand factor glycosylation mediates resistance to ADAMTS13 proteolysis.**

  Rachel T. McGrath, Maartje van den Biggelaar, Barry Byrne, Emily McRae, Orla Rawley, Richard O'Kennedy, Jan Voorberg, James S. O'Donnell.

  XXIII Congress of the International Society on Thrombosis and Haemostasis, 2011.

- **A critical role for N- and O-linked carbohydrate determinants in modulating von Willebrand factor clearance *in vivo*.**

  Emily McRae, Hendrick Nel, Orla Rawley, Rachel T. McGrath, Gudmunder Bergsson, Alain Chan, Roger Preston, Padriac Fallon and James S. O'Donnell.

  53rd American Society of Haematology Annual Meeting, 2010.

  Oral presentation.

- **Specific N- and O-linked carbohydrate structures mediate von Willebrand factor interaction with galectins -1 and -3.**

  Orla Rawley, Jamie M O'Sullivan, Gudmunder Bergsson, Alain Chan, Rachel T. McGrath, Martje van den Biggelaar, Jan Voorberg, P. Vince Jenkins, James S. O'Donnell.

  53rd American Society of Haematology Annual Meeting, 2011.

  Poster presentation.

- **The N-linked glycans of von Willebrand factor mediate novel interactions with the carbohydrate recognition domains of galectins-1 and -3.**

  Jamie O'Sullivan, Orla Rawley, Gudmundur Bergsson, Emily McRae, Rachel McGrath, Alain Chan, and James O'Donnell.

  Haematology Association of Ireland, Annual Meeting 2010.

  Oral poster presentation.

  Winner: Best Oral Poster.

Publications

- **Altered glycosylation of platelet-derived von Willebrand factor confers resistance to ADAMTS13 proteolysis**

  McGrath RT, van den Biglaar M, Byrne B, O'Sullivan JM, Rawley O, O'Kennedy R, Voorberg J, Preston RJ, O'Donnell JS.

  Blood 2013;122(25):4107-10.

- **Elucidating the role of carbohydrate determinants in regulating haemostasis – insights and opportunities.**

  Roger J.S. Preston, Orla Rawley, Eimear M. Gleeson and James S. O'Donnell

  Blood 2013;121(19):3801-10

- **Elevated factor VIII levels and risk of venous thrombosis.**

  Jenkins PV, Rawley O, Smith OP, O'Donnell JS.

  Br J Haematol 2012; 157(6):653-63.

Submissions

- **Arginine 1205 substitutions enhance macrophage-mediated clearance of von Willebrand factor.**

  Orla Rawley*, Jamie M. O'Sullivan*, Alain Chion, Sean Keyes, Michelle Lavin, Nico van Rooijen, Roger J. Preston, Teresa Brophy, Padraic Fallon and James S. O'Donnell.

  Blood Short Report

Abbreviations

ADAMTS13: A Disintegrin and Metalloprotease with Thrombospondin repeats

AGE: Advanced glycation end products

AIC: Akaike information criteria

$\alpha_M\beta_2$: alpha-M beta-2 integrin; Cd11b/Cd18; Mac-1

ASGPR: Asialoglycoprotein Receptor

ASOR: Asialo-orosomucoid protein

β2-GP1: β2-glycoprotein 1

BOEC: Blood Outgrowth Endothelial Cell

BSA: Bovine Serum Albumin

Ca^{2+}: Calcium

cAMP: Cyclic adenosine monophosphate

CLEC4M: C-Type Lectin Domain Family 4, Member M; DC-SIGNR

CRD: Carbohydrate Recognition Domain

CTCK: C-terminal Cysteine Knot Domain

DDAVP: 1-desamino-8-D arginine vasopressin; desmopressin

EC: Endothelial cells

ELISA: Enzyme-linked immunosorbent assay

ER: Endoplasmic reticulum

FVIII: Factor VIII

Gal: Galactose

GalNAc: N-acetylgalactosamine

$GdCl_3$: Gadolinium chloride

GlcNAc: N-acetylglucosamine

GPIbα: Platelet glycoprotein Ibα

GPIIb/IIIa: Platelet glycoprotein IIb/IIIa

HEK: Human Embryonic Kidney cell

HMWM: High Molecular Weight Multimers

HUVECs: Human Umbilical Vein Endothelial cells

LacNAc: N-acetyllactosamine

LDL: Low Density Lipoprotein

LLG: Leucine - Leucine - Glycine adhesion motif

LMWM: Low Molecular Weight Multimer

LRP: Low density lipoprotein related receptor

MRT: Mean Residence Time

NeuAc: N-acetylneuraminic acid; sialic acid

NIF: Neutrophil Inhibitory Factor

OPG: Osteoprotegerin

Pd: Plasma-derived

Plt: Platelet

PMA: Phorbol Myristate Acetate

PSGL-1: P-selectin glycoprotein ligand 1

RAP: Receptor Associated Protein

RGD: Arginine - Glycine - Aspartic Acid motif

Siglec: Sialic acid binding immunoglobulin-like lectin

SNP: Single Nucleotide Polymorphism

SPR: Surface Plasmon Resonance

TGF-β: Transforming Growth Factor

THP-1: human acute monocytic leukaemia cell

TNF: Tumour Necrosis Factor

TRAIL: Tumour necrosis factor-related apoptosis-inducing ligand

TSP-1: Thrombospondin-1

UL: Ultra-large

VNTR: Variable Number Tandem Repeat

VWD: von Willebrand Disease

VWF: von Willebrand Factor

VWF:Ag von Willebrand Factor Antigen

VWF:CBA von Willebrand Factor Collagen Binding Activity

VWFpp: von Willebrand Factor Propeptide

VWF:RCo von Willebrand Factor Ristocetin Cofactor Activity

WPB: Weibel-Palade Bodies

WT: Wild-type

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1.0 Introduction

1.1 VWF Biosynthesis

Von Willebrand factor (VWF) is a large multimeric sialoglycoprotein that exerts two critical roles in maintaining normal haemostasis. First, VWF acts as a molecular bridge mediating platelet capture to exposed subendothelial collagen at sites of vascular injury. Second, VWF acts as a chaperone for procoagulant factor VIII (FVIII) prolonging its half-life within the circulation (Lollar 1991). In normal plasma, VWF circulates at a concentration of ~10μg/ml, though this can vary over a wide range (from 5-20μg/ml). Qualitative or quantitative VWF deficiency is responsible for the most common inherited bleeding disorder, von Willebrand disease (VWD), which occurs in up to 1% of the general population. Conversely, elevated plasma VWF levels are associated with increased risk of myocardial infarction (Crawley et al. 2008), ischaemic stroke (Bongers et al. 2006) and venous thromboembolism (Nossent et al. 2006).

1.1.1 The *VWF* gene

The *VWF* gene was cloned and characterized in 1985 by four independent groups (Ginsburg et al. 1985; Girma et al. 1990; Berndt et al. 1992; Nichols et al. 1995). Located on the short arm of chromosome 12 (12p13.3), it spans 178kb over 52 exons which range in size from 1.3kb (exon 28) to 40bp (exon 50) (Ginsburg et al. 1985; Bowie et al. 1986). The VWF signal peptide and propeptide (VWFpp) are encoded by the first 17 exons; while the mature VWF subunit and the 3' noncoding region are encoded by

the remaining 35 exons. A partial, unprocessed pseudogene also exists and is located on chromosome 22 (22q11-13) (Mancuso et al. 1991). The pseudogene is 21-29kb in length and duplicates the *VWF* gene sequence from exons 23 to 34 with 97% sequence homology.

1.1.2 Tissue distribution of VWF expression

In-vivo, VWF biosynthesis is limited to endothelial cells and megakaryocytes (Tannenbaum et al. 1989). Our current knowledge of VWF biosynthesis is largely based on endothelial cell culture experiments due to difficulties with *in vitro* cultivation of megakaryocytic cell lines. However, both primary guinea pig (Schick et al. 1997) and human megakaryocytes (Sporn et al, 1985) have been shown to produce significant amounts of VWF. In endothelial cells, VWF mRNA and antigen (VWF:Ag) are heterogeneously expressed and differential expression varies with the vascular bed of origin. Quantitative RT-PCR and in-situ hybridization studies of *VWF* gene expression in CB6 mice have demonstrated that VWF mRNA concentrations vary up to 50 fold in endothelial cells of different tissues (Yamamoto et al. 1998). The highest concentrations were found in lung, spleen, aorta, and brain, with lower levels observed in kidney, skeletal muscle, gut, and liver. Accumulating evidence also suggests that VWF expression differs significantly between venous and arterial endothelial cells. Immunohistochemical analysis of porcine blood vessels demonstrated that VWF was expressed in all venous endothelial cells, with the exception of the pulmonary vein. In contrast, VWF was consistently absent from the majority of porcine arterial endothelial cells (Rand et al. 1987).

1.1.3 Regulation of gene expression

The regions of the VWF gene responsible for regulating endothelial-cell-specific expression have been characterized. A 734 base pair (bp) fragment from -487bp upstream of the transcription start site to +247bp was originally identified as the cell-type-specific promoter in bovine aortic endothelial cells (Jahroudi and Lynch 1994). It consists of a core promoter fragment (-90 to +22bp), a strong negative regulatory element upstream of the core promoter (-313 to -487bp) and a cell-specific positive regulatory region located downstream of the core promoter in the first exon. The activity of the core promoter is not cell type-specific, and the negative regulatory element is required to inhibit its activity in all cell types (Jahroudi and Lynch 1994). The inhibitory effect of the negative regulatory element has been attributed to interactions with an NF1-like cis-acting element and Oct-1 (Jahroudi et al. 1996; Schwachtgen et al. 1998). Inhibition is relieved by the positive regulatory region only in endothelial cells which results in endothelial-cell-specific gene expression (Jahroudi and Lynch 1994). This process requires an intact GATA binding element within the positive regulatory region (Jahroudi and Lynch 1994). Additionally, the VWF promoter contains a polymorphic dinucleotide repeat $(GT)_n$ which has been demonstrated to regulate VWF promoter activity under conditions of shear stress (Hough et al. 2008). Furthermore, polymorphic variation in GT repeat length was also shown to correlate with VWF plasma levels in normal individuals (Hickson et al. 2011).

Additional factors also contribute to the regulation of endothelial VWF expression. In transgenic mice expressing a fragment of the VWF gene

containing 2,182bp of the 5′ flanking sequence, the first exon, and first intron, VWF expression was detected only in endothelial cells of the brain, heart, and skeletal muscle. This limited distribution of expression suggests that cis-acting elements located outside the promoter region contribute to the transcriptional regulation of the VWF gene with an additional role for the local microenvironment (Aird et al. 1997).

1.1.4 *VWF* translational product

VWF is synthesized as a pre-pro-polypeptide. The primary translational product is 2813 amino acids in length comprising a 22 amino acid signal peptide, a large 741 amino acid propeptide, and the 2050 amino acid mature subunit.

1.2 Post translational modification

Prior to secretion the pre-pro-VWF primary translational product undergoes extensive post-translational modification. This includes signal peptide cleavage, C-terminal dimerization, glycosylation, sulfation, N-terminal multimerization and endoproteolytic cleavage of the propeptide.

1.2.1 N-linked glycosylation

N-linked glycosylation begins as a co-translational event in the endoplasmic reticulum (ER). Pre-assembled mannose-rich oligosaccharide core structures ($Glc_3Man_9GlcNAc_2$) are attached to the nascent polypeptide chain by the enzyme oligosaccharyltransferase (OST). These mannose-rich 14-sugar cores are transferred to specific asparagine (Asn) residues located in the

consensus sequence Asn-X-Ser/Thr, where X may be any amino acid except proline (Kelleher et al. 1992). Each mature VWF subunit contains 12 such sites with an additional 4 sites located within the VWF propeptide (VWFpp) region (Titani et al. 1986). The mature VWF subunit also carries an atypical Asn-X-Cys consensus sequence in the D3 domain, thus carrying a total of 13 potential N-linked sites (Titani et al. 1986). Ten of these sites have found to be occupied (Figure 1.0) (Canis et al. 2012).

The 14-sugar core unit is rapidly remodelled by a series of glycosyltranfersases and glycosidases as the protein passes through the ER (Schwarz and Aebi 2011). Glucosidase I and II remove two terminal glucose moieties and the resulting structure becomes a ligand for the resident ER lectin chaperones, calreticulin and calnexin (Hebert et al. 1995). When the protein is correctly folded, the final glucose residue is trimmed facilitating release from the chaperones and signalling that the protein is ready to continue to the Golgi.

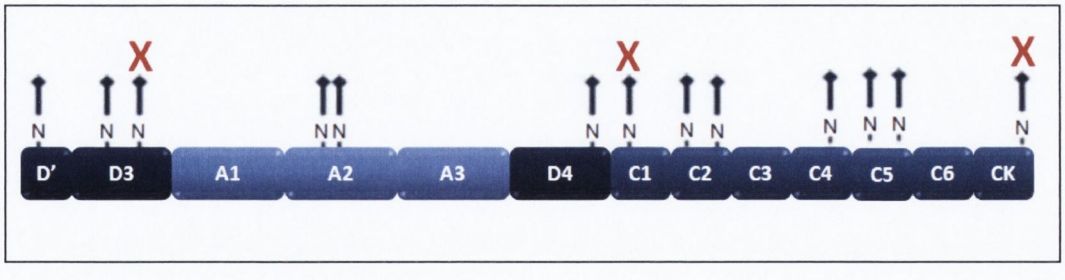


Figure 1.0 Domain structure of monomeric VWF depicting locations of potential N-linked glycan sites.
Potential N-linked sites are located at Asn857, Asn1147, Asn1231, Asn1515, Asn1574, Asn2223, Asn2290, Asn2357, Asn2400, Asn2546, Asn2585, Asn2635 and Asn2790. X indicates N-linked sites whose glycosylation has not yet been defined.

1.2.2 Dimerization

Following N-linked glycosylation, pro-VWF subunits dimerize through C-terminal disulfide bonding in the ER. The neutral pH of the ER and the presence of resident chaperones provide an optimal environment for disulfide bond formation. Dimerization requires only the final 150 residues of the C-terminus, the last 90 residues of which comprise the VWF C-terminal cystine knot (CTCK) domain that is homologous to the "cystine knot" superfamily. Mutagenesis of specific cysteine residues within this domain identified both Cys2771 and Cys2773 as residues critical to the dimerization process (Katsumi et al. 2000).

Dimerization is dependent on the prior attachment of N-linked glycan structures. Treatment of endothelial cells with the antibiotic tunicamycin, which prevents N-linked glycosylation *in vitro*, resulted in the accumulation of pro-VWF monomers in the ER, demonstrating that only dimeric VWF can leave the ER and continue onto the Golgi compartments for further processing (Wagner et al. 1986). Exit from the ER appears to be the rate-limiting step of VWF biosynthesis.

1.2.3 Modification of the N-linked glycans

When pro-VWF dimers reach the Golgi, the high-mannose glycan chains are further processed. Galactose (Gal) and N-acetylglucosamine (GlcNAc) residues are sequentially added by glycosyltransferases to produce complex type carbohydrate structures (figure 1.1) (Kornfeld and Kornfeld 1985). In addition, these structures may also be capped with terminal sialic acid (NeuAc) moieties. NeuAc moieties are transferred to terminal non-

reducing sugars by the action of a family of 20 different Golgi membrane-bound glycosyltransferases known as sialyltransferases (Harduin-Lepers et al. 2005). Human sialyltransferases are classified into three subfamilies according to linkage specificity (Harduin-Lepers et al. 2001) α2-3 sialyltransferases mediate the transfer of sialic acid with an α2-3 linkage to terminal Gal or GalNAc (N-acetylgalactosamine) residues. Similarly α2-6, and α2-8 sialyltransferases transfer sialic acid moieties with α2-6 and α2-8 linkages respectively. VWF N-linked sialic acid is predominantly α2-6 linked to penultimate galactose residues and accounts for ~80% of total VWF sialic acid expression (McGrath et al. 2010).

The N-linked glycans of VWF have been characterized and represent a heterogeneous population of sugars. Over 300 distinct structures have been identified, the most common of which is a monosialylated biantennary complex-chain that accounts for ~78% of all N-linked chains (Matsui et al. 1992; Canis et al. 2012). Tri- and tetra-antennary complex chains have also been described and a proportion of the N-linked glycans (less than 1%) were found to exist as high-mannose type structures (figure 1.1). In addition, glycomic analysis has identified numerous distinct structural features that are responsible for the marked the heterogeneity of the VWF N-glycome. These include core fucosylation, lactosaminic extensions, and bisecting GlcNAc residues that give rise to elongated and highly branched glycan chains (Canis et al. 2012).

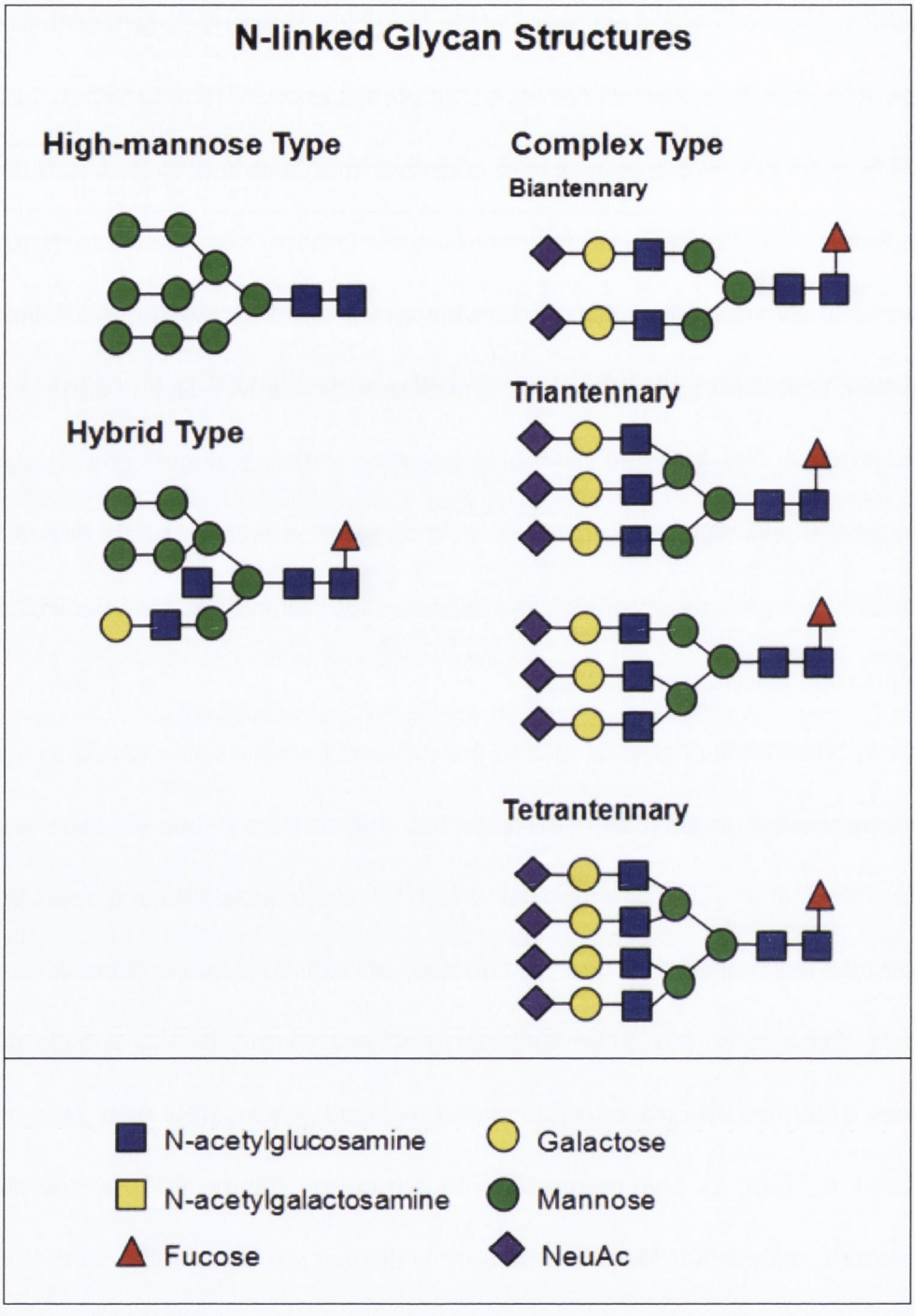


Figure 1.1 Examples of typical N-linked glycan structures expressed on VWF.

1.2.4 Addition of ABO(H) blood group antigens

Unusually, 13% of the N-linked glycans are further modified by the addition of terminal ABO(H) blood group antigens (Matsui et al. 1992). In the endothelium, fucosyl transferase (FUT1) competes with sialyl transferases to add fucose to the terminal end of the glycan chain, thus creating the H-antigen [Fuc (α1-2) Gal (β1-4) GlcNAc (β1-)]. The H-antigen which corresponds to blood group O is the precursor structure for the addition of group A and group B antigens. Group A (GalNAc) and group B (Gal) antigens are created by A and B glycosyltransferase enzymes, that catalyse the addition of either A (GalNAc) or B (Gal) sugars to the terminal β-galactose of the H-antigen precursor structure (Yamamoto 2000). In individuals with the rare Bombay blood group the H antigen is not expressed (Bhende et al. 1994). Consequently, no terminal A or B antigens can be added.

Characterization of the N-linked glycans has demonstrated that H-antigen expression is not localized to particular glycan chains. MALDI-TOF analysis revealed that H-antigen is expressed at all but one of the N-linked glycan sites (Asn1147) though its abundance is not uniform (Canis et al. 2012). H-antigen expression was lowest at sites Asn857, Asn2290 and Asn2585 being expressed on only 1-3% of glycan antennae. In contrast, expression was greatest at Asn2635, with H-antigen expressed on ~16% of glycan antennae. However, the molecular mechanisms governing ABO(H)-antigen distribution on VWF N-linked glycans remain unknown.

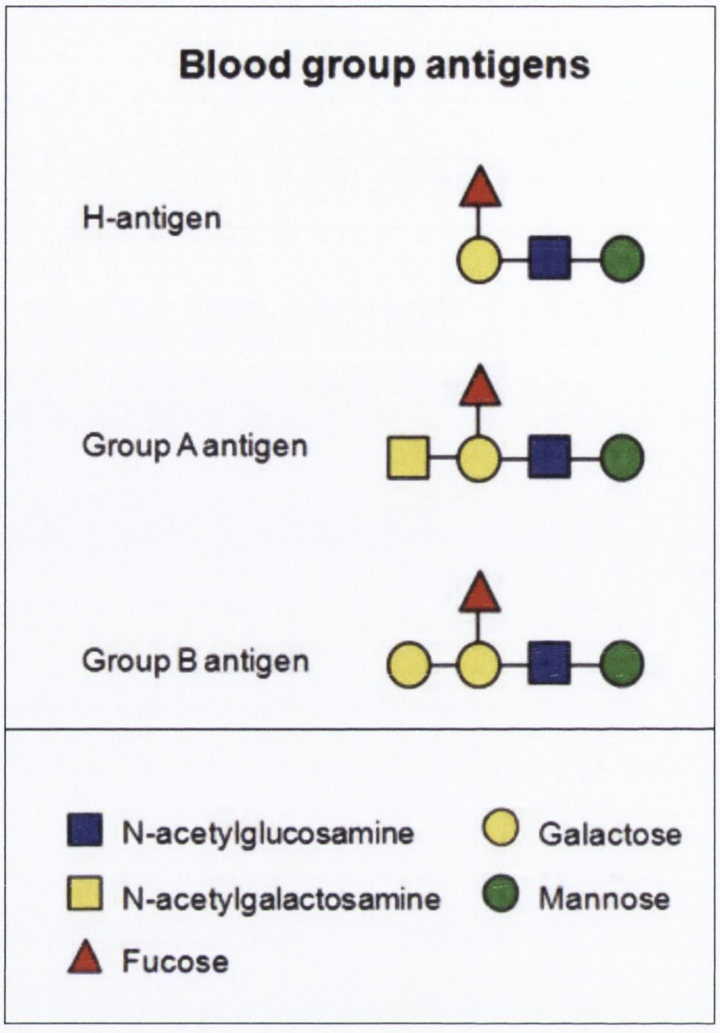


Figure 1.2 Structures of blood group antigens expressed on VWF glycans

1.2.5 O-linked glycosylation

O-linked glycosylation also occurs in the golgi. O-linked glycans are added directly to serine or threonine residues of VWF. Though O-linked glycosylation does not require a specific consensus sequence, glycosylation of Ser or Thr is more common if Ser/Thr residues are present in clusters, or are located in proline or alanine rich regions (Wilson et al. 1991). O-linked glycan synthesis involves the sequential addition of monosaccharide units by specific glycosyltransferases, rather than transfer of a preassembled core. The mature VWF subunit possesses 10 putative O-linked glycan sites, 8 of which are

clustered around the A1 and A2 domains. The remaining two O-glycan sites are located within the A3 and D4 domains (Figure 1.3).

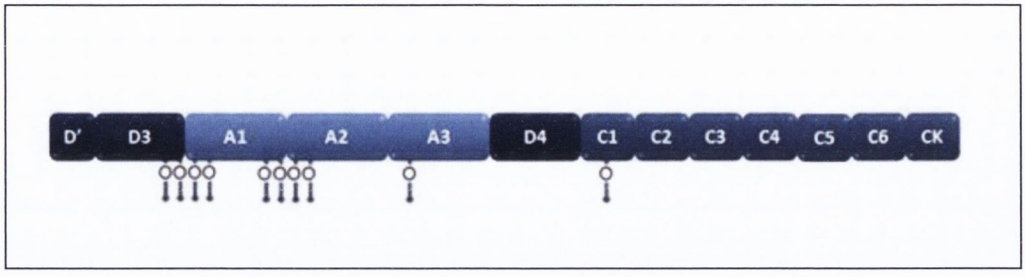


Figure 1.3 VWF domain structure highlighting 10 potential O-linked sites. Potential O-linked sites are located at Thr1248, Thr1255, Thr1256, Ser 1263, Thr1468, Thr1477, Ser1486, Thr1487, Ser1679 and Ser2298.

The VWF O-glycome is comprised of a heterogeneous population of sialylated core 1 and core 2 structures (Canis et al. 2010). The predominant O-linked structure is the sialylated T-antigen (Figure 1.5), a di-sialyl core 1 structure; [NeuAc (α2-3)] Gal (β1-3) [NeuAc(α2-6)]GalNAc (Samor et al. 1989). Recently, mass spectroscopic analysis has revealed the existence of ABO(H) blood group antigens on a proportion of the core-2 structures accounting for approximately 1% of the total VWF O-glycan population (Canis et al. 2010). Surprisingly, a large proportion (~35%) of the core-1 structures were also found to express unusual disialosyl motifs, in which 2 sialic acid moieties are linked, though the functional significance of this is presently unclear.

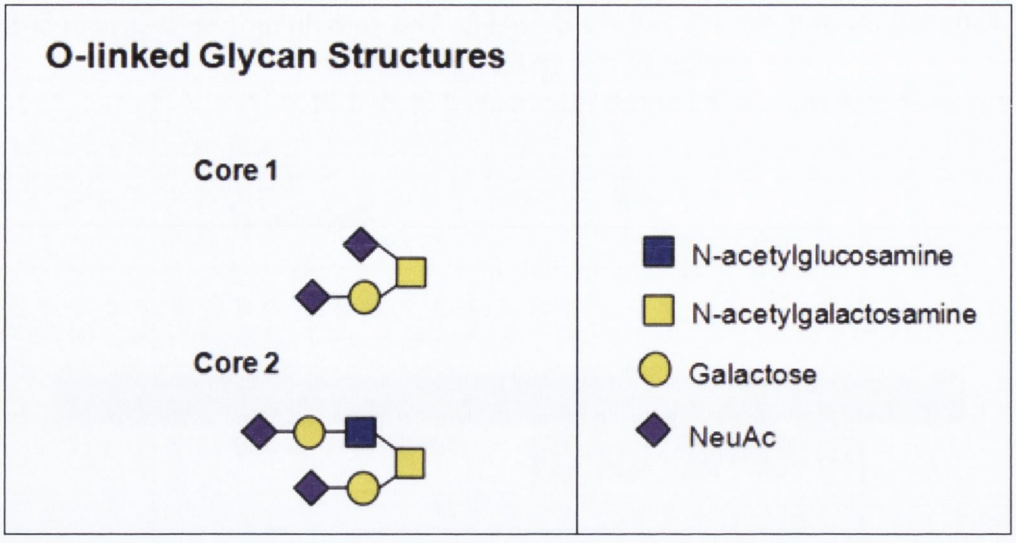


Figure 1.4 Examples of typical O-linked glycan structures expressed on VWF.

1.2.6 Sulfation

VWF is reported to carry sulfated glycans at Asn1147 and/or Asn1231 in the VWF D3 domain (Carew et al. 1990). Sulfation occurs in the trans-Golgi network and pulse chase analysis has demonstrated that it occurs prior to multimerization of pro-VWF dimers and propeptide cleavage (Vischer and Wagner 1994). Interestingly, mass spectroscopic analysis revealed that sulfation occurs on sialylated antennae (Canis et al. 2012). The functional significance of VWF sulfation however, remains unclear as inhibition of sulfation via sodium chlorate treatment was shown to have no effect on VWF multimerization, Weibel-Palade body (WPB) storage or susceptibility to ADAMTS13 proteolysis (Carew et al. 1990). The VWFpp is also reported to carry sulphated N-linked glycans (Fay et al. 1986).

1.2.7 Multimerization

Despite the acidic pH of the Golgi and lack of known molecular chaperones, pro-VWF dimers form 'head-to-head' multimers through N-terminal disulfide bonding. D3 domain cysteine residues Cys1099 and Cys1142 were identified as critical to this process as alanine mutagenesis of either of these residues abrogated multimer assembly (Purvis et al. 2007). VWF multimerization in the Golgi is strictly pH-dependent. Treatment of HUVECS (human umbilical vein endothelial cells) with ammonium chloride to raise the pH of the Golgi was found to completely abolish multimerization (Wagner et al. 1986). An optimal pH of 5.8 was defined for VWF multimerization *in vitro* (Mayadas and Wagner 1989). Since the Golgi lacks molecular chaperones, VWF multimerization involves a unique oxidoreductase mechanism that requires the participation of the VWFpp (Huang et al. 2009). At acidic pH, the propeptide functions as an endogenous chaperone facilitating inter-molecular disulfide formation. The propeptide contains vicinal cysteine residues (Cys159-Gly160-Leu161-Cys162 and Cys521-Gly522-Leu523-Cys524) that provide the required oxidoreductase activity (Mayadas and Wagner 1992).

A recent landmark publication provided new insight into the process of VWF multimerization and tubular packing for storage (Zhou et al. 2011). Electron microscopy revealed that at acidic pH equivalent to that of the Golgi, pro-VWF dimers zipped up to form "dimeric bouquets". Pro-VWF dimeric bouquets were zipped C-terminally from the A2 domain to the CTCK domain and at the N terminus by D1D2 homodimerization (figure 1.5). It was suggested that zipping positions the N-terminal D'D3 domains of pro-VWF dimers in close proximity to enable inter-molecular disulfide formation and

concomitant tubular assembly. This arrangement would also facilitate furin-mediated cleavage of the VWF propeptide which is the final intracellular VWF processing event.

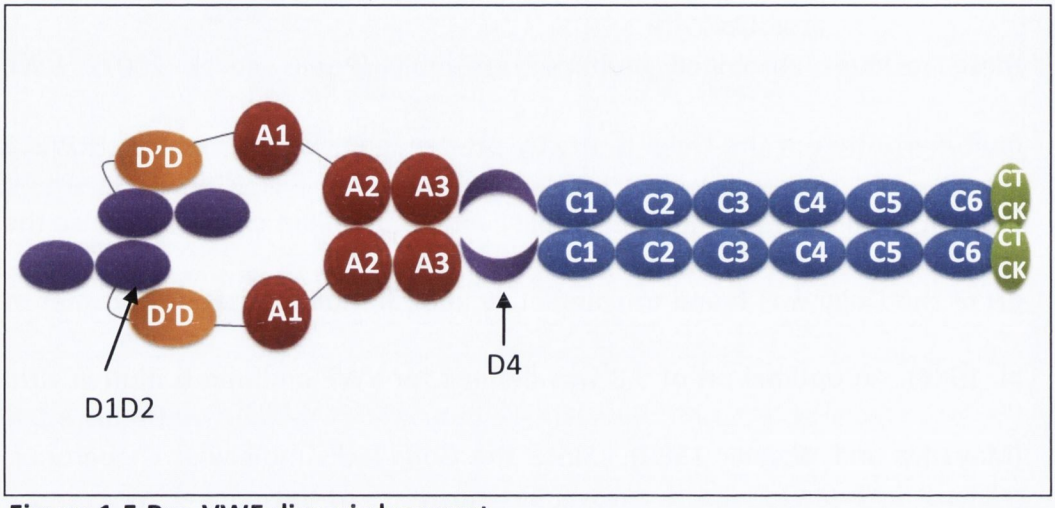


Figure 1.5 Pro-VWF dimeric bouquet.
At acidic pH equivalent to that of the Golgi, pro-VWF dimers zip up to form bouquet structures. Zipping facilitates multimer formation and tubular packaging.

1.2.8 Tubular packaging

In the trans-Golgi network, pro-VWF dimers are assembled into helical tubules (figure 1.7) that constitute an immature form of the Weibel-Palade body (WPB) (Zenner et al. 2007). Tubular assembly is both pH and Ca^{2+}-dependent and is necessary for ordered storage and compaction of long VWF multimers and also for efficient secretion. Interestingly, only the VWFpp and disulfide-linked D'D3 dimers are required for packaging (Huang et al. 2008). VWF tubules contain a repeating unit of one D'D3 dimer and two propeptides, arranged in a right-handed helix. Once formed, multiple VWF tubules align in

parallel giving mature WPBs their characteristic cigar-like shape (Berriman et al. 2009).

1.2.9 Propeptide cleavage

Endoproteolytic cleavage of the propeptide constitutes the final step in the post-translational modification of VWF. Propeptide cleavage appears to occur in the trans-Golgi network and WPBs, and is mediated by the Ca^{2+}-dependent enzyme furin (Rehemtulla and Kaufman 1992). Furin cleaves at a dibasic site between Arg763-Ser764 of the Lys762-Arg763-Ser764 motif present at the C-terminal end of the VWF D2 domain. Once cleaved, the propeptide is released into the plasma in equimolar amounts with mature VWF (Wagner et al. 1987). However, approximately 1% of circulating VWF contains uncleaved propeptide which prevents FVIII binding (Fay et al. 1986; Bendetowicz et al. 1998).

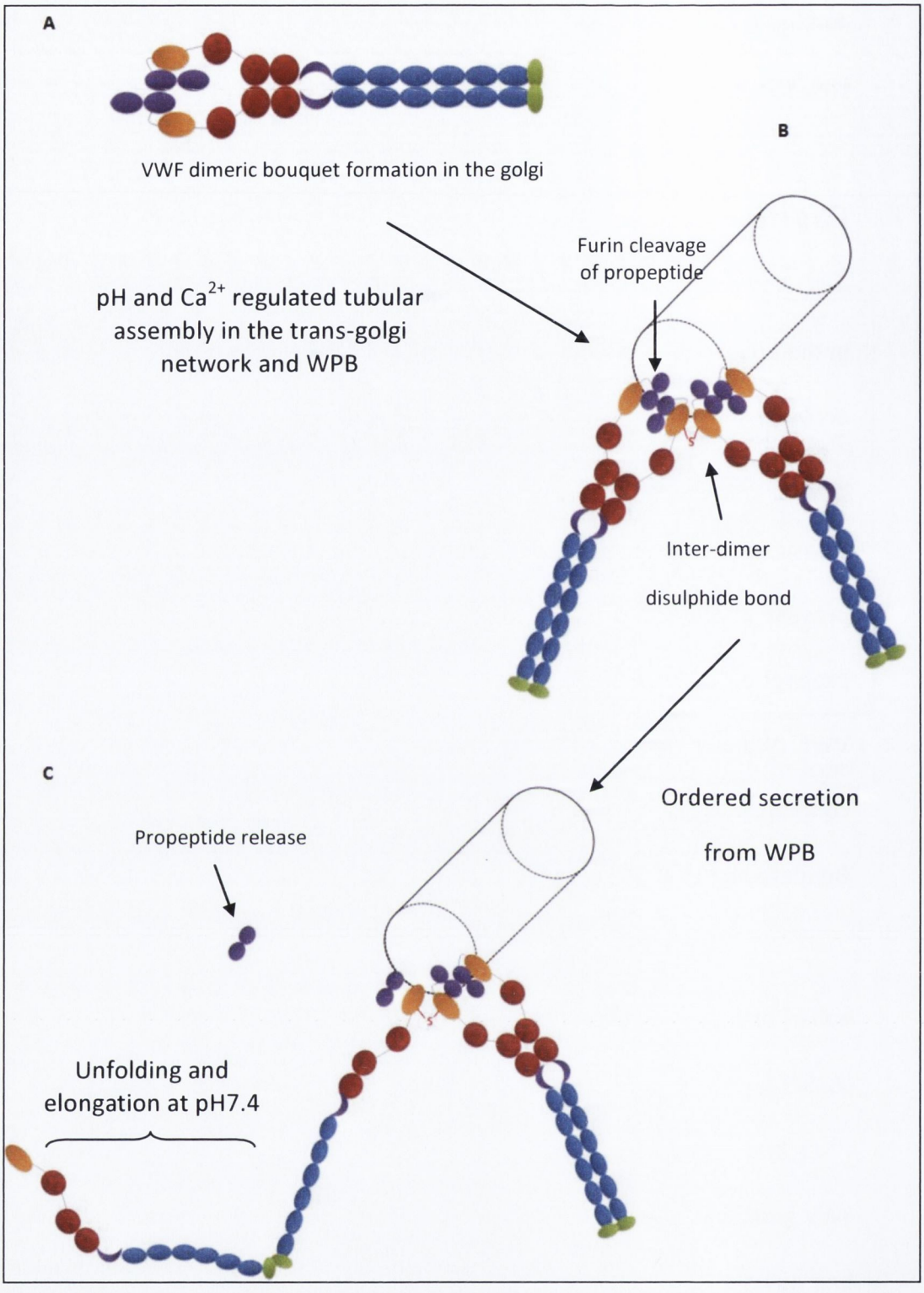


Figure 1.6 Overview of VWF multimerization and tubular packaging.
A) pH dependent dimeric bouquet. B) Assembly of dimeric bouquets into helical tubules. Helical assembly positions D'D3 domains in close proximity for multimer formation and concomitant tubular packaging. C) Ordered secretion of multimeric VWF and VWF propeptide from WPB.

1.3 VWF storage and secretion

Following biosynthesis, VWF is stored in the WPBs of endothelial cells and in platelet α-granules. Both organelles store HMW-VWF, which is then secreted through a regulated pathway following stimulation. Alternatively however, VWF produced by endothelial cells may also undergo constitutive secretion. Approximately 90% of the VWF present in endothelial cells was thought to be released via this constitutive secretory pathway serving to maintain circulating VWF levels at ~10μg/ml (Sporn et al. 1986). However, identification of an additional secretory pathway has demonstrated that the majority of endothelial cell derived VWF (~80%) actually undergoes basal secretion (Giblin et al. 2008). Basal secretion does not require stimulation and occurs from a post-Golgi organelle, presumably the WPB.

1.3.1 Weibel Palade Bodies

WPBs are large cigar-shaped, membrane bound organelles that are unique to endothelial cells. WPBs may be up to 0.3μm wide and 1-5μm in length (Valentijn et al. 2008) and their formation is dependent on VWF expression as endothelial cells of VWF deficient animals lack WPBs (Denis et al. 2001; Haberichter et al. 2005). As well as VWF, WPBs also contain a number of other proteins, including P-selectin (Bonfanti et al. 1989), osteoprotegerin (Zannettino et al. 2005), angiopoietin-2 (Fiedler et al. 2004) and others.

As VWF undergoes tubular packaging it is inserted into nascent WPBs via a process dependent on the presence of a clathrin coat and the adaptor protein AP-1 (Lui-Roberts et al. 2005; Lui-Roberts et al. 2008). Immature WPBs

are then trafficked to the periphery of the cell along microtubules where they become anchored to facilitate maturation (Manneville et al. 2003). Exocytic fusion with the plasma membrane occurs upon secretagogue stimulation and VWF is then released. Both WPB maturation and exocytic fusion are critically dependent on members of the Rab GTPase family (Hannah et al. 2003; Nightingale et al. 2009; Zografou et al. 2012).

Exocytosis from WPBs is tightly regulated but can be induced by a variety of secretagogues including thrombin (Birch et al. 1992), histamine (Hamilton and Sims 1987), phorbol myristate acetate (PMA) (Reinders et al. 1987)) and calcium ionophores (Loesberg et al. 1983) all of which raise intracellular calcium levels. In addition, VWF exocytosis can be triggered by increases in intracellular cyclic adenosine monophosphate (cAMP) levels using agents such as DDAVP (1-desamino-8-D arginine vasopressin). DDAVP is a synthetic analogue of the hormone vasopressin and is a specific agonist for vasopressin V2 receptors on endothelial cell membranes. DDAVP activates cAMP signalling to release VWF from WPB storage and is used often as a therapeutic agent in the management of VWD (Kaufmann et al. 2000). Qualitative differences in WPB secretion have been observed in response to intracellular Ca^{2+} and cAMP agonists (Vischer et al. 2000). While intracellular Ca^{2+} agonists induce release of both peripheral and central WPBs, cAMP-mediated signalling involves only WPBs that are located at the periphery of the cell. Thus cAMP-mediated signalling is likely to play a physiological role in regulating VWF plasma levels by releasing only fully mature VWF into the circulation.

1.3.2 Platelet α-granules

Platelet α-granules are the most abundant platelet secretory organelle, comprising ~10% of the total platelet volume (Blair and Flaumenhaft 2009). Each platelet contains 50-80 α-granules that range in size from 200-500nm (Frojmovic and Milton 1982). Upon platelet activation, α-granules fuse with the plasma membrane to release their cargo in a SNARE-dependent manner (Polgar et al. 2002). Immunoelectron microscopy demonstrated that VWF is located in the peripheral electronluscent zone of α-granules (Cramer et al. 1985). Within the α-granules, VWF has been shown to co-localize with other haemostatic proteins including fibrinogen, fibronectin and thrombospondin (Wencel-Drake et al. 1985). VWF release is triggered by several different agonists, including ADP, collagen and thrombin and proceeds via the regulated secretory pathway though constitutive secretion of VWF by primary guinea pig megakaryocytes has been demonstrated *in vitro* (Schick et al. 1997). However, the significance of constitutive VWF secretion from megakaryocytes in the context of maintaining VWF plasma levels is not known.

1.4 VWF structure and function

Following secretion, mature VWF circulates as a series of multimers containing a variable number of subunits (2-40), with the largest multimers having molecular weights in excess of 20,000kDa (Chopek et al. 1986). Larger VWF multimers are more haemostatically active than lower molecular weight forms. Because of this, the multimer distribution of circulating VWF is tightly

controlled by the action of metalloprotease ADAMTS13. ADAMTS13 cleaves haemostatically active high molecular weight VWF (HMW-VWF) into less active low molecular weight VWF (LMW-VWF). Thus mature VWF circulates in plasma as a heterogeneous mix of multimers.

1.4.1 VWF domain structure

Each VWF monomer exhibits considerable internal homology and is composed of a series of repeated domains (A-D). Historically the VWF domain structure was given as NH2-D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-COOH. Recently however, structural analysis of VWF via electron microscopy as well as sequence alignment analysis has led to the re-annotation and sub-division of the VWF domains (Figure 1.7) (Zhou et al. 2012).

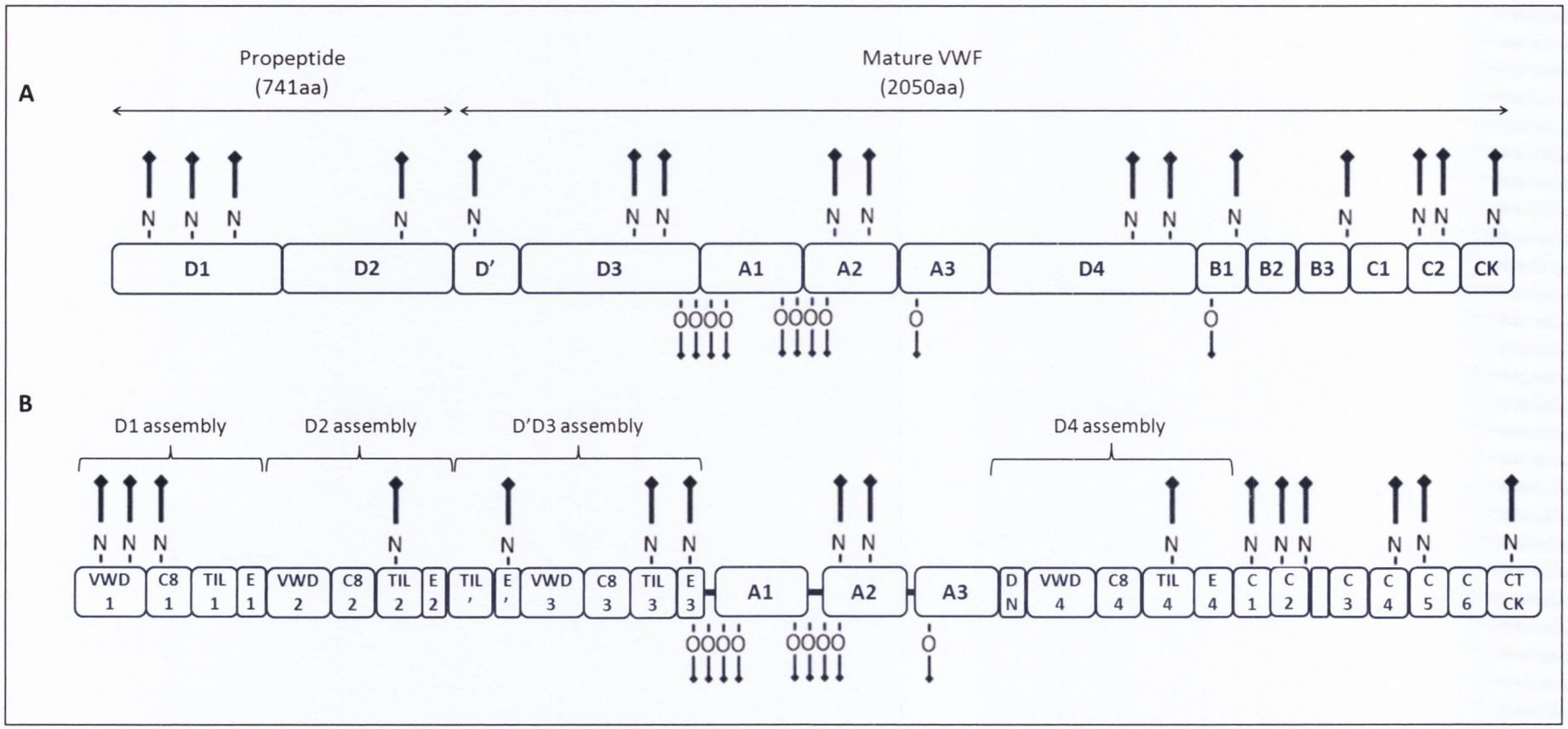


Figure 1.7 Domain structure of VWF.

A) Historical annotation of the VWF domains. B) Re-annotated VWF domain architecture.

1.4.1.1 D domains

The VWF D1 and D2 domains comprise the VWFpp while the mature subunit includes a truncated D' domain and complete D3 and D4 domains. Based on sequence alignment analysis the VWF D domains were subdivided into VWD, C8, TIL and E modules and are now referred to as D assemblies. D' is a truncated assembly containing only the TIL and E subdomains. The D1, D2 and D3 assemblies comprise VWD, C8, TIL and E subdomains, while the D4 assembly lacks the E subdomain but carries a unique subdomain structure designated D4N.

The major FVIII binding site has been localized to the first 310 residues of the D'D3 assembly (Foster et al. 1987; Takahashi et al. 1987). Mutations within this region inhibit FVIII binding and result in type 2N VWD (Jacquemin 2009). Interestingly, a significant number of type 2N VWD causing mutations have been found to cluster within the TIL' subdomain. Homology modelling revealed that the majority of these mutations are located at or near cysteine residues involved in intra-molecular disulfide bonding suggesting that structural conformational of the TIL' domain is critical for FVIII binding (Castro-Nunez et al. 2013). In addition, alanine mutagenesis identified both Ser764 and Lys773 of the TIL' domain as important FVIII binding residues.

1.4.1.2 A domains

The VWF A domain is the prototype for a protein superfamily. Von Willebrand A (VWA) domains are found in various plasma proteins including collagens VI, VII, XII and XIV, the I-domain of integrins and complement factors B, C2, CR3 and CR4 (Perkins et al. 1994). The VWF A1, A2 and A3 domains are well characterized and their crystal structures have been determined (Huizinga et al. 1997; Emsley et al. 1998; Zhang et al. 2009). Both the A1 and A3 domains contain intra-molecular disulfide bonds (A1 Cys1272-Cys1458; A3 Cys1686-Cys1872), which results in the formation of interchain loops of ~185 amino acid residues. The A2 domain is similar to A1 and A3 at the primary sequence level but lacks the disulfide bonded loop. Instead, the VWF A2 domain expresses a rare vicinal disulfide bond between Cys1669-Cys1670 that plays a key role in stabilizing the domain (Luken et al. 2010). In addition, the presence of a Ca^{2+} binding site also serves to confer stability (Zhou et al. 2011; Xu and Springer 2012). Other unique features of the VWF A2 domain include the presence of an "α4-less loop" and a *cis*-peptide bond between Trp1644-Pro1645 that are thought to enable A2 to act as force-sensing domain (Zhang et al. 2009).

Binding sites for platelet GPIbα, heparin, and collagens I, III and VI have been localized to the A1 domain. The GPIbα binding site appears to comprise several distinct residues and sequences which are involved in GPIbα binding either directly as contact sites, or indirectly via conformational effects. The use of inhibitory peptides identified two short peptide sequences residues 1237-1251 and 1458-1471 located on either side of the interchain loop that are likely to be involved (Mohri et al. 1988). In addition, a cluster of

residues on the surface of the A1 domain was also identified, and six mutations in this region showed reduced ability to support platelet adhesion under flow (Cruz et al. 2000).

The heparin binding site has been localized to a 23 amino acid sequence between Tyr1328-Ala1350 within the A1 disulfide loop (Fujimura et al. 1987; Sobel et al. 1992). A second lower-affinity heparin binding site was also mapped to the first 272 residues of the mature subunit, adjacent to the FVIII binding site (Mohri et al. 1989). The VWF A1 domain also contains binding sites for collagens I, III and VI (Hoylaerts et al. 1997). While the major collagen III binding site has been localized to the VWF A3 domain, domain deletion studies have shown that the VWF A1 domain also supports collagen III binding (Lankhof et al. 1996). Mutagenesis studies also identified an important role for His1786 of the VWF A3 domain, as mutation of this residue abolished collagen III binding (Romijn et al. 2001).

The VWF A2 domain contains the proteolytic cleavage site for metalloprotease ADAMTS13. The scissile bond is located between Tyr1605-Met1606, cleavage of which releases two VWF fragments of 140kDa and 176kDa respectively (Berkowitz et al. 1987). This cleavage site constitutes a unique site for ADAMTS13 as a deletion mutant lacking the VWF A2 domain (ΔA2-VWF) displayed complete resistance to ADAMTS13 proteolysis (Lankhof et al. 1997).

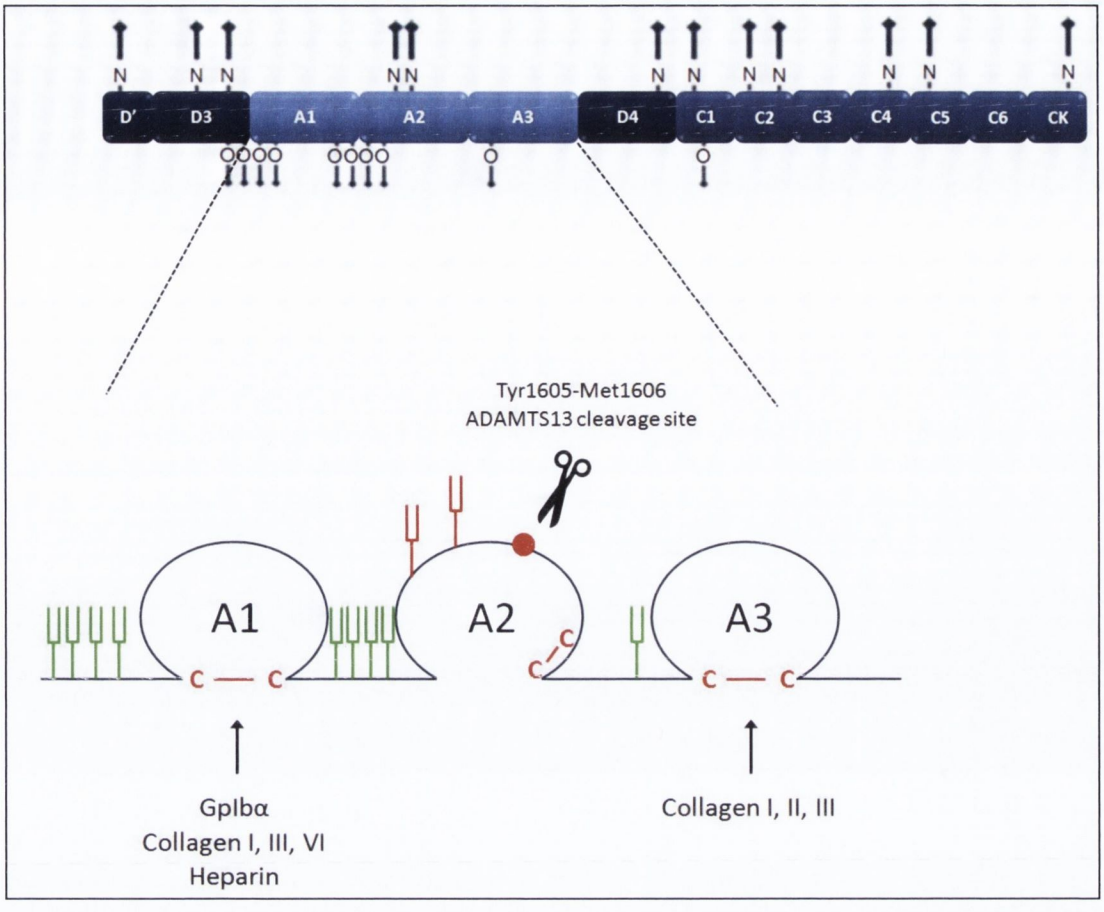


Figure 1.8 Domain diagram of the VWF A domains.
Disulphide bonded A1 and A3 loops are shown as well as the vicinal cysteines of the A2 domain. Major haemostatic ligands for each domain are indicated and the ADAMTS13 cleavage site is also highlighted. O-linked glycans are depicted in green, and N-linked glycans are depicted in red.

1.4.1.3 C domains

The former VWF B and C domains were re-annotated recently as 6 tandem von Willebrand C (VWC) and VWC-like domains (Zhou et al. 2012). VWC domains constitute one of the most common protein domain structures and have been identified in approximately 70 human proteins (Zhang et al. 2008). VWC domains are found in several highly glycosylated mucins and are also particularly common in matrix proteins that modulate growth factor responses by binding to bone morphogenetic proteins of the TGF-β family

(Probst et al. 1992; Xu et al. 1992; Bork 1993; Zhang et al. 2008). The VWF C1, C3 and C5 domains are classified as VWC domains by sequence homology while the intervening domains C2, C4 and C6 are designated the VWC-like domains (Bork 1993). The VWF C4 domain contains an RGD sequence that mediates binding to integrin $\alpha_{IIb}\beta_3$ (GpIIb-IIIa) on the surface of activated platelets (Beacham et al. 1992). The C4 domain RGD sequence has also been shown to mediate endothelial cell adhesion (Dejana et al. 1989).

VWF is extremely cysteine rich, with cysteine residues comprising 8.2% of the total amino acid content (Miseta and Csutora 2000). Cysteines are mainly found clustered in the N- and C- termini where they are involved in extensive intra- and inter-molecular disulfide bonding. Until recently, free cysteines were thought not to exist within the VWF molecule suggesting that all cysteine residues were involved in disulfide bonding (Shapiro et al. 2013). However, a proportion of both pd-VWF and recombinant VWF molecules have been reported to contain several unpaired cysteine residues (Choi et al. 2007; Ganderton et al. 2011). Mass spectroscopic analysis localized these to 9 sites, 7 of which are located in the VWF C domains; C2448, C2451, C2453, C2490, C2491, C2528 and C2533. Indeed, C2453 of the VWF C3 domain was identified as the most abundant unpaired cysteine in recombinant VWF (Ganderton et al. 2011). Despite these data, the presence of free cysteines within VWF remains the subject of some controversy. Nevertheless, it has been suggested that free cysteines may function to modulate lateral self-association of VWF via thiol-disulfide exchange (Ganderton et al. 2011). It has also been reported that initial pairing of these potential free cysteines is critical to ensure proper

folding within the ER and that unpairing must occur following exit from the ER or secretion into the plasma (Shapiro et al. 2013).

1.4.1.4 C-terminal cystine knot domain

The C-terminal cystine knot (CTCK) is a structural motif that contains six conserved cysteines arranged in a knot-like topology. CTCK domains are found in a diverse array of proteins including Norrie disease protein, mucins, connective tissue growth factor, and TGF-β. The VWF CTCK domain contains 11 cysteine residues, 8 of which have been chemically assigned and have been shown to mediate intra-molecular disulfide formation (Katsumi et al. 2000). Of the remaining cysteines, 2 are predicted to form an additional intra-molecular disulfide though this has not been confirmed, while the final cysteine Cys2773 mediates inter-molecular disulfide formation to facilitate C-terminal dimerization (Meitinger et al. 1993; Katsumi et al. 2000).

1.4.2 Additional VWF interactions

In addition to binding to proteins of the haemostatic system, VWF also binds a diverse array of matrix proteins as well as circulating plasma proteins. Though the functional significance of some of these interactions is not known, these interactions implicate roles for VWF outside of the haemostatic system.

VWF colocalizes with cell-adhesion molecule P-selectin in the WPBs of endothelial cells and the α-granules of platelets (Bonfanti et al. 1989). Following endothelial activation, P-selectin is expressed at the cell surface where it was proposed to function as an anchor for newly secreted VWF strings (Padilla et al. 2004). However this hypothesis was opposed by the observation that P-selectin inhibition had no effect on VWF string formation

(Huang et al. 2009). Subsequently it was suggested that $\alpha_V\beta_3$ integrin may constitute the major physiological ligand for endothelial tethering. VWF also binds another WPB and α-granule resident osteoprotegerin (OPG) (Zannettino et al. 2005; Chollet et al. 2010). OPG is a member of the tumour-necrosis factor (TNF) receptor family that acts as a decoy receptor for pro-apoptotic TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) to negatively regulate apoptosis. Once secreted, VWF and OPG circulate in complex via interactions mediated by the VWF-A1 domain, which serve to abolish the inhibitory effect of OPG on TRAIL-induced apoptosis (Shahbazi et al. 2007; Baud'huin et al. 2009). β2-Glycoprotein I (β2-GPI) also binds to the VWF A1 domain, and competitively inhibits binding to GPIbα, resulting in decreased platelet aggregation (Hulstein et al. 2007). Thrombospondin-1 (TSP-1) is an adhesive matrix glycoprotein that binds the VWF A3 domain (Pimanda et al. 2004; Wang et al. 2010). Binding of TSP-1 to ultra-large VWF multimers has been shown to prevent ADAMTS13-mediated proteolysis via competitive inhibition (Bonnefoy et al. 2006; Wang et al. 2010).

VWF also directly interacts with several proteins involved in inflammation. VWF interacts with P-selectin glycoprotein-1 (PSGL-1) and activated β_2 integrins to facilitate leukocyte recruitment (Pendu et al. 2006). PSGL-1 promotes initial transient binding, while β_2 integrins participate in stable adhesion. VWF also has a critical role in platelet-mediated infected erythrocyte adhesion to activated endothelial cells, which may be important in the pathogenesis of cerebral malaria (Bridges et al. 2010). In addition, VWF binds to *Staphylococcus aureus* protein A during endovascular infection which may facilitate colonization (Hartleib et al. 2000; O'Seaghdha et al. 2006).

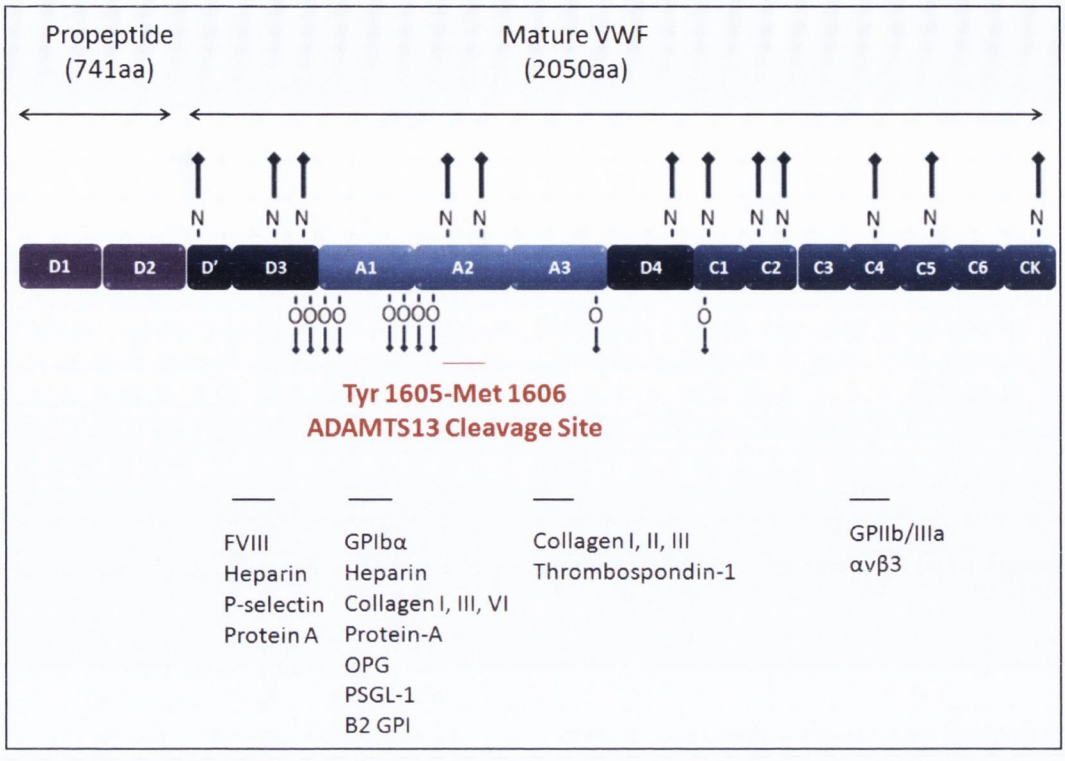


Figure 1.9 VWF structure-function.
VWF domain structure is shown with functional binding sites indicated.

1.5 Platelet-VWF

VWF is synthesized by both endothelial cells and megakaryocytes. Platelet-VWF (plt-VWF) is released from the α-granules upon platelet activation and constitutes 10–25% of the total VWF antigen present in normal platelet-rich plasma (Howard et al. 1974; Nachman and Jaffe 1975; Zucker et al. 1979). Though plt-VWF demonstrates similar subunit composition to plasma derived VWF (pd-VWF), a number of important differences have been described between plt-VWF and pd-VWF in terms of post-translational modification and functional activity (Williams et al. 1994). Multimer gel analysis has demonstrated that plt-VWF is enriched in haemostatically active ultra-large multimers not found in pd-VWF (Fernandez et al. 1982). In addition, plt-VWF was found not to exhibit the triplet pattern associated with ADAMTS13 proteolysis that is characteristic of pd-VWF (Fernandez et al. 1982). The glycosylation profiles of plt- and pd-VWF also differ significantly (Williams et al. 1994; Kagami et al. 2000). In particular, plt-VWF was found to express up to 50% less sialic acid and 50% galactose than its plasma-derived counterpart (Williams et al. 1994). Furthermore, while up to 13% of the N-linked and ~1% of the O-linked glycans of pd-VWF express terminal ABO(H) blood group antigens, they have not been described on the glycan structures of plt-VWF. Although, ABO(H) expression is a major determinant of VWF plasma levels, it appears not to have no effect on plt-VWF concentration.

The functional properties of plt-VWF have been compared to those of pd-VWF (Williams et al. 1994). Though plt-VWF and pd-VWF bound collagen I with similar affinities, plt-VWF bound GPIbα significantly less well than pd-VWF. In contrast, platelet-VWF displayed enhanced affinity for both GpIIbIIIa

and unfractionated heparin. Additionally, plt-VWF exhibits significant resistance to ADAMTS13 mediated proteolysis compared with pd-VWF (McGrath et al. 2013). The mechanisms responsible for the differences in functional activity of plt-VWF compared to pd-VWF have not been defined, but it seems likely that glycosylation differences may play a key role.

1.6 Shear-dependent regulation of VWF function.

VWF-mediated platelet capture at sites of vascular injury is initiated by interactions between VWF and components of the subendothelial matrix. VWF binds subendothelial collagen via binding sites in its A1 and A3 domains and once tethered, captures platelets via GPIbα of the GPIb-IX-V complex (figure 1.11). This ultimately leads to formation of a platelet plug and cessation of bleeding. However, the haemostatic function of VWF is critically dependent upon shear stress, as platelet binding requires exposure of the cryptic GPIbα binding site buried within the VWF A1 domain. At low levels of shear stress (10-1000s^{-1}), VWF circulates as a globular 'ball of string' of approximately 2μM in size (Schneider et al. 2007). In this conformation VWF does not bind readily to components of the vessel wall or to platelets. As shear rates increase from low physiological rates (less than 600 s^{-1}) to high physiological rates (up to ~1,500^{s-1}) to pathological rates (up to ~10,000s^{-1}), VWF undergoes elongational conformational change to form an extended chain of linked subunits. Elongation exposes the cryptic GPIbα binding site which is crucial for high-shear platelet binding. Initially, platelets tether to and translocate across immobilized VWF, but do not firmly adhere (Savage et al. 1996). Firm adhesion requires platelet activation that switches platelet

receptor GPIIb/IIIa to a high-affinity state. Binding of GPIIb/IIIa to tethered VWF is more secure and leads to platelet spreading and aggregation, and ultimately to the formation of a stable platelet plug (Ikeda et al. 1991).

Counter-intuitively, high shear-stress enhances platelet adhesion to tethered VWF. Atomic force microscopy has delineated the mechanism by which shear-stress regulates the VWF/GPIbα interaction and has also demonstrated the biphasic catch/release nature of the interaction (Yago et al. 2008). Under conditions of shear stress VWF binds GPIbα. As shear forces approach an optimal level, VWF/GPIbα bond lifetimes are prolonged (catch bonds) facilitating platelet-rolling and translocation across the VWF surface. Platelet-rolling serves to decrease platelet velocity, increasing contact time with VWF to facilitate GPIIb/IIIa-mediated firm adhesion. As shear forces increase beyond a critical threshold, bond lifetimes become shortened (slip bonds) leading to faster platelet rolling, preventing platelet accumulation.

A number of "gain-of-function" mutations in the VWF A1 domain have been demonstrated that overcome the requirement for shear-stress activation. These variants therefore spontaneously bind to GPIbα with enhanced affinity. Type 2B VWD is characterized by decreased HMW-VWF and fluctuating levels of thrombocytopenia with the presence of circulating platelet aggregates (Casari et al. 2013).

In vitro, the effects of shear stress may be mimicked by utilizing ristocetin. Ristocetin is a glycopeptide antibiotic that binds to proline rich sequences Cys1237–Pro1251 and Glu1463–Asp1472 flanking the A1 disulfide loop (Girma et al. 1990; Berndt et al. 1992; Azuma et al. 1993). Though the exact mechanism is unknown, ristocetin binding is thought to induce allosteric

changes in VWF that expose the GPIbα binding site in the absence of shear stress (Dong et al. 2001). In addition, ristocetin binding also exposes the cryptic ADAMTS13 cleavage site located in the VWF A2 domain (Chen et al. 2012).

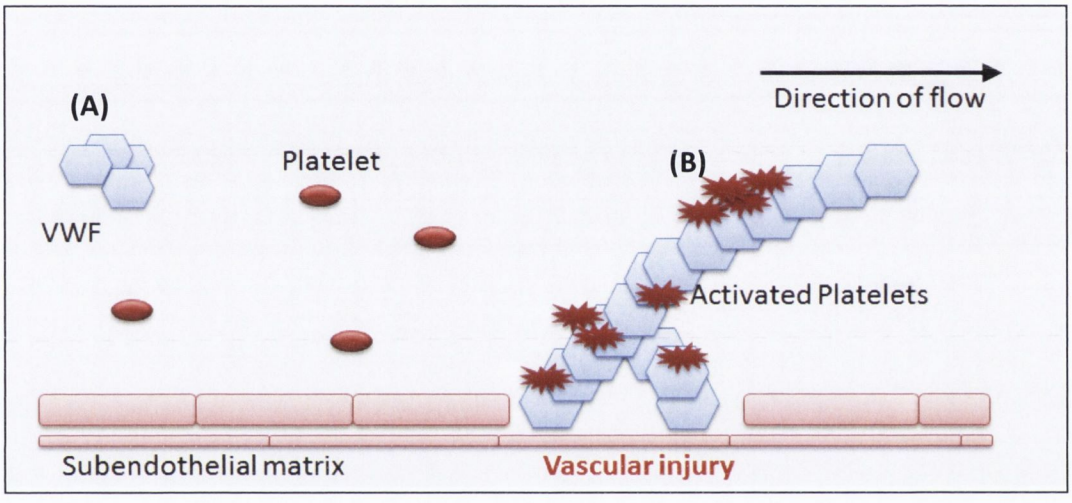


Figure 1.10 VWF-mediated platelet capture at sites of vascular injury.
A) In the absence of shear-stress VWF circulates in its globular form. B) At sites of vascular injury, exposure of subendothelial collagen causes VWF to become tethered. VWF then unwinds in a shear stress dependent manner to adopt an elongated conformation to mediate platelet capture.

1.7 VWF glycosylation and function

Accumulating data demonstrate that VWF glycosylation is of direct physiological relevance. In particular, VWF glycan structures influence circulatory half-life, and modulate susceptibility to ADAMTS13 mediated proteolysis. However, the molecular mechanisms through which VWF glycans modulate these effects remain unclear.

1.7.1 Terminal glycosylation modulates VWF clearance

1.7.1.1 ABO blood group

VWF terminal glycosylation regulates survival in the plasma. Importantly, even minor modification of terminal glycan structures dramatically impacts VWF survival. In humans, VWF clearance is regulated by terminal expression of ABO(H) blood group antigens on VWF. Approximately 13% of the N-linked and 1% of the O-linked VWF glycans carry these ABO(H) structures which differ only with respect to the addition of a single terminal sugar residue to the core H structure. Despite this relatively minor change in carbohydrate structure ABO blood group has a profound effect on plasma VWF levels. Blood group O individuals have 25% less circulating VWF compared with non-O individuals (group A, B and AB). VWF plasma levels are further reduced in individuals with the rare Bombay phenotype who do not express the core H structure (O'Donnell et al. 2005; Gallinaro et al. 2008). Convincing evidence suggests that expression of ABO(H) blood group determinants modulate VWF survival. For example, normal individuals with blood group O have a significantly shorter VWF plasma half-life following DDAVP infusion compared with non-O individuals (Group O vs non-O: 10.0 vs 25.5 hours respectively). Moreover, group O individuals also demonstrate significantly higher VWF:pp/VWF:Ag ratios, which are also consistent with enhanced VWF clearance (Haberichter et al. 2006; Gallinaro et al. 2008). Though the mechanisms underlying this ABO effect on VWF clearance remain unknown, recent studies have demonstrated significant over-representation

of blood group O in patients with type 1 VWD (Goodeve et al. 2007; James et al. 2007).

1.7.1.2 Sialic acid

The importance of terminal sialic acid expression in modulating VWF plasma clearance has been illustrated in a number of animal models. Removal of terminal sialic acid with subsequent exposure of penultimate galactose residues, targets VWF for rapid clearance by the asialoglycoprotein receptor (ASGPR). Consequently, enzymatic desialylation of pd-VWF markedly reduced VWF half-life in New Zealand White rabbits (Sodetz et al. 1977). Additionally, in transgenic mice, aberrant VWF glycosylation was also shown to cause enhanced VWF clearance. Genetic inactivation of the sialyltransferase ST3Gal-IV in C57Bl mice resulted in a 2-fold reduction in the VWF half-life (Ellies et al. 2002). ST3Gal-IV is the enzyme is responsible for terminal addition of α2-3 linked sialic acid to a penultimate Gal or GalNAc residues. Inactivation of the enzyme, with subsequent exposure of subterminal galactose residues led to enhanced ASGPR-mediated VWF clearance. Further evidence supporting the importance of VWF sialylation in regulating plasma clearance was also reported in the RIIIS/J mouse strain. In these mice, aberrant expression of a specific N-acetylgalactosaminyltransferase, B4GALNT2 in endothelial cells resulted in pathological VWF glycosylation, significantly enhanced hepatic clearance, and ultimately resulted in VWF plasma levels that were reduced by 20-fold compared to other inbred mouse strains (Mohlke et al. 1999).

1.7.2 VWF glycosylation modulates susceptibility to ADAMTS13 proteolysis

1.7.2.1 ABO blood group

In addition to modulating VWF clearance, terminal expression of ABO(H) blood group antigens also regulates susceptibility to ADAMTS13 mediated proteolysis. Blood group O VWF is cleaved more rapidly than non-O VWF in the order O≥B≥A≥AB (Bowen 2003). Furthermore, Bombay VWF is proteolysed significantly faster than group O VWF (O'Donnell et al. 2005). Interestingly however, terminal ABO(H) antigen expression may only indirectly affect proteolysis since enzymatic removal of terminal sialic acid ablates the differential ABO effect.

1.7.2.2 Sialic acid

Expression of terminal sialic also influences VWF susceptibility to ADAMTS13 proteolysis. While α2-6 linked sialic acid expression was shown to protect VWF against proteolysis by plasma serine and cysteine proteases such as chymotrypsin and cathepsin B, it specifically enhanced susceptibility to cleavage by ADAMTS13 (McGrath et al. 2010). Moreover, differential sialic expression on plt-VWF compared with pd-VWF confers specific resistance to ADAMTS13 proteolysis (McGrath et al. 2013).

Site directed mutagenesis has demonstrated that glycan variation at particular N-linked sites may also be of specific importance in the context of ADAMTS13 mediated proteolysis. Mutation of Asn1574, with subsequent elimination of the associated glycan chain resulted in markedly enhanced

susceptibility of VWF to ADAMTS13 proteolysis. In contrast, mutation of neighbouring glycan Asn1515 had no such effect (McKinnon et al. 2008).

1.8 VWF-binding lectins

Despite the critical role played by VWF glycans structures in modulating functional activity, the ability of VWF to interact with carbohydrate binding proteins (lectins) in normal plasma is not well described. Recently however, a number of novel VWF/lectin interactions have been reported that may be important in this setting.

1.8.1 Galectins

Galectins are a family of carbohydrate binding proteins characterized by their affinity for β-galactoside containing glycans. They are ubiquitously expressed and function both intra- and extracellularly. Though many galectins are secreted, they lack a signal peptide required for classical secretion and are thought to be secreted via a non-classical secretory pathway (Hughes 1999). The galectin carbohydrate recognition domain (CRD) is approximately 130 amino acids in length and unlike the CRD of C-type lectins, binds carbohydrate moieties in a Ca^{2+}-independent manner. The CRDs of individual galectins differ substantially in their carbohydrate binding specificity though all recognize the basic N-acetyllactosamine (LacNAc) unit (Hirabayashi et al. 2002). Glycan determinants that constitute preferential ligands for galectin binding are abundantly expressed on both the N- and O-linked glycans of VWF. Indeed recent *in vitro* binding studies have identified both galectins -1 and -3 as novel binding partners for VWF (Saint-Lu et al. 2012).

1.8.1.1 Galectin-1

Encoded by *LSGALS1* on the long arm of chromosome 22 (22q13.1) galectin-1 is classed as a prototype galectin. It is expressed as a 14 kDa monomer, but functions as a non-covalent homodimer with two equivalent CRDs. Galectin-1 contains 6 cysteine residues which under oxidizing conditions form 3 intra-molecular disulfide bonds. In its oxidized form galectin-1 possesses no lectin binding activity though it plays important roles in central nervous system regeneration following injury (Outenreath and Jones 1992). Under reducing conditions, galectin-1 has been shown to mediate a diverse array of biological functions including T cell homeostasis and platelet activation in a carbohydrate dependent manner (Perillo et al. 1995; Pacienza et al. 2008).

1.8.1.2 Galectin-3

Galectin-3 encoded by *LGALS3* located on chromosome 14q22.3 is the sole chimera type galectin. As well as its CRD, galectin-3 carries a unique proline- and glycine-rich N-terminal collagen-like domain. Galectin-3 is expressed as a monomer but forms higher order multimers up to pentamers for high avidity glycan binding (figure 1.11) (Ahmad et al. 2004). Until recently, higher order multimers were thought to be formed exclusively through N-terminal interactions. However, it has recently been demonstrated that ligand binding can also induce galectin-3 oligomerization through the CRD (Lepur et al. 2012). The galectin-3 N-terminal domain contains two serine phosphorylation sites at positions 6 and 12 and phosphorylation status dictates sugar binding affinity. Phosphorylation of Ser6 led to an 85%

reduction in sugar binding compared with de-phosphorylated galectin-3, suggesting that phosphorylation acts as an "on-off" switch for lectin activity (Huflejt et al. 1993; Mazurek et al. 2000).

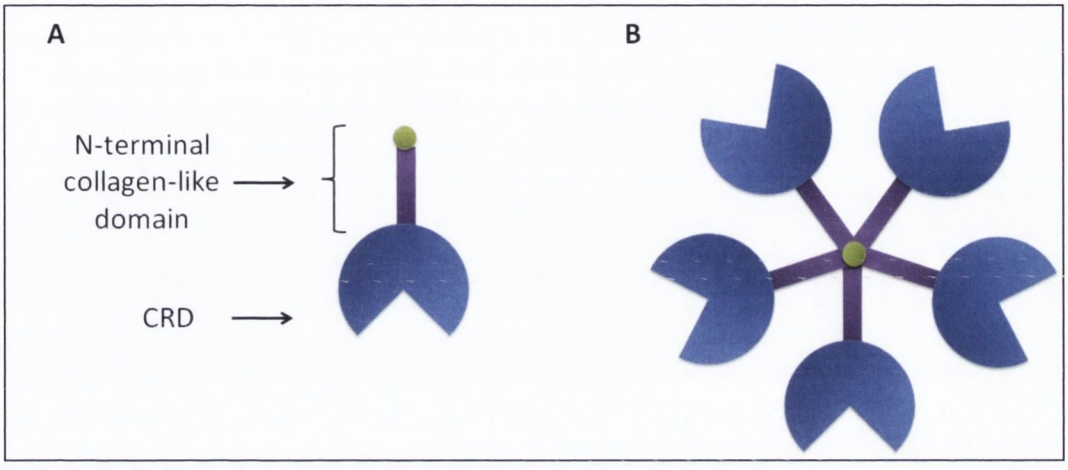


Figure 1.11 Domain diagram for chimera-type galectin-3.
A) Galectin-3 is composed of a C-terminal CRD as well as a unique N-terminal collagen-like domain. B) Galectin-3 forms higher order multimers predominantly through its N-terminal domains.

1.8.2 Galectins in thrombosis and haemostasis

Both galectin-1 and galectin-3 have been proposed to mediate an array of diverse biological processes including cell adhesion, inflammation and immunity (Hughes 2001; Almkvist and Karlsson 2004; Rabinovich and Toscano 2009). Several studies have identified galectin-1 as a key regulator of T-cell activation and function (Perillo et al. 1995; Chung et al. 2000), while galectin-3 has been identified as an important mediator of angiogenesis (Nangia-Makker et al. 2000; Markowska et al. 2010). In addition, galectin-3 was previously shown to function as an opsonin to enhance macrophage-mediated clearance

of apoptotic neutrophils (Karlsson et al. 2009). More recently, putative roles for specific members of the galectin family have also been described in relation to thrombosis and haemostasis (Romaniuk et al. 2010; Romaniuk et al. 2012; Saint-Lu et al. 2012; Zappelli et al. 2012). Galectin-1 was identified as a novel mediator of platelet activation, triggering platelet activation in a carbohydrate-dependent manner, via the α_{IIb} subunit of $\alpha_{IIb}\beta_3$ (Romaniuk et al. 2012). Thus, galectin-1 deficient mice demonstrated prolonged bleeding times as well as impaired platelet $\alpha_{IIb}\beta_3$ signalling (Romaniuk et al. 2012).

A recent report described galectins-1 and -3 as novel binding partners for VWF (Saint-Lu et al. 2012). Both galectin-1 and galectin-3 bound immobilized VWF in a dose-dependent manner and glycan-dependent manner and directly modulated VWF functional activity. Enzymatic removal of the VWF N-linked glycans via exoglycosidase digestion demonstrated that the N-linked glycans were the predominant mediators of the interaction. Additional *in vitro* experiments demonstrated colocalization of VWF with galectin-1 and galectin-3 within endothelial cells. In addition, immunoprecipitation studies revealed that VWF circulated in plasma in complex with both galectin-1 and galectin-3. Moreover, *in vivo* studies demonstrated that VWF acts as a chaperone molecule for both galectins within the circulation. $VWF^{-/-}$ mice were shown to have a two-fold reduction in circulating galectin-1 and galectin-3 compared to wild-type mice. However, plasma galectin levels were corrected following hydrodynamic gene transfer of VWF. Importantly, galectin binding was shown to directly modulate VWF function. In the presence of galectin inhibitor lactose, significantly more VWF-dependent platelet strings were observed on the surface of cultured human umbilical vein endothelial

cells (HUVECs). In addition, more platelets bound per string were noted. Cumulatively, these data suggest a novel role for the galectins as negative modulators of VWF-platelet string formation at the endothelial cell surface.

These *in vitro* findings were further supported by *in vivo* experiments. Intra-vital microscopy revealed that fewer platelet strings formed in the mesenteric venules of Gal-1/Gal-3 dual knockout mice compared to wild-type mice upon endothelial stimulation. Finally, galectins also appear to contribute to early stage VWF-dependent thrombus formation *in vivo*. In a ferric-chloride induced thrombosis model Gal-1/Gal-3 dual knockout mice formed thrombi more rapidly than wild-type mice suggesting that more efficient VWF-platelet string formation in the absence of galectins promotes faster thrombus formation in these mice.

1.9 VWF clearance

Though the biochemical pathways underlying VWF synthesis and secretion are well defined, there is limited knowledge available regarding the pathways governing VWF clearance. While significant progress has recently been made with regard to identifying cell types and receptors involved in the VWF clearance pathway, the molecular mechanisms underlying clearance remain unclear. In particular, the pathological mechanisms responsible for modulating enhanced VWF clearance remain unknown despite the fact that enhanced VWF clearance has emerged as an important pathogenic mechanism in Type 1 VWD.

1.9.1 Factors affecting VWF plasma levels

VWF circulates in plasma at a concentration of 10μg/ml though a wide range is observed within the normal population. VWF plasma levels can vary from 5-20μg/ml and are influenced by a variety of genetic and acquired factors. This variation has significant clinical implications as it is often difficult to distinguish normal individuals at the lower end of the VWF distribution curve from those with milder forms of VWD.

1.9.1.1 Acquired determinants of VWF plasma levels

The effects of several acquired or environmental factors on VWF plasma levels are well described. VWF is one of several haemostatic proteins that respond as acute phase reactants. Consequently, elevated plasma levels are observed in patients with acute infectious illness (Pottinger et al. 1989). Transient VWF elevation has also been documented following periods of increased physical activity (Prentice et al. 1972). Chronic elevation of VWF plasma levels is seen in patients with hyperthyroidism (Liu et al. 1993), while hypothyroidism is associated with lower plasma levels (Dalton et al. 1987). Increased VWF levels have also been reported in response to increasing levels of oestrogens following hormone replacement therapy, oral contraceptive use and during pregnancy (Clark et al. 1998; Kadir et al. 1999). In addition, VWF plasma levels increase by 1–2% per year of age. This has been documented in normal individuals as well as in patients with Type 1 VWD in whom the age-related rise eventually serves to normalize plasma VWF levels (Conlan et al. 1993; Coppola et al. 2003).

1.9.1.2 Genetic regulation of VWF plasma levels

VWF plasma levels are strongly influenced by genetic determinants though estimates of the heritability of plasma VWF levels range from ~30% to 75% (de Lange et al. 2001; Souto et al. 2003). Early twin studies demonstrated that up to 66% of the variability in VWF plasma levels was genetic, with 30% of this variation directly attributable to ABO blood group locus (Orstavik et al. 1985). More twin recent studies have yielded conflicting results with the estimated heritability of plasma VWF levels as high as 75% in British twins (de Lange et al. 2001) or low as 53% in Danish twins (Bladbjerg et al. 2006). Additional analysis in Spanish families calculated heritability of just 32% (Souto et al. 2003).

1.9.1.2.1 Polymorphic variation in the VWF gene regulates VWF plasma levels

The *VWF* gene is highly polymorphic containing more than 1,100 single nucleotide polymorphisms (SNP). To date, over 150 unique SNPs have been identified within the VWF coding sequence or in closely flanking intronic sequences (Hampshire et al. 2010). Consequently, several studies have examined the impact of polymorphic variation in the *VWF* gene on the regulation of VWF plasma levels (Smith et al. 2010; van Loon et al. 2012; Desch et al. 2013). Though the results of these studies are somewhat conflicting, they have consistently demonstrated significant association between SNP rs1063856 in the *VWF* gene and VWF plasma levels. This SNP in exon 18 results in a threonine to alanine substitution at residue 789 in the

VWF D' domain and is significantly associated with increased VWF plasma levels (Lacquemant et al. 2000; Klemm et al. 2005).

SNPs located in the non-coding region of the VWF also contribute to the genetic variability in VWF plasma levels. For example, polymorphic variation in the 5' regulatory region of the VWF gene has been shown to of importance in this regard. Three specific single nucleotide polymorphisms (SNPs) were identified in the VWF promoter (C/T -1234, A/G -1185, and G/A -1051) that displayed strong linkage disequilibrium and segregated as two distinct haplotypes (Haplotype 1: C1234 /A1185 / G1051 and Haplotype 2: T1234 / G1185 / A1051). Plasma VWF levels varied according to SNP genotype, such that significantly higher plasma VWF levels were found in individuals homozygous for haplotype 1 compared with heterozygotes. The lowest plasma VWF levels were described in haplotype 2 homozygotes (Keightley et al. 1999). More recently, eight novel VWF promoter changes were described in Type 1 index cases by the large Canadian multi-centre study (James et al. 2007). Three further novel changes were subsequently described by the European Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand Disease (MCMDM-1VWD) study confirming a role for polymorphic variation in the 5' regulatory region of the VWF gene in modulating VWF plasma levels (Goodeve et al. 2007).

A GT-tandem repeat element in the VWF promoter region has also been shown to contribute to genetic variation in VWF plasma levels (Hickson et al. 2011). Individuals homozygous for small alleles with (repeat length <20) had significantly lower plasma VWF levels compared with individuals homozygous for larger alleles (repeat length >20). This is in keeping with

previous reports that the length of GT-tandem repeat influences shear-stress enhancement of VWF promoter activity. Longer GT repeats were shown to induce larger increases in VWF promoter activity compared with shorter repeats under conditions of shear stress (Hough et al. 2008). Taken together, the above reports suggest that transcriptional regulation is an important genetic determinant of VWF plasma levels.

1.9.1.2.2 Contribution of ABO blood group to the regulation of VWF plasma levels

The ABO blood group association with VWF plasma levels is well defined and has been robustly confirmed by a number of linkage and association studies (Smith et al. 2010; Desch et al. 2013). Importantly, pedigree-based linkage analysis has shown that the *ABO* locus exerts a direct functional effect on VWF plasma levels, rather than acting in linkage disequilibrium with another modulatory locus (Souto et al. 2000). Consequently differential VWF plasma levels are likely to result from ABO(H) antigen expression on the VWF N-linked and O-linked glycans. Indeed, a dosage effect of the *ABO* locus has been demonstrated. Individuals heterozygous for the O allele (AO and BO individuals) had lower plasma levels than those not carrying an O allele (AA, BB, AB) (Shima et al. 1995). Furthermore, among group A individuals, the quantity of A antigenic determinant present on VWF was directly related to *ABO* genotype (O'Donnell et al. 2002). The highest levels of A-antigen expression were found in *A1A1* homozygotes, who also had significantly higher VWF plasma levels compared with *A1O1* and *A2O1* subjects.

Though several lines of evidence suggest that differential VWF clearance is responsible for the quantitative ABO effect on VWF plasma levels, the receptor responsible has not been identified. However, it is clear that the quantitative ABO effect on VWF plasma levels is of direct clinical relevance. Several reports have demonstrated increased risk in non-O individuals for ischaemic heart disease and venous thromboembolism (Koster et al. 1995). Conversely, significant over-representation of group O individuals has been reported in type 1 VWD (Goodeve et al. 2007; James et al. 2007).

1.9.1.2.3 Contribution of additional genetic loci to the regulation of VWF plasma levels

More recently, the contribution of genetic loci other than the VWF and ABO loci to the variability of VWF plasma levels has been established. GWAS studies identified several novel candidate loci that were significantly associated with VWF plasma levels including *STXBP5, STX2, TCN2, SCARA5, STAB2,* and *CLEC4M* (Smith et al. 2010). Three of the six novel loci (*SCARA5, STAB2,* and *CLEC4M*) identified encode scavenger receptors that may modulate VWF plasma levels by influencing VWF clearance. Interestingly, the total variance in plasma VWF levels explained by the significant loci in this study was estimated to be ~12.8%, which is considerably less than the estimate of 75% that was previously reported (de Lange et al. 2001). Additional linkage analysis, based on sibling structure subsequently identified chromosome region 2q12–2p13 as a major genetic determinant of VWF plasma levels (Desch et al. 2013). Importantly, the contribution of this region was found to be as important as that of the ABO blood group locus and

accounted for a significant proportion of the reported disparity in genetic variability. Despite this, candidate genes in these regions that contribute to the variation in VWF levels have not been identified. Similarly, significant associations between VWF plasma levels and chromosome regions 1p36.13, 2q23.2, 5q31.1, 6p22.3, and 22q11.1 have previously been reported but the identities of the genes responsible remain unknown (Souto et al. 2003).

The contribution of additional genetic loci to the regulation of VWF plasma levels is of particular significance given the results of recent type 1 VWD cohort studies. These studies consistently showed that while *VWF* gene mutations are observed in ~65% of Type 1 index cases, mutations were significantly more common in patients with plasma VWF levels less than 0.3U/mL. *VWF* gene mutations were identified in 75% of index cases with plasma VWF levels <0.30 U/mL, but only in 50% of cases with VWF levels >0.30 U/mL (James et al. 2007). Linkage analysis subsequently demonstrated that reduced plasma VWF levels in this latter cohort are mediated by mechanisms that are independent of the *VWF* gene (Eikenboom et al. 2006; James et al. 2006). Though the absolute contribution of individual genetic loci (other than the ABO locus) to the regulation of VWF plasma levels is minor (<5%), genetic variation at these loci may play a significant role in modulating plasma VWF levels in these patients.

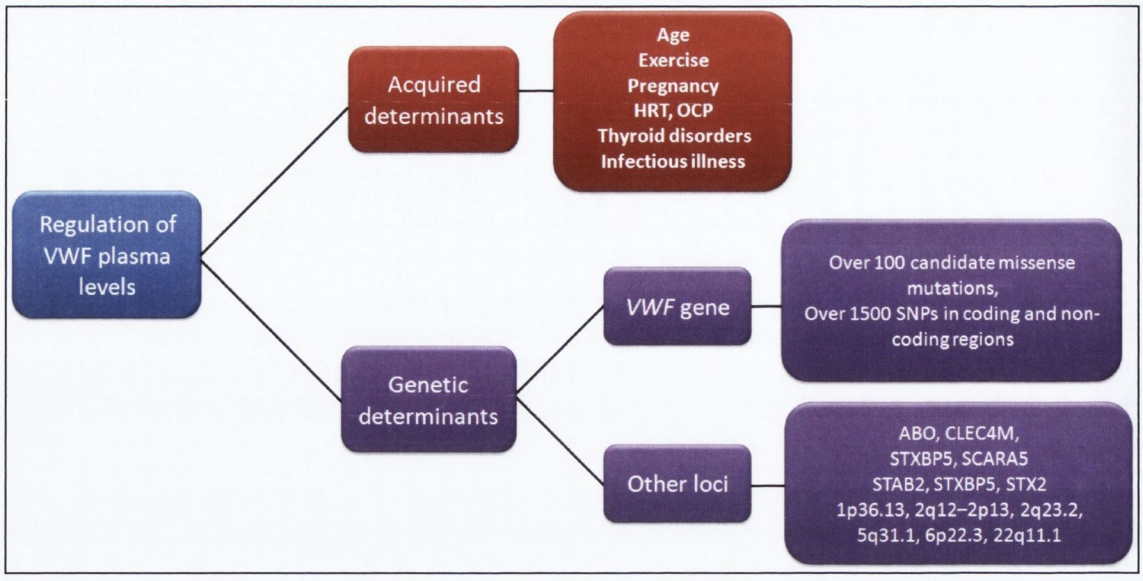


Figure 1.12 Overview of regulation of VWF plasma levels.
Acquired and genetic factors contribute to the regulation of VWF plasma levels.

1.9.2 Cell types involved in VWF clearance

Both the liver and spleen were previously identified as the most important organs involved in VWF clearance (Lenting et al. 2004; van Schooten et al. 2008). Biodistribution studies utilizing radiolabelled VWF demonstrated significant accumulation of radioactivity within the liver compared to other organs (Lenting et al. 2004). Indeed 40-60% of the total organ radioactivity was attributable to the liver (van Schooten et al. 2008). However, by comparing the relative efficiency with which organs take up VWF (VWF uptake per weight of tissue) the spleen was also identified as an efficient participant in the VWF clearance pathway. Immunohistochemical analysis of livers and spleens of $VWF^{-/-}$ mice following VWF infusion demonstrated colocalization of VWF with the macrophage populations of these organs (van Schooten et al. 2008). In particular, VWF colocalized with CD68+ Kupffer cells in the liver and with F4/80 macrophages in the red pulp of

the spleen. VWF was subsequently shown to directly interact with monocyte-derived and THP-1 macrophages *in vitro* (van Schooten et al. 2008). Binding was followed by rapid VWF internalization and subsequent degradation. Furthermore, macrophages were identified as key mediators of VWF clearance *in vivo*. Chemical inactivation of macrophages via gadolinium chloride ($GdCl_3$) treatment resulted in significantly prolonged VWF survival. VWF half-life was prolonged ~ 1.5 fold in $GdCl_3$-treated $VWF^{-/-}$ mice compared with saline-treated controls. These data suggest that hepatic macrophages or Kupffer cells may play a critical role in regulating VWF clearance *in vivo*.

1.9.3 Candidate VWF clearance receptors

To date, several candidate VWF clearance receptors have been described. These include both glycan-dependent and glycan-independent receptors. Interestingly, however the absolute contribution of these candidate receptors to VWF clearance remains unclear. Clearance studies utilizing transgenic mice deficient in these receptors have demonstrated only modest increases in VWF plasma levels. VWF clearance therefore appears to represent a complex pathway, involving a network of receptors that mediate both physiological and pathological VWF clearance.

1.9.3.1 Asialoglycoprotein receptor

The asialoglycoprotein receptor (ASGPR) was the first VWF clearance receptor to be identified (Grewal et al. 2008). ASGPR is a Ca^{2+}-dependent lectin receptor that is abundantly expressed on the vascular face of

hepatocytes. Encoded by separate genes (*ASGR1* and *ASGR2*) on the short arm of chromosome 17, ASGPR is a hetero-oligomeric receptor composed of two homologous transmembrane polypeptides (Spiess and Lodish 1985). Isoforms of both subunits also exist due to alternative splicing of the primary transcripts. *ASGR1* gives rise to two alternatively spliced transcripts, the shorter of which lacks a transmembrane domain and is secreted as a soluble protein (Liu et al. 2010). *ASGR2* gives rise to five alternative transcripts encoding four different isoforms (a-d).

Asgr-1 and Asgr-2 contain four distinct domains; an N-terminal cytoplasmic domain; a transmembrane domain; an extracellular stalk region; and a CRD. Lectin binding activity is predominantly associated with the Asgr-1 subunit, and the crystal structure of the human Asgr-1 CRD has been described (Meier et al. 2000). It was shown to comprise three conserved disulfide bonds and three integral Ca^{2+} ions that are directly involved in ligand binding. Terminal β-linked galactose (Gal) and N-acetylgalactosamine (GalNAc) residues constitute preferential ASGPR ligands. However, ASGPR can also recognise particular sialylated structures including terminal α2-6 linked sialic acid which is abundantly expressed on both the N- and O-linked glycans of VWF (Ishibashi et al. 1994).

Although a role for ASGPR as a clearance receptor for exogenous asialoglycoproteins has been well defined for some time, until recently its physiological role remained elusive. Although mice deficient in either Asgr-1 or Asgr-2 exhibited decreased clearance of desialylated exogenous glycoproteins, they appeared not to accumulate endogenous asialoglycoproteins within the circulation (Ishibashi et al. 1994; Braun et al.

1996). The first endogenous ligands of the ASGPR were only recently described as components of the haemostatic system, including VWF and platelets (Grewal et al. 2008). ASGPR was shown to bind and remove VWF from the circulation and thereby to regulate normal VWF homeostasis. Mice lacking the Asgr-1 but not the Asgr-2 subunit had VWF plasma levels that were increased 1.5 fold compared to wild-type littermates (Grewal et al. 2008). However, increased plasma VWF levels were not associated with elevated levels of asialo-VWF in the circulation suggesting that the ASGPR also modulates clearance of normal sialylated VWF.

1.9.3.2 C-type lectin domain family 4 member M

Genetic variation in the *CLEC4M* locus has been significantly associated with plasma VWF levels in normal individuals (Smith et al. 2010). The *CLEC4M* locus located on chromosome 19 encodes CLEC4M (L-SIGN; liver/lymph node-specific intracellular adhesion molecules-3 grabbing non-integrin), a Ca^{2+}-dependent mannose-specific lectin (Soilleux et al. 2000). CLEC4M is homologous to another receptor DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin).

CLEC4M and DC-SIGN both function as pathogen recognition receptors and adhesion receptors. However, they differ with respect to ligand-binding ability and tissue distribution. CLEC4M is expressed by liver sinusoid endothelial cells and lymph nodes while DC-SIGN is expressed on immature dendritic cells and on specialized macrophages (Bashirova et al. 2001; Soilleux et al. 2002). Both lectins are organized into three distinct domains; an N-terminal transmembrane domain for cellular signalling, a C-type lectin CRD for

ligand binding; and a neck region that is essential for receptor tetramerization at the cell surface. The CLEC4M neck region is highly polymorphic and contains variable numbers of tandem repeats (VNTRs) of a highly conserved 23 amino acid sequence (Bashirova et al. 2001). VNTRs of 3 to 9 repeats (VNTR allele 3-9) have been described. Importantly, CLEC4M VNTR polymorphism significantly impacts ligand binding ability and thus modulates genetic susceptibility to a number of pathogens including HIV-1 (Liu et al. 2006; Li et al. 2012). Though the role of DC-SIGN as an endocytic receptor is well described, until recently CLEC4M was thought to function only as a pathogen recognition and adhesion receptor. However, convincing evidence now demonstrates that CLEC4M also functions as an endocytic receptor, binding and internalizing VWF to remove it from the circulation (Rydz et al. 2013).

Initial *in vitro* binding studies identified VWF as a novel ligand for CLEC4M. Binding of pd-VWF to immobilized recombinant CLEC4M was both dose-dependent and glycan-dependent, with a specific role for the VWF N-linked glycans (Rydz et al. 2013). Cell based studies subsequently demonstrated the ability of CLEC4M to internalize VWF. Human embryonic kidney 293 (HEK293) cells stably expressing CLEC4M (VNTR allele 7) efficiently bound and internalized VWF compared to control cells. Moreover, VWF and CLEC4M were found to colocalize with early endosomal antigen-1 confirming endocytosis of VWF/CLEC4M complex. Furthermore, *in vivo* studies also supported a role for CLEC4M in modulating VWF clearance. Hydrodynamic expression of the *CLEC4M* cDNA in C57/BL6 mice resulted in a 2-fold decrease in VWF plasma levels.

CLEC4M VNTR polymorphism modulates ligand binding. In keeping with this, genotyping of patients with type 1 VWD together with unaffected family members, demonstrated that CLEC4M polymorphism status is associated with VWF plasma levels (Rydz et al. 2013). Family-based association analysis revealed that the CLEC4M VNTR 6 was associated with reduced risk for VWD. VNTR 6 was associated with higher VWF plasma levels and VWF activity, and was more frequently found in unaffected family members compared with type 1 individuals. Importantly, although DC-sign was also shown to bind VWF *in vitro*, there is no evidence to suggest that DC-sign contributes to the genetic variation in VWF plasma levels.

1.9.3.3 Sialic acid Ig-like lectin 5

Siglec-5 (sialic acid Ig-like lectin 5) has been shown to mediate clearance of both VWF and FVIII *in vivo* (Pegon et al. 2012). Encoded by the *SIGLEC5* gene on the long arm of chromosome 19 (19q13.3), Siglec-5 is classified is an I-type lectin that contains four extracellular Ig-like domains (Cornish et al. 1998). The N-terminal V-set domain mediates sialic acid recognition and is followed by three C2-set domains. Determination of the crystal structure of the N-terminal extracellular domains has provided insight in to the linkage specificity of Siglec-5 (Zhuravleva et al. 2008). Siglec-5 was shown to bind α2-3 and α2-6 linked sialic acid and also recognized α2-8 linked sialic acid though to a lesser extent (Brinkman-Van der Linden and Varki 2000; Zhuravleva et al. 2008).

Siglecs are selectively expressed in cells of hematopoietic origin. Siglec-5 expression is limited to monocytes, macrophages, and neutrophils. It

is also weakly expressied on THP-1 and U937 monocytic cell lines (Cornish et al. 1998; Jandus et al. 2011). Pegon *et al* demonstrated that both VWF and FVIII bind Siglec-5 *in vitro* and that Siglec-5 functions as a clearance receptor for both proteins *in vivo*. *In vitro* binding assays revealed that both VWF and FVIII bound immobilized Siglec-5 in a dose-dependent and reversible manner (Pegon et al. 2012). Binding of Siglec-5 to VWF was sialic acid dependent as removal of VWF sialic acid via sialidase digestion resulted in >90% reduction in binding. In contrast however, Siglec-5 bound plasma-derived FVIII in a sialic acid independent manner. Enzymatic removal of FVIII sialic acid had no effect on Siglec-5 binding. Moreover, binding to recombinant B-domain deleted FVIII which lacks ~85% of the FVIII glycans chains was similar to that of plasma-derived FVIII. Siglec-5 was previously shown to bind Group B streptococcus β-protein in a sialic acid independent manner (Carlin et al. 2009). This interaction was mediated via the N-terminal V-set domain suggesting that the Siglec-5 lectin domain may also be involved in mediating protein-protein interactions.

Cell binding assays demonstrated binding of VWF and FVIII to cell-surface expressed Siglec-5 (Pegon et al. 2012). Immunofluorescent staining showed that VWF and FVIII bound significantly better to the surface of Siglec-5 expressing HEK293 cells compared with non-transfected controls. Furthermore, pre-treatment of Siglec-5 expressing 293 cells with sialidase to remove interfering sialic acid expressed by neighbouring cell surface glycoproteins served to significantly enhance VWF binding. Conversely, sialidase pre-treatment had no effect on the FVIII Siglec-5 interaction. Siglec-5 expressing 293 cells were also shown to internalize VWF and FVIII. Proximity

ligation analysis demonstrated colocalization of both VWF and FVIII with early endosomal marker EEA-1, a finding that was subsequently confirmed by confocal microscopy (Pegon et al. 2012). Furthermore, hydrodynamic expression of the Siglec-5 gene in C57Bl mice led to a significant reduction in VWF and FVIII plasma levels. VWF plasma levels were reduced ~70% in Siglec-5 positive mice compared to wild-type littermates while FVIII plasma levels were reduced by ~40% demonstrating that Siglec-5 is an endocytic receptor that modulates VWF and FVIII plasma levels *in vivo*.

1.9.3.4 Low density lipoprotein receptor-related protein 1

Macrophage low density lipoprotein receptor-related protein 1 (LRP1) was recently identified in two independent studies as a VWF clearance receptor (Castro-Nunez et al. 2012; Rastegarlari et al. 2012). LRP1 is a ubiquitously expressed scavenger receptor that participates in several cellular processes including lipid metabolism and intracellular signalling (Beisiegel et al. 1991; Bacskai et al. 2000). It consists of a 515kDa α-subunit and an 85kDa membrane-spanning β-subunit the larger of which lacks a transmembrane domain and remains associated with the smaller subunit via non-covalent interactions (Herz et al. 1990). The receptor is composed of five common structural domains; a ligand binding cysteine-rich complement-type repeats, epidermal growth factor (EGF) receptor-like cysteine-rich repeats; the β-propeller (YWTD) domains; a transmembrane domain; and a cytoplasmic domain. The ligand binding cysteine-rich complement-type repeats occur in clusters containing between two and eleven individual repeats. The major ligand-binding sites have been localized to clusters II and IV (Neels et al.

1999). No ligands besides LRP1 antagonist RAP (Receptor Associated Protein) are known to bind cluster I or cluster III.

To date, approximately 30 LRP1 ligands have been identified including coagulation factor FVIII (Herz and Strickland 2001). LRP1 was previously found to function as an endocytic clearance receptor that regulated FVIII plasma levels in mice. Several studies demonstrated that RAP mediated LRP1 antagonism significantly prolonged FVIII half-life in mice (Saenko et al. 1999; Schwarz et al. 2000; Turecek et al. 2000). Further clearance experiments in conditional LRP1-deficient mice confirmed a role for LRP1 in the FVIII clearance pathway. Conditional liver-specific LRP1 deletion prolonged FVIII half-life 1.5 fold and also resulted in increased plasma FVIII levels (Bovenschen et al. 2003). Surprisingly, elevated plasma VWF levels were also observed in these mice despite previous reports that VWF was unable to interact with LRP1 *in vitro* (Lenting et al. 1999; Saenko et al. 1999). Subsequent *in vitro* studies revealed that VWF does indeed interact with LRP1, but this VWF/LRP1 interaction is critically dependent upon shear-stress (Castro-Nunez et al. 2012; Rastegarlari et al. 2012). Perfusion experiments involving VWF-coated beads demonstrated shear stress-dependent adhesion to LRP1 coated surfaces (Rastegarlari et al. 2012). Furthermore, primary monocyte-derived macrophages bound and internalized VWF only under conditions of shear stress. Shear stress was also required for FVIII uptake in the presence of VWF (Castro-Nunez et al. 2012). A role for LRP1 in mediating VWF clearance was further demonstrated in mice with a macrophage specific LRP1 deficiency (macLRP1$^-$). MacLRP$^-$ mice had 1.5 fold increased plasma VWF levels and a ~1.7 fold prolongation in VWF half-life compared to macLRP$^+$ mice

(Rastegarlari et al. 2012). Interestingly, hydrodynamic expression of RAP in macLRP$^-$ mice had no additional effect in elevating plasma VWF levels, suggesting that macrophage LRP1 and not LRP1 expressed on other cell types is the primary modulator of VWF clearance in these mice.

Hydrodynamic expression of αMβ2-intergrin inhibitor NIF in both macLRP$^+$ and macLRP$^-$ mice also highlighted a potential role for αMβ2-intergrins in the VWF clearance pathway (Rastegarlari et al. 2012). NIF expression led to increased plasma VWF and FVIII levels in macLRP$^+$ mice but had no effect on VWF or FVIII plasma levels in macLRP$^-$ mice. These data suggest that αMβ2-intergrins may modulate VWF clearance, and that they to do so in an LRP1-dependent manner. This is consistent with previous reports that VWF is a binding partner for β_2 integrins and that LRP1 is required for optimal β_2 integrin function (Spijkers et al. 2005; Pendu et al. 2006).

VWF clearance is a complex process that involves multiple cellular pathways. Though several candidate VWF clearance receptors have now been identified, the molecular mechanisms governing VWF clearance for the most part remain unknown. The contribution of individual receptors to physiological and/or pathological VWF clearance has yet to be elucidated. In addition, the clearance receptor(s) responsible for the differential clearance of group O versus non-O VWF have not yet been identified. While VWF glycan variation and sequence variation are well defined regulators of VWF clearance, it is now clear that polymorphic variation in the activity or expression of candidate VWF clearance receptors may also regulate VWF clearance.

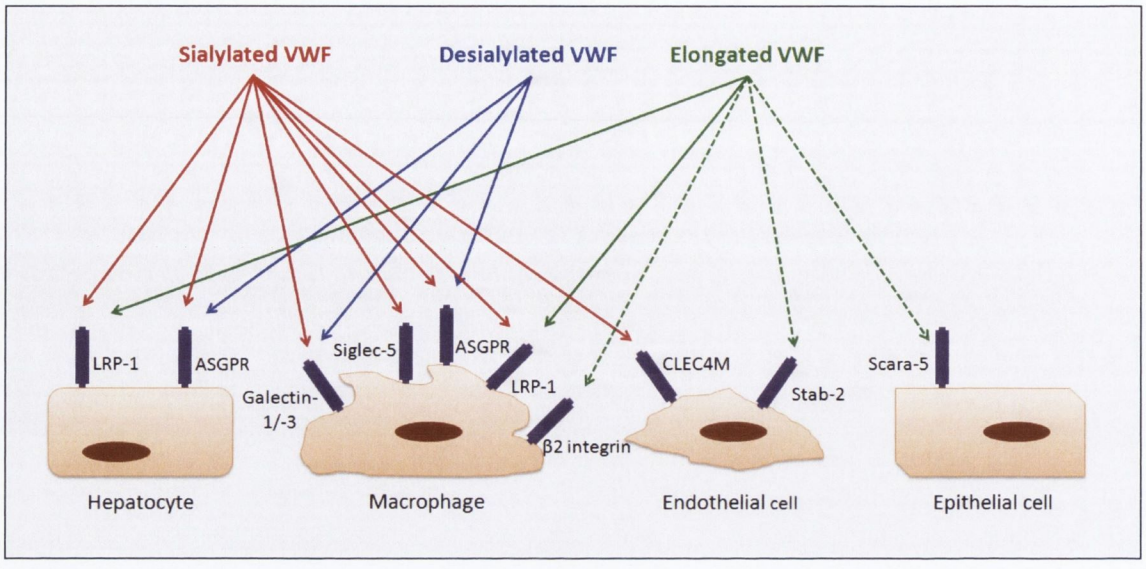


Figure 1.13 Cellular expression of candidate VWF clearance receptors.
Potential clearance pathways for sialylated, desialylated and elongated VWF are shown.

Candidate clearance receptor	Receptor class/family	Cellular Expression	Preferential ligands/binding motifs	VWF binding sites	Effect on VWF clearance
ASGPR	C-type lectin	Hepatocytes Macrophages	Galactose, α2-3, α2-6 linked sialic acid	N-linked glycans O-linked glycans	Impaired VWF clearance in ASGPR$^{-/-}$ mice.
LRP1	LDL receptor family	Hepatocytes Macrophages	Undetermined polypeptide motif	Not determined	Impaired VWF clearance in mice with macrophage-specific or liver-specific LRP deficiency
Siglec-5	I-type lectin	Macrophages	α2-3, α2-6, α2-8 linked sialic acid	N-linked glycans O-linked glycans	Reduction in VWF plasma levels following hydrodynamic *SIGLEC5* expression.
β_2 integrins	Integrin family	Macrophages	LLG motif	D3 and A2 domains	Impaired VWF clearance following β2 integrin inhibition.
Galectin-1	S-type lectin	Macrophages	*N*-acetyllactosamine	N-linked glycans	VWF plasma levels unaffected in dual galectin-1/galectin-3$^{-/-}$ mice.
Galectin-3	S-type lectin	Macrophages	*N*-acetyllactosamine	N-linked glycans	VWF plasma levels unaffected in dual galectin-1/galectin-3$^{-/-}$ mice.
CLEC4M	C-type lectin	Endothelial cells	Mannose	N-linked glycans	Reduction in VWF plasma levels following hydrodynamic *CLEC4M* expression.
Stabilin-2	Scavenger receptor class H	Endothelial cells	Undetermined polypeptide motif	Not determined	Not determined.
Scara-5	Scavenger receptor class A	Epithelial cells	Undetermined polypeptide motif	Not determined	Not determined.

Table 1.0 Cellular expression of candidate VWF clearance receptors.

1.9.4 Role of multimer composition in regulating VWF clearance

Several lines of evidence suggest that VWF clearance is independent of multimer size. In healthy controls, the ratio of VWF collagen binding activity (VWF:CB) to VWF:Ag, which is used as a measure of multimeric distribution is not significantly different in group O and non-O individuals, suggesting that VWF clearance from the plasma is independent of multimer distribution (Gallinaro et al. 2008). *In vivo* clearance studies also support this hypothesis. In $VWF^{-/-}$ mice, clearance of HMW-VWF (high molecular weight VWF) and LMW-VWF (low molecular weight VWF) proceeded at a similar rate. HMW-VWF was cleared with a MRT (mean residence time) of ~3 hours while LMW-VWF was cleared with a MRT of ~2.3 hours. Moreover, multimer analysis of mouse plasma showed no evidence of proteolytic conversion of large multimers to smaller multimers. This is consistent with previous reports that type 2A VWF is cleared at the same rate as wild-type VWF in rats (Stoddart et al. 1996).

1.9.5 Domain-dependent VWF clearance

To investigate the role of individual VWF domains in regulating clearance, a series of VWF domain truncations have been studied in VWF-/- mice (Lenting et al. 2004). While truncated fragment A1A2A3-VWF was cleared at the same rate as full-length wild-type VWF, N-terminal addition of the D'D3 domains (D'A3-VWF) served to significantly prolong circulatory half-life. Moreover, clearance of the D'D3-VWF fragment alone proceeded even more slowly suggesting the presence of a clearance inhibitory element within the VWF D'D3 domains. In contrast C-terminal addition of the D4-CK domains

to A1A2A3-VWF to create A1CK-VWF served to significantly enhance clearance. These data demonstrate the presence of a clearance receptor interactive site within the VWF A1A2A3 domains as well as a clearance modulatory site within the D4-CK domains.

1.9.6 Increased clearance as a pathological mechanism in type 1 VWD

Type 1 VWD comprises up to 75% of all cases of VWD, and is characterized by a partial quantitative VWF deficiency. The majority cases are caused by single amino acid substitutions and over 100 candidate mutations have been identified throughout the coding region of the *VWF* gene. These mutations result in a variety of pathogenic mechanisms, including reduced synthesis and/or secretion. Recently however, patient data has also identified increased VWF clearance as an important pathogenic mechanism in type 1 VWD. To date, over 20 mutations that cause enhanced clearance have been identified (Casari et al. 2013). These mutations appear to cluster within the VWF D'D3 and A1 domains and were identified on the basis of elevated VWFpp/VWF:Ag ratios (Figure 1.14).

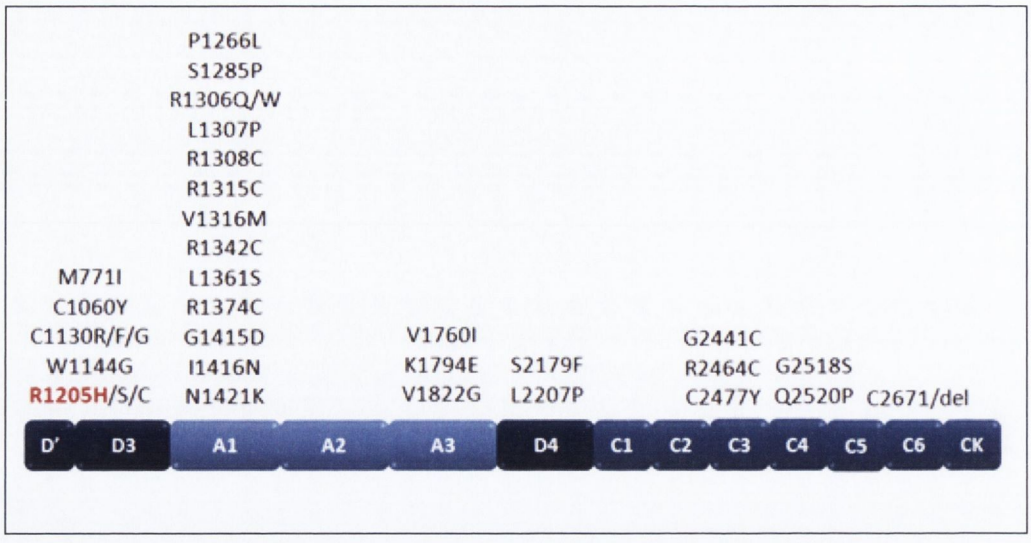


Figure 1.14 Candidate VWF mutations leading to enhanced clearance.
Mutations were identified on the basis of elevated VWFpp/VWF:Ag ratios (Casari et al. 2013).

1.9.7 VWFpp/VWF:Ag ratio as a marker for increased clearance

Though VWF and VWFpp are co-secreted from endothelial cells (1:1 ratio) they are cleared independently. VWFpp is cleared with a significantly shorter half-life of 2-3 hours compared with a half-life of 8-12 hours for VWF. Because of this, the ratio between VWFpp and VWF:Ag may be used to assess VWF survival in the plasma (Haberichter et al. 2006). Increased VWF clearance but normal VWFpp clearance, results in an increased VWFpp/VWF:Ag ratio and a ratio greater than 2.3 is considered indicative of enhanced VWF clearance (Eikenboom et al. 2013). Interestingly, mutations that give rise to increased VWFpp/VWF:Ag ratios are not only associated with type 1 VWD. Increased VWFpp/VWF:Ag ratios have also been described in patients with type 2B VWD suggesting that type 2B VWD comprises both qualitative and quantitative defects (Casonato et al. 2010).

1.10 VWD Vicenza

Of the mutations known to cause enhanced VWF clearance, the best characterised is the "Vicenza" mutation (R1205H). The Vicenza mutation is the most common cause of highly penetrant type 1 VWD and has been identified in up to 6% of type 1 patients (Cumming et al. 2006; Goodeve et al. 2007; James et al. 2007). VWD Vicenza is characterized by markedly enhanced VWF clearance resulting in plasma VWF antigen levels that may be as low as 0.1U/L. Low plasma antigen levels are associated with a proportionate reduction in VWF activity and normal platelet VWF content. VWD Vicenza is associated with the highest described VWFpp/VWF:Ag ratio (mean ± SD 15.8 ± 11.2) but wide variation within the Vicenza cohort suggests that factors other than the R1205H mutation may also modulate enhanced clearance in this population (Casari et al. 2013). The drastic reduction in VWF plasma half-life that is associated with the R1205H mutation has important clinical implications. Though patients with VWD Vicenza typically have a normal or enhanced response to DDAVP, the increase in VWF plasma levels is short-lived, and plasma levels return to baseline within just 4 hours of DDAVP administration (Casonato et al. 2002; Castaman et al. 2008).

1.10.1 Functional properties of VWF-R1205H

Several *in vitro* studies have examined whether the R1205H mutation influences the functional properties of VWF. Despite its location in the VWF D3 domain, surface plasmon resonance based analysis has shown that the R1205H mutation does not affect binding to the FVIII light chain, GPIbα, or collagen type III (Lenting et al. 2004). In addition the mutation has no affect

on VWF proteolysis by ADAMTS13 (Pruss et al. 2011). Interestingly however, the presence of the mutation results in sub-optimal VWFpp binding at low pH. In the Weibel Palade bodies VWFpp binds to the VWF D'D3 domains to facilitate helical packaging for regulated secretion (Huang et al. 2008; Madabhushi et al. 2012). This interaction is strictly pH dependent. Thus, sub-optimal interaction of R1205H-VWF with the propeptide at low pH may have implications for tubular packaging of VWF though this has not been confirmed (Lenting et al. 2004).

In vivo studies have also investigated the functional properties of VWF-R1205H. In particular, hydrodynamic expression studies have facilitated comparison of the *in vivo* thrombogenic potential of R1205H compared to WT-VWF. Importantly, in a ferric chloride injury model, both time to occlusion and total platelet accumulation were similar for both R1205H and WT-VWF (Pruss et al. 2011).

1.10.2 Ultra-large VWF Multimers in VWD Vicenza

UL-VWF multimers and a reduction in VWF satellite bands are recognized features of the VWD Vicenza phenotype though the underlying molecular mechanism has not been fully elucidated. Recent data suggests these UL-VWF multimers arise due to the enhanced rate of clearance associated with VWD Vicenza (Gezsi et al. 2010). Using a mathematical model that incorporated VWF Vicenza synthesis, cleavage and secretion, Gézsi et al demonstrated a multimer profile that tended towards UL-VWF Furthermore, reduced proteolytic remodelling predicted less prominent satellite bands, also a feature of VWD Vicenza. Conversely, a faster rate of proteolysis shifted the

plasma distribution towards smaller proteolysed multimers, a pattern frequently observed in Type 2A VWD (Figure 1.16).

Based on this mathematical modelling, it would appear that decreasing the VWF half-life by a factor of 10 was sufficient to produce the UL-VWF multimer distribution that is characteristic of VWD Vicenza. Indeed, reducing the VWF half life from 12 hours (normal VWF) to 1.2 hours (VWD Vicenza) accurately recapitulated the UL-VWF Vicenza multimer profile. This multimer transition zone appears to represent a narrow window. For example, reduction of VWF half-life to just 4 hours predicted VWF plasma levels of just 37%, but produced a multimer pattern that was difficult to distinguish from normal. The existence of such a threshold may account for the fact that UL-VWF multimers are present only in a proportion of Vicenza patients.

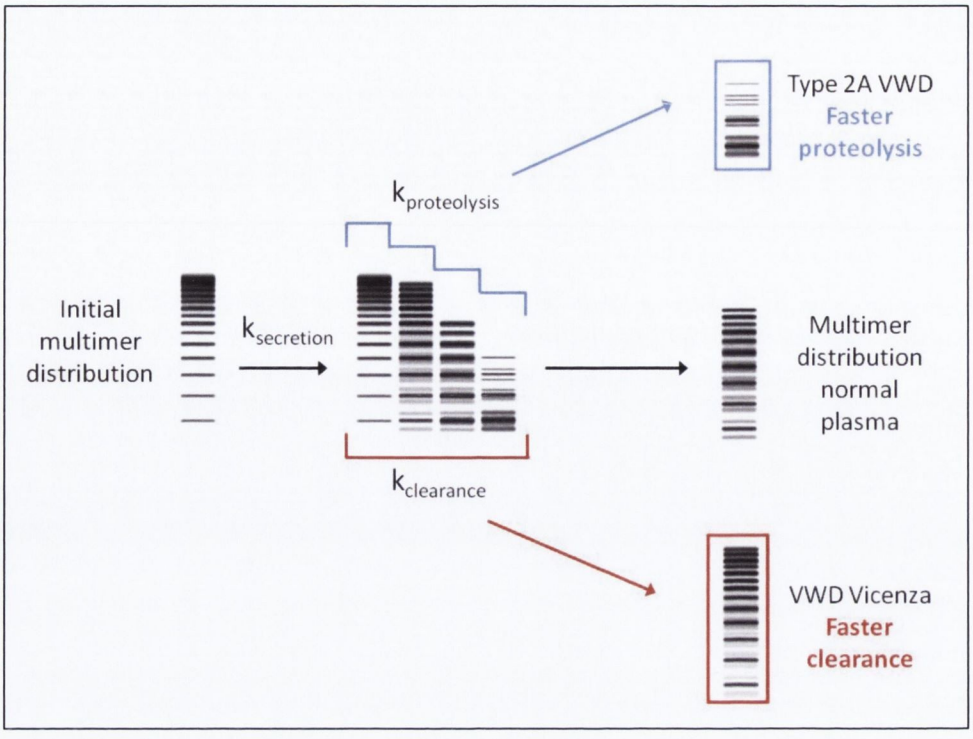


Figure 1.15 Pharmacokinetic modelling of VWD Vicenza.
(adapted from Sadler et al. 2005).

The multimer composition of pd-VWF is a critical determinant of its functional activity. High molecular weight multimers bind collagen and platelets with significantly higher affinity than low molecular weight VWF (Arya et al. 2002). Paradoxically, the UL-VWF multimers observed in patients with VWD Vicenza are not associated with increased haemostatic activity (d'Alessio et al. 1992). Both ristocetin co-factor activity and collagen binding activity which are sensitive to the presence of large multimers have consistently been shown to be normal in Vicenza patients when expressed as ratios though the reason for this is not known (Casonato et al. 2002).

1.10.3 Role of platelet-VWF in VWD Vicenza

Despite that marked reduction in VWF plasma levels in VWD Vicenza, the clinical phenotype is relatively mild (Casonato et al. 2002). Initially it was suggested that enhanced haemostatic activity of UL-VWF multimers and normal platelet VWF content compensated for low plasma VWF levels. However enhanced haemostatic activity of UL-VWF multimers was ruled out when perfusion experiments examined the relative contribution of UL-VWF multimers and platelet-VWF to the haemostatic process (d'Alessio et al. 1992). No significant increase in collagen binding or platelet adhesion was observed when plasma from Vicenza patients containing UL-VWF was perfused over collagen coated surfaces compared to type 1 VWD controls. However, normal collagen adhesion upon perfusion of VWD Vicenza platelets confirmed that normal platelet-VWF content sufficiently compensated for low VWF plasma levels in VWD Vicenza. This is not surprising given that the local concentration of platelet VWF:Ag released following platelet activation has been estimated to be in the region of ~52IU/mL (Fernandez et al. 1982). The finding that platelet-VWF is capable of supporting haemostasis in the presence of reduced plasma VWF:Ag levels is also consistent with several animal transplantation studies that have established an important role for platelet-VWF in mediating haemostasis. Development of chimeric animals expressing only platelet-or plasma-VWF has demonstrated that platelet-VWF can sustain haemostasis in the presence of limiting amounts of plasma-VWF (Bowie et al. 1986; Nichols et al. 1990; Nichols et al. 1995; Kanaji et al. 2012).

1.10.4 ABO regulation of VWF plasma levels in VWD Vicenza

As previously discussed, the ABO blood group effect on VWF plasma levels is well established. Interestingly, in patients with VWD Vicenza, baseline VWF plasma levels do not differ significantly between the groups (group O 9IU/ml versus non-O 8IU/ml). In addition, no significant difference in VWFpp/VWF:Ag ratio was observed for group O versus non-O Vicenza patients (VWFpp/VWF:Ag 10.2 group O versus 9.7 non-O), suggesting that VWF clearance proceeded at a rate so rapid that ABO differential clearance was prevented (Castaman et al. 2010). In contrast however, following DDAVP infusion, ABO differential VWF plasma levels became apparent in Vicenza patients (Castaman et al. 2010). Non-O Vicenza patients demonstrated an increased initial response to DDAVP, as well as a longer VWF half-life compared to group O Vicenza patients. Nevertheless, the longer VWF half-life and the greater increase in absolute VWF plasma levels observed for non-O Vicenza patients failed to reach clinically useful levels as the half-life values remained severely compromised. Cumulatively, these data suggest that though ABO blood group is a critical modulator of VWF plasma levels, the R1205H mutation is a more potent modifier.

1.10.5 Additional mutations responsible for enhanced clearance

In addition to the R1205H mutation, two other amino acid mutations have also been identified at position 1205 in patients with VWD (R1205C and R1205S respectively). Interestingly, the R1205C and R1205S mutations were both associated with significant reductions in plasma VWF survival following DDAVP administration in these individuals (elimination half-life of 3.3 hours

and 1.9 hours compared to 11.4 hours in controls) (Millar et al. 2008). Although both of these 1205 mutations were associated with enhanced VWF clearance, the R1205H mutation was associated with the shortest post-DDAVP survival and the greatest increase in VWFpp/VWF:Ag ratio.

1.10.6 Vicenza-type pseudo VWD

Intriguingly, the existence of a Vicenza type pseudo-VWD has been reported (Casonato et al. 2006). The authors described a patient with the hallmark features of VWD Vicenza, including the presence of UL-VWF multimers and reduced post-DDAVP VWF survival ($t_{1/2}$ elimination 2.01 hours). Despite this, the R1205H mutation was not detected upon exon 27 sequencing. Most interestingly, enhanced clearance of exogenous VWF ($t_{1/2}$ elimination 1.20 hours) was also described in this patient in the absence of any detectable anti-VWF antibodies. Mutation of putative VWF clearance receptors leading to enhanced activity could potentially account for the dramatically enhanced clearance of both endogenous and exogenous VWF in this particular patient.

1.11 Purpose of investigation

VWF plasma levels vary widely in the normal population and this variation is of direct clinical significance, as VWF deficiency is responsible for von Willebrand disease, the most common inherited bleeding disorder. Several factors contribute to the variation in VWF plasma levels including a variety of genetic and environmental determinants. Glycosylation is a major determinant of VWF plasma levels as both ABO blood group antigens and sialic acid have been shown to influence the rate of VWF clearance. However, the molecular mechanisms through which the glycan affects are mediated remain unknown, and the ability of VWF to interact with lectins in normal plasma is poorly characterized. The first aim of this study therefore, was to define the biochemical basis through which VWF interacts with specific lectins within the circulation. In particular, this thesis focuses on characterization of the glycan determinants responsible for mediating the recently documented novel interaction between VWF and galectin-3.

In addition to glycosylation, sequence variation also contributes to variation in VWF plasma levels. Missense mutations in the *VWF* coding sequence are responsible for the majority of cases of Type 1 VWD and lead to a variety of pathogenic mechanisms including enhanced VWF clearance. Nevertheless, the biology underlying pathological VWF clearance remains unclear. In particular, the receptors responsible for mediating enhanced clearance in VWD Vicenza remain unknown. Consequently, another objective of this thesis was to elucidate the molecular mechanisms underlying enhanced clearance of VWF-R1205H and to identify the cell types and receptors involved. Following extensive *in* vitro and *in* vivo characterization of

VWF-R1205H, this work also aims to examine the clinical significance of enhanced VWF clearance in a VWD Vicenza patient cohort.

Importantly while sequence variation is a key determinant of VWF plasma levels in the majority of patients with Type 1 VWD, several cohort studies have recently revealed that a significant portion of Type 1 patients do not have a *VWF* gene mutation. Indeed, *VWF* gene mutations are significantly less common in patients with intermediate reductions in plasma VWF:Ag levels (0.3 - 0.5U/mL) and the genetic and molecular mechanisms regulating variation in plasma VWF:Ag levels in these patients appear to be complex. Therefore, the final objective of this study was to investigate the pathogenic mechanisms leading to reduced VWF plasma levels in this particular patient cohort.

2.0 Materials and Methods

2.1 Purification of plasma-derived 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 as well as a number of excipients. Size exclusion chromatography (gel filtration) was used to purify plasma-derived VWF (pd-VWF) from Haemate P®.

2.1.1.1 Gel filtration

Gel filtration (size exclusion) chromatography separates proteins on the basis of size. Larger proteins elute from the column more rapidly than smaller proteins. Consequently, this methodology can be exploited for the separation of high, intermediate and low molecular weight multimers of VWF. A Sepharose 2B-CL gel filtration column (600mm x 26mm, 320mL volume, Amersham Pharmacia, UK) was pre-equilibrated with two column volumes of Tris-Citrate (TC) buffer (20mM Tris, 10mM sodium citrate, pH7.4). Reconstituted Haemate P® (approximately 10mL) was applied to the column at a flow rate of 0.5mL/min. Pd-VWF was then eluted with TC buffer at a flow rate of 1mL/min. Fractions were collected (figure 2.0) and VWF concentration

was determined by VWF antigen ELISA (section 2.8.1). VWF function and multimer composition were assessed by collagen binding assay (section 2.8.2).

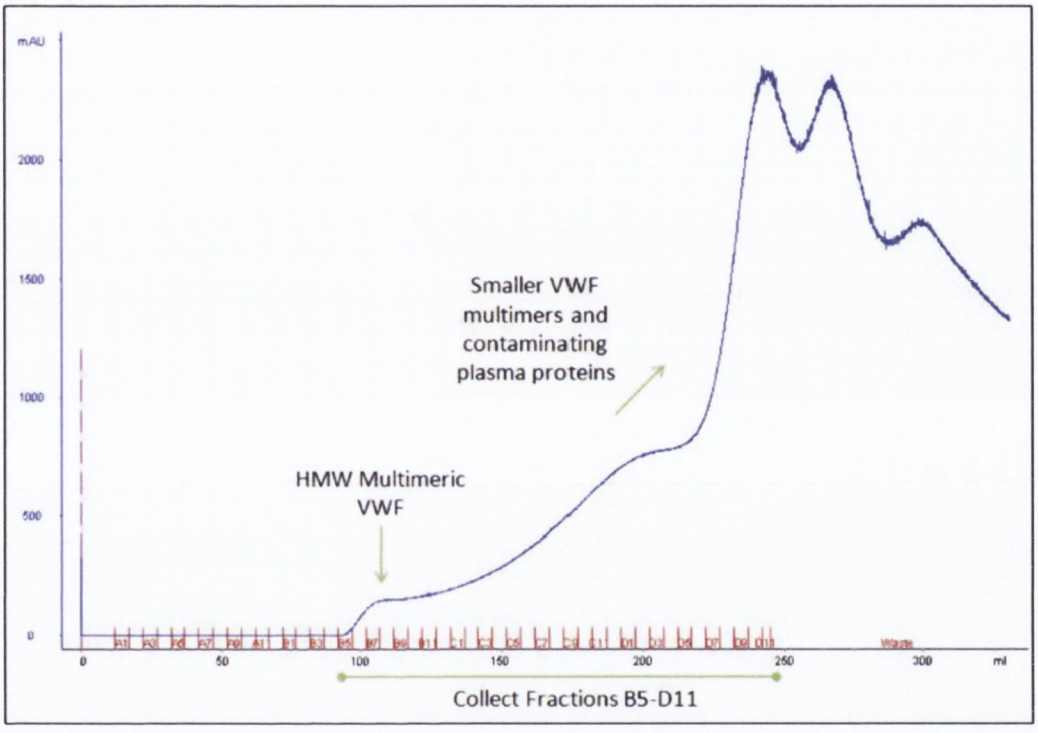


Figure 2.0 Purification of pd-VWF via gel filtration.
High purity, high molecular weight multimeric VWF is eluted first, followed by smaller VWF multimers and contaminating plasma proteins.

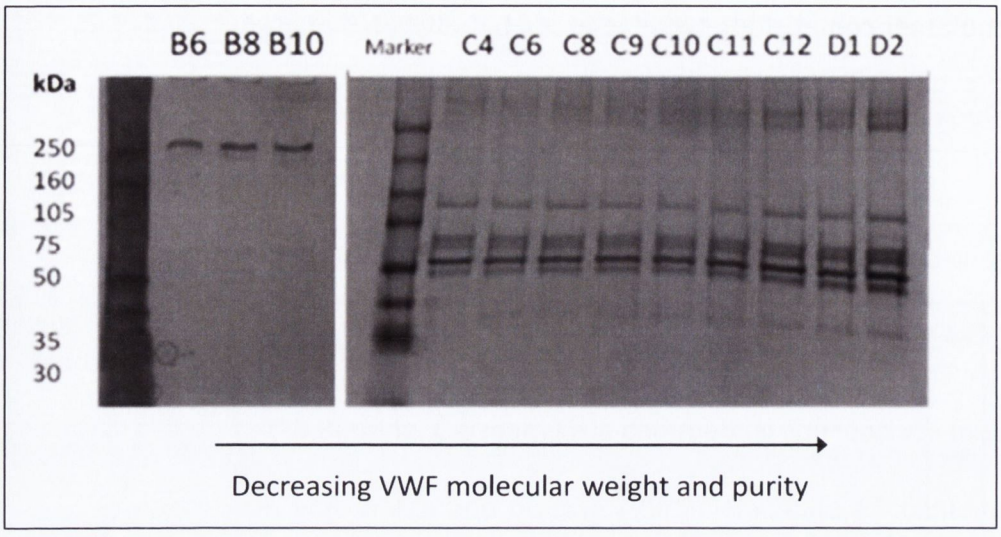


Figure 2.1 SDS-PAGE assessment of VWF purity following gel filtration.
Purity of fractions collected after gel filtration chromatography was analysed by SDS-PAGE followed by silver staining. Two representative purifications are shown. The purity of VWF, present at ~250kDa decreases with increasing fraction number.

2.1.1.2 Purification of blood group specific VWF from plasma

Blood group specific VWF (group O, group AB and Bombay respectively) was purified from plasma by cryoprecipitation. Cryoprecipitation exploits the phenomenon that various circulating proteins precipitate out of solution at 4°C. Fresh, frozen plasma (FFP) was obtained from the Irish Blood Transfusion Service and allowed to thaw for 24 hours at 4°C. Two units of blood group specific plasma (~300mL per unit) were pooled in a pre-chilled glass beaker at 4°C and were slowly stirred. 50% (v/v) ethanol, pre-chilled to 4°C was added drop wise to the stirring plasma to a final concentration of 8% (v/v). The mixture was stirred for an additional 30 minutes before being decanted into pre-chilled 50mL centrifuge tubes on ice. The resulting precipitate was harvested by centrifugation; 3500rpm for 30 minutes at 4°C. The cryo-supernatant was decanted off and the VWF rich pellets were then resuspended in 12mL of TC buffer 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 section 2.1.1.1. VWF concentration was subsequently determined by VWF antigen ELISA (section 2.8.1).

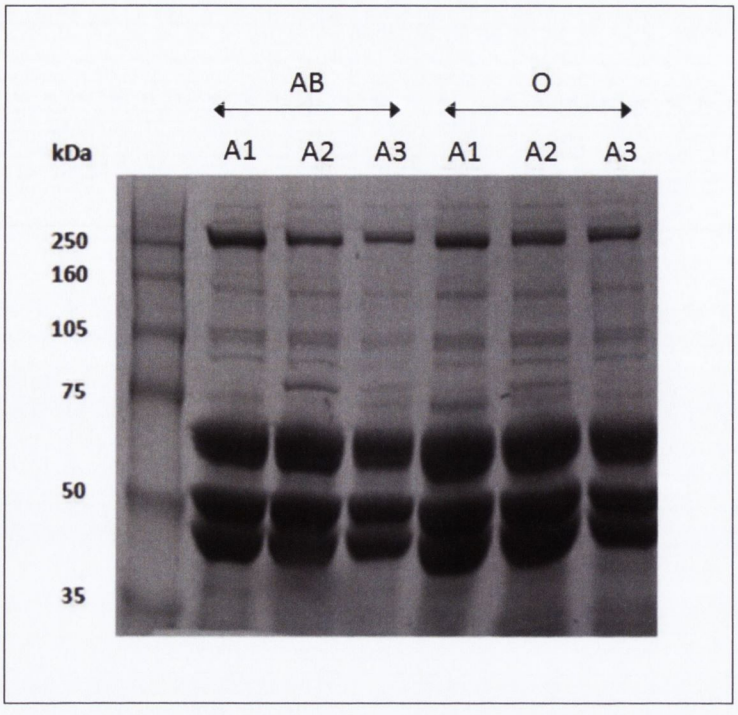


Figure 2.2 SDS-PAGE assessment of VWF purity following cryoprecipitation and gel filtration purification of blood group-specific VWF.
Purity of blood group-AB fractions A1, A2, A3 and blood group-O fractions A1, A2, A3 collected after cryoprecipitation and size exclusion chromatography were analysed by SDS-PAGE followed by Coomassie staining.

2.2 Purification of platelet-derived VWF

2.2.1 Isolation of platelets

Surplus platelets were obtained from the Irish Blood Transfusion Service. Two units of pooled platelets in leukocyte-depleted plasma (~460mL) were decanted into 50mL tubes (~30mL per tube). Platelets were separated from plasma by centrifugation; 1500g for 10 minutes at room temperature. The plasma supernatant was discarded and the platelet pellet washed by gentle resuspension 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 discarded and the platelet wash step was repeated a further three times.

2.2.2 Platelet lysis by snap freeze-thawing

The resulting platelet pellets were resuspended in buffer A and platelet lysis was carried out by repeated snap freeze-thaw cycles. Platelets were rapidly frozen in liquid nitrogen followed by rapid defrosting at 37°C in a water bath. The platelet mixture was then vortexed on a bench top mixer (Vortex Genie® 2, Scientific Industries Inc, USA) at a speed of approximately 2500rpm for 3 minutes. Freeze-thaw cycles were repeated a further 3 times.

The platelet mixture was then centrifuged at 10,000g for 30 minutes twice and the resulting platelet lysate was stored at -80°C.

2.2.3 Purification of platelet-VWF by immunoaffinity chromatography

Immunoaffinity chromatography was carried out in collaboration with Dr. Jan Voorberg (Department of Plasma Proteins, Sanquin Research, Amsterdam, The Netherlands) as previously described (Schwarz et al. 2000). VWF was purified from platelet lysate by immunoaffinity chromatography using the monoclonal antibody CLB-RAg20 coupled to CNBr sepharose 4B (Amersham Biosciences, UK). Platelet VWF was eluted using 50mM HEPES, 0.1M NaCl, pH 7.4. Platelet-VWF containing fractions were dialyzed against 20mM Tris, 0.15M NaCl, pH 7.4 and VWF concentration was subsequently determined by VWF antigen ELISA 9 (section 2.8.1).

2.3 Modification of pd-VWF glycosylation profile

2.3.1 Glycosidase digestions

Modification of pd-VWF glycan structures was performed using a series of specific exoglycosidase enzymes (Table 2.0). Digestions were carried out overnight at 37°C with pd-VWF at a final concentration of 75µg/mL in phosphate buffered saline, pH 7.4 (PBS). Quantitative changes in VWF carbohydrate expression following glycosidase digestion were assessed by lectin ELISA and compared to untreated pd-VWF controls.

Enzyme	Concentration (per μg of VWF)	Buffer	Supplier
PNGase F (*Flavobacterium meningosepticum*)	312U	0.5M Sodium Phosphate, pH 7.5	New England Biolabs
O-Glycosidase (*Streptococus pneumoniae*)	0.50 mU	0.25M Sodium Phosphate, pH 5.0	Sigma Aldrich
α2-3,6,8,9 Neuraminidase (*Arthrobacter ureafaciens*)	1.25mU	0.25M Sodium Phosphate, pH 6.0	Calbiochem
α2-3 Neuraminidase (*Streptococus pneumoniae*)	0.60 mU	0.25M Sodium Phosphate, pH 6.0	Sigma Aldrich

Table 2.0 Exoglycosidase enzymes used to modify the glycan structures of pd-VWF.

2.3.2 Lectin ELISA

Untreated pd-VWF and glycan modified VWF were serially diluted in PBS and immobilised onto a 96-well MaxiSorb plate (Nunc, UK) at 37°C for 2 hours. The plate was washed with PBS-T (phosphate buffered saline with containing 0.05% Tween, pH 7.4) and biotinylated lectins (Table 2.1; Vector Laboratories, UK) diluted to a final concentration of 1μg/mL in PBS were incubated on the plate for 1 hour at 37°C. The plate was again washed with PBS-T and then incubated with streptavidin-HRP (R&D Systems, UK), diluted 1:200 in PBS-T for 1 hour at 37°C. After further washing in PBS-T, 100μl horseradish peroxidise (HRP) substrate 3,3',5,5'-Tetramethylbenzidine (TMB; R&D Systems,UK) was added to the plate for colour development. The reaction was stopped with 1M H_2SO_4, and absorbance at 450nm was measured using the VERSAmax microplate reader (Molecular Devices, UK).

Levels of specific glycan expression pre- and post- glycosidase treatment were expressed as a percentage of the untreated controls.

Lectin	Specificity
Concanavalin A	N-linked glycans
Jacalin	O-linked glycans (mono- and di-sialylated T-antigen)
Sambucus nigra	Terminal α2-6 sialic acid (to a lesser degree α2-3 linked sialic acid)
Maackia amurensis II	α2-3 sialic acid

Table 2.1 Lectins used to quantify glycan structures on VWF and their specificity.

2.4 Expression vectors for recombinant protein production

2.4.1 VWF expression vectors

2.4.1.1 pcDNA VWF expression vector

The VWF expression vector pcDNA VWF (Figure 2.2) was a kind gift from Dr. Tom McKinnon (Imperial College London, UK) and was previously described (McKinnon et al. 2008). pcDNA VWF carries a unique BamHI restriction site in the D3 domain and a unique KpnI restriction in the A2 domain as the KpnI site in the D1 domain was removed via mutagenesis. In addition to the VWF cDNA this vector includes an ampicillin resistance gene for selection in bacterial cells and a PUC origin which allows high-copy number replication in *E.coli*. The vector also incorporates a cytomegalovirus (CMV) immediate-early promoter to drive high-level gene expression in mammalian cells; a neomycin resistance gene to facilitate selection in mammalian cells; and a SV40 origin to drive episomal replication in cells expressing the SV40 large T antigen.

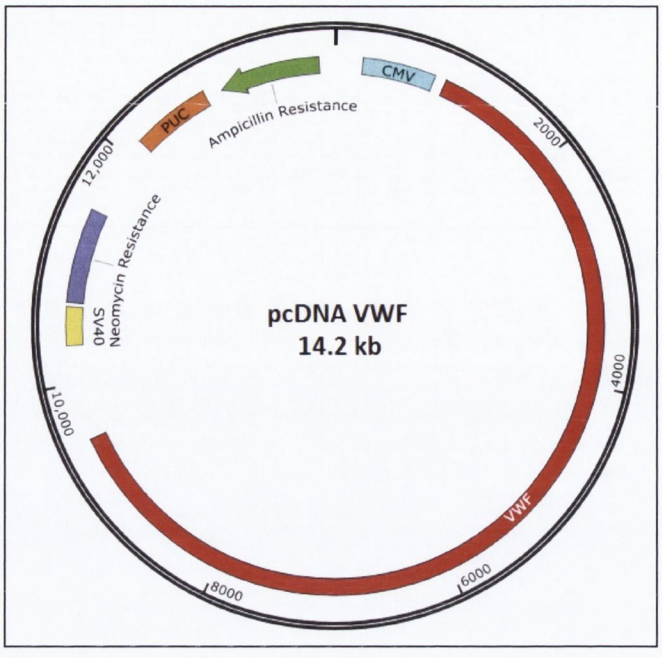


Figure 2.3 Full length VWF expression vector pcDNA VWF.

2.4.1.2 pcDNA VWF ΔA1 expression vector

The pcDNA VWF ΔA1 expression vector was a gift from Dr. Tom McKinnon (Imperial College London, UK). pcDNA VWF ΔA1 is similar to pcDNA VWF except that it lacks a 241 amino acid segment (residues 1241-1482) that corresponds to the VWF A1 domain. The expressed protein is unable to bind heparin or platelets but retains the ability to bind collagen (Sixma et al. 1991).

2.4.1.3 pcDNA D'A3 VWF expression vector

The pcDNA D'A3 VWF expression vector was previously established in our laboratory by Dr. Alain Chan. The D'A3 coding region spans amino acid residues 764-1878 and the expressed protein carries a C-terminal polyhistidine tag.

2.4.1.4 pcDNA A1A2A3 VWF

The pcDNA A1A2A3 VWF expression vector was a kind gift from Raimondo De Cristofaro (Catholic University School of Medicine, Rome, Italy) and was previously described (De Cristofaro et al. 2006). The A1A2A3 VWF coding region spans amino acid residues 1260-1874 and the expressed protein carries an N-terminal polyhistidine tag as well as a C-terminal V5 tag.

2.4.2 Galectin-3 Expression Vector

The expression vector pET 151/D TOPO (figure 2.3) was used to produce recombinant galectin-3 with an N-terminal polyhistidine tag. pET 151/Gal-3 was constructed by inserting the galectin-3 cDNA (Origene, MD, USA) into the pET 151/D TOPO expression vector (Invitrogen, UK).

2.4.2.1 pET 151/D TOPO® bacterial expression vector

pET 151/D TOPO is a linearized topoisomerase I-activated vector that facilitates directional cloning without the need for restriction enzymes or DNA ligases. It incorporates a T7*lac* promoter for high-level, IPTG-inducible gene expression in *E.coli*, as well as a *lacI* gene encoding the lac repressor to reduce basal transcription from this promoter. In addition, the vector also includes an ampicillin resistance gene for selection in bacterial cells.

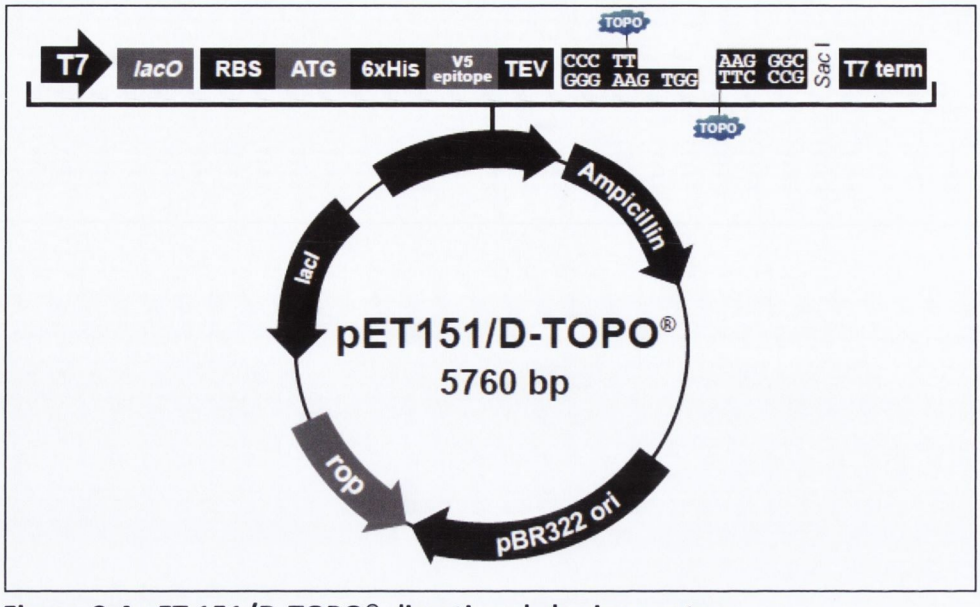


Figure 2.4 pET 151/D-TOPO® directional cloning vector.

The TOPO® directional cloning system exploits the inherent ligase activity of DNA topoisomerase I. Topoisomerase I is covalently attached to the free 3' end of each DNA strand of the pET151/D TOPO® vector and where a topoisomerase recognition sequence (CCCTT) is also carried. In addition, the vector contains a single-stranded GTGG overhang on the 5′ end. In this system PCR products are directionally cloned by adding a four base sequence (CACC) to the 5′ end of the forward primer (Figure 2.4). The GTGG overhang of the TOPO® vector invades the double-stranded DNA of the PCR product and anneals to the CACC sequence added by the primer. Topoisomerase I then ligates the PCR product in the correct orientation.

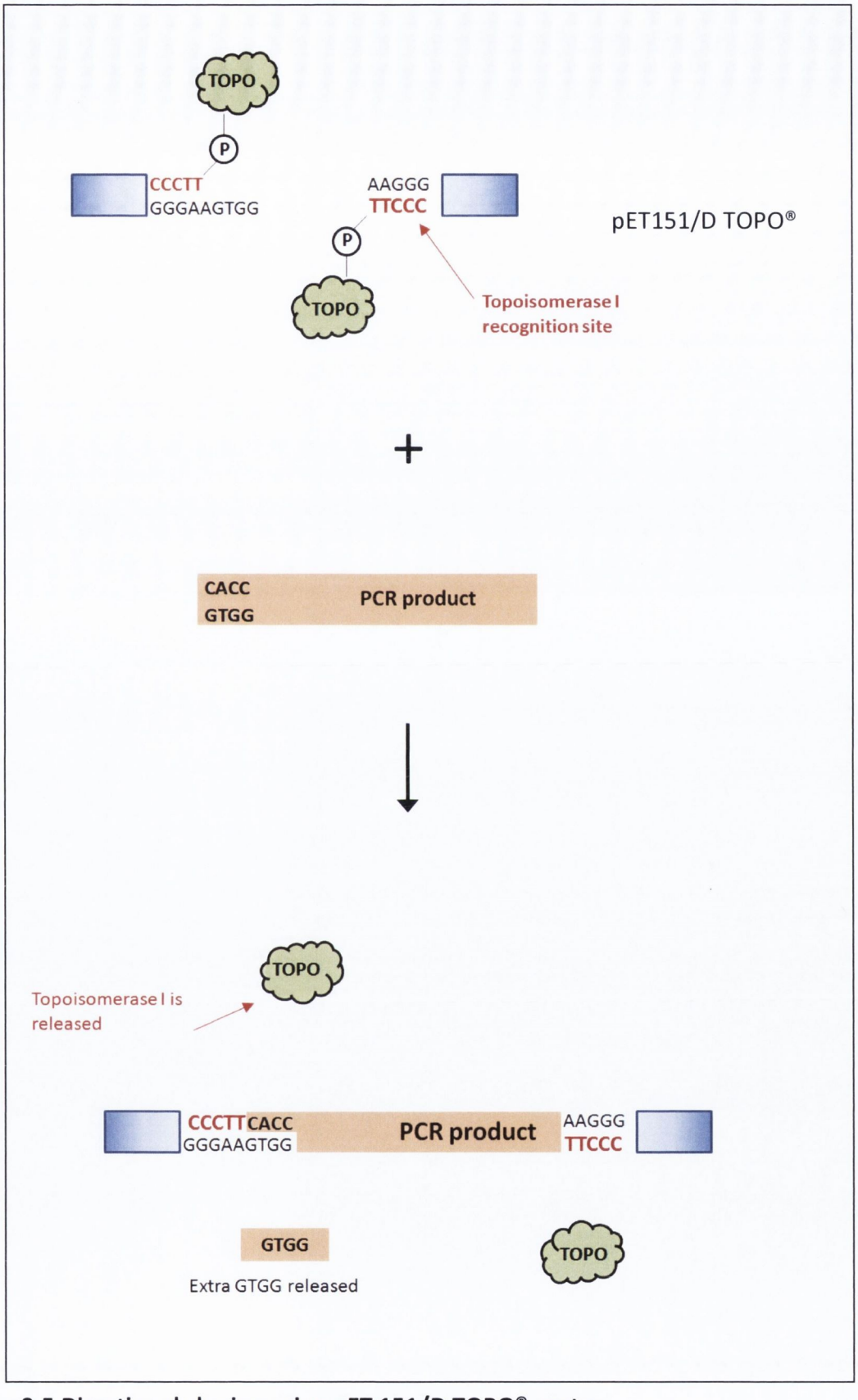


Figure 2.5 Directional cloning using pET 151/D TOPO® vector.

2.4.2.2 Amplification of the galectin-3 cDNA

The galectin-3 cDNA was amplified utilizing the primers described in table 2.2 and the KOD Hot Start DNA Polymerase (Novagen®, UK). To enable directional cloning, the forward polymerase chain reaction (PCR) primer was designed to contain the CACC sequence at the 5' end. The reverse primer incorporated a stop codon.

Primer ID	Primer sequence 5'→3'
Forward 151 Gal-3	**CACC**ATGGCAGACAATTTTTCGCTC
Reverse 151 Gal-3	**TCA**TATCATGGTATATGAAGCACT

Table 2.2 PCR primers for galectin-3 amplification.

Reactions were set up in thin-walled, dome-capped PCR tubes and PCR cycling was performed using the MJ Research PTC 200 Thermo Cycler with heated lid (GMI, MN, USA). Cycling parameters are outlined in Table 2.3.

Step	Temperature	Time	Cycles
Denaturation	95°C	2 minutes	1
Denaturation	95°C	20 seconds	25
Annealing	60°C	Lowest primer Tm for 10 seconds	
Extension	68°C	20seconds/kb	

Table 2.3 PCR cycling parameters for galectin-3 amplification using KOD Hot Start DNA Polymerase.

2.4.2.3 Agarose gel purification

The resulting ~750bp PCR product was purified via agarose gel electrophoresis followed by gel extraction. 0.7% agarose (w/v) was dissolved in Tris/Borate/EDTA (TBE) buffer by heating. GelRed™ (Cambridge Bioscience, UK), a sensitive ethidium bromide substitute with low toxicity was then added at a 1/10,000 dilution. The agarose mixture was poured into a BRL 58 Horizontal Gel Electrophoresis Apparatus minitank (Gibco, UK) and allowed to set. The PCR product was subsequently electrophoresed in TBE buffer at 90V against the 1kb DNA ladder (New England Biolabs, UK). DNA bands were visualised for excision under ultra-violet (UV) light using the BioSpectrum GelDoc imaging system (UVP, CA, USA).

2.4.2.4 Gel extraction

Once excised, bands of the correct size were purified using the QIAquick Gel Extraction Kit (QIAGEN, UK). This kit utilizes spin-column technology to purify DNA on a silica membrane. DNA adsorbs to the membrane under high salt conditions, while contaminants pass through the column. DNA is then eluted from the membrane using a Tris based buffer. This method can be used to purify up to 10 µg of DNA.

The excised gel fragment was weighed and 3 volumes of Buffer QG were added to 1 volume of gel (~100µl to 100mg of gel). The mixture was incubated at 50°C for 10 minutes with occasional vortexing. Once the agarose had dissolved, 1 gel volume of isopropyl alcohol was added to the sample and mixed well. The sample was then applied to a QIAquick spin column and was centrifuged at 17,800g for 1 minute. The flow-through was discarded and the

membrane was washed with 0.75mL Buffer PE. The column was centrifuged for 1 minute at 17,800g, the flow through was discarded and the column was centrifuged for an additional minute to remove residual ethanol. The QIAquick column was then placed in a clean 1.5mL microcentrifuge tube. 30µL elution buffer was applied to the centre of the QIAquick membrane and was incubated at room temperature for 4 minutes. To elute the DNA, the column was centrifuged for at 17,800g for 1 minute. The eluted DNA was visualized by agarose gel electrophoresis to confirm its size/purity. DNA was quantified using the Nanodrop 2000 microvolume spectrophotometer (Thermo Scientific, UK).

2.4.2.5 TOPO® cloning

Following DNA quantification, the TOPO® cloning reaction was set up as in table 2.4. The reaction was mixed gently and incubated at room temperature for 30 minutes. The sample was then placed on ice.

Reagent	Volume
PCR product	0.5-4µL
Salt solution (1.2 M NaCl ,0.06 M $MgCl_2$)	1µL
Nuclease free water	Xµl
TOPO® vector	1µL
Total volume	**6µL**

Table 2.4 Reagents and volumes required for TOPO® cloning reaction.

In order to calculate the appropriate amount of PCR product to include in the ligation reaction the following equation was used. A 2:1 PCR product:vector molar ratio was utilized.

$$\frac{(\text{amount of vector in ng}) \times (\text{size of insert in kb})}{(\text{size of vector in kb})} \times \text{insert:vector molar ratio} = \text{amount of insert in ng}$$

2.4.2.6 Transformation of competent *E.coli*

High-efficiency competent *E.coli* were used to propagate the TOPO® cloning reaction. One vial of NEB 10-beta competent *E.coli* (New England Biolabs, UK) containing 50µL cells was thawed on ice. 2µL of the TOPO® reaction was added to the vial, mixed gently and incubated on ice for 30 minutes. Cells were heat shocked at 42°C for 30 seconds and then returned to ice for 2 minutes. 100µL pre-heated SOC medium (super optimal broth with catabolite repression; Promega, UK) was then added to the vial containing transformed cells and incubated at 37°C for 45 minutes with vigorous shaking (~150rpm). Approximately 100µL of the resulting culture was spread on an LB-ampicillin agar plate which was then incubated at 37°C overnight. Potential transformants were picked and used to inoculate 5mL cultures in LB broth with ampicillin (Fisher Scientific, Ireland). 5mL cultures were grown at 37°C overnight with vigorous shaking (~150rpm).

2.4.2.7 Isolation of plasmid DNA

Plasmid DNA was isolated from bacterial cultures in order to screen for potential transformants. Screening was carried out on the basis of plasmid size. pET 151D/TOPO is a 5,760bp linearized vector. Insertion of the gal-3 cDNA (~750bp) via TOPO® cloning creates an expression plasmid 6510bp in length and results in a visible migration shift upon agarose gel electrophoresis. Plasmid DNA was isolated using the GeneJet Plasmid Miniprep Kit (Thermo Scientific, UK). This kit combines silica membrane and spin column technology and recovers up to 20 µg of high copy plasmid DNA per isolation procedure.

The overnight culture was centrifuged at 17,800g for 5 minutes to pellet bacterial cells. The resulting cell pellet was resuspended in 250µL Resuspension Solution by vortexing to produce a homogeneous solution. Bacterial cells were lysed by addition of 250µL Lysis Solution with careful mixing. 350µl of the neutralization solution was then added and mixed thoroughly by inverting the tube 4-6 times. The solution was centrifuged at 17,8000g for 5 minutes to pellet cellular debris and chromosomal DNA. The resulting supernatant was transferred to the silica membrane of a GeneJet Spin Column and was centrifuged for 1 minute at 17,800g. The flow-through was discarded and the membrane was washed with 500µL of the ethanol-based Wash Solution. The column was centrifuged again at 17,800g for 1 minute and the flow-through was discarded. The wash procedure was then repeated. To ensure complete removal of ethanol from the silica membrane the column was subsequently centrifuged for an additional minute at 17,800g. The GeneJet column was transferred to a fresh 1.5mL microcentrifuge tube and 30µL Elution Buffer was added to the centre of the membrane. Elution

Buffer was incubated on the membrane for 2 minutes at room temperature before centrifugation at 17,800g for 2 minutes to elute the plasmid DNA. Plasmid DNA was then subjected to agarose gel electrophoresis as described in section 2.4.2.3. Plasmid DNA of the correct size was selected and the presence of the galectin-3 insert was confirmed by sequencing.



Figure 2.6 Bacterial expression vector pET 151/Gal-3.

2.5 Shuttle vector for VWF mutagenesis

Due to the large size of the pcDNA VWF expression plasmid (~14.2kb), a mutagenesis strategy utilizing an intermediate VWF shuttle vector (pGEM-VWF) was employed. Shuttle vector pGEM-VWF comprises the XhoI- KpnI fragment of pcDNA VWF (residues 786-1584) and served as a mutagenesis template.

2.5.1 pGEM-T vector

pGEM-VWF was prepared by amplifying the *Xho*I-*Kpn*I fragment of pcDNA VWF and inserting the resulting PCR product into the pGEM®-T vector (Promega, UK) via TA cloning. pGEM®-T is linearized vector with a single 3′-terminal thymidine at both ends. Addition of a single adenine to the 3′ ends of a PCR product via incubation with *Taq* polymerase facilitates ligation into the pGEM®-T vector without the need for restriction enzymes.

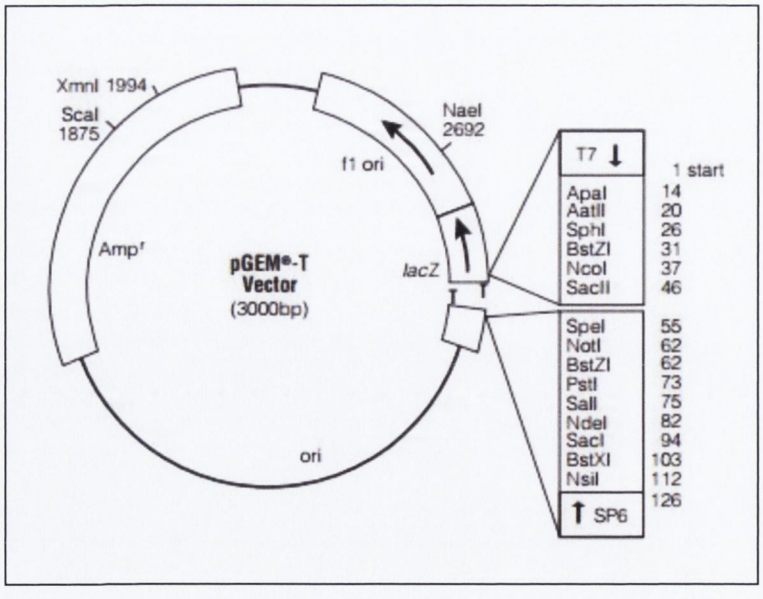


Figure 2.7 pGEM®-T vector.

2.5.2 Amplification of *Xho*I- *Kpn*I fragment of pcDNA VWF

The *Xho*I-*Kpn*I fragment was amplified using the KOD Hot Start DNA Polymerase (Novagen®, UK) as described in section 2.4.2.2. Following amplification, the PCR product was incubated with *Taq* DNA polymerase (New England Biolabs, UK) for 30 minutes at 70°C to facilitate non-template dependent addition of 3' adenine overhangs. The PCR product was then gel purified (section 2.4.2.3). The DNA fragment was visualized by agarose gel electrophoresis to confirm its size and purity, and was quantified using the Nanodrop 2000 microvolume spectrophotometer (Thermo Scientific, UK).

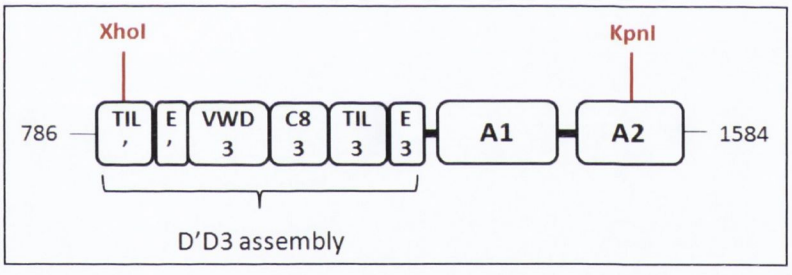


Figure 2.8 The *Xho*I-*Kpn*I fragment of pcDNA VWF comprising residues 786-1584.

2.5.3 TA cloning reaction

The *Xho*I-*Kpn*I VWF fragment was inserted into the pGEM®-T vector via TA cloning. Ligation reactions were set up as described in table 2.5 and incubated at room temperature for 1 hour. A 3:1 insert:vector molar ratio was employed.

Reaction component	Ligation reaction
2X Rapid Ligation Buffer	5µL
pGEM-T vector	1µL
PCR product	XµL
T4 DNA Ligase	1µL
Nuclease-free water	XµL
Total	**10µL**

Table 2.5 Reaction components and volumes required for pGEM®T TA cloning.

2.5.4 Selection of transformants utilizing blue/white screening

Following ligation, NEB 10-beta competent *E.coli* (New England Biolabs, UK) were transformed with 2µL of the ligation reaction as detailed in section 2.4.2.6. Transformed cells were spread on IPTG/X-Gal/LB-ampicillin agar plates (for blue/white screening of recombinant colonies) which were then incubated at 37°C overnight. IPTG/X-gal/LB-ampicillin agar plates were prepared by spreading 100µl of 100mM IPTG (Sigma-Aldrich, MO, USA) and 20µL of 50mg/mL X-gal (Sigma-Aldrich, MO, USA) across the surface of LB-ampicillin agar plates. The mixture was allowed to absorb for 30 minutes at 37°C prior to use.

Blue/white screening is based on α-complementation of the *lac*Z gene. In this system, the host *E. coli* strain carries a specific *lacZ* mutation (N-terminal deletion of residues 11-41; *lacZΔM15*) that is unable to express a functionally active β-galactosidase enzyme. The plasmid carries the *lacZα* sequence which encodes the first 59 residues of β-galactosidase. When the plasmid containing the *lacZα* sequence is transformed into *lacZΔM15* cells, β-galactosidase function is restored. β-galactosidase

expression leads to the formation blue-coloured colonies on X-gal agar. White colonies are produced via insertional activation of the *lac*Zα gene. Cloning of a PCR product into the pGEM®-T multiple cloning site disrupts the *lac*Zα sequence and functional β-galactosidase is not expressed. Consequently, white colonies represent successful transformants.

White colonies were picked from IPTG/X-Gal/LB-ampicillin agar plates and were used to inoculate 5mL LB broth with ampicillin. 5mL cultures were incubated at 37°C overnight with vigorous shaking. Plasmid DNA was isolated as previously described (section 2.4.2.7) and the presence of the *Xho*I-*Kpn*I insert was confirmed by sequencing.

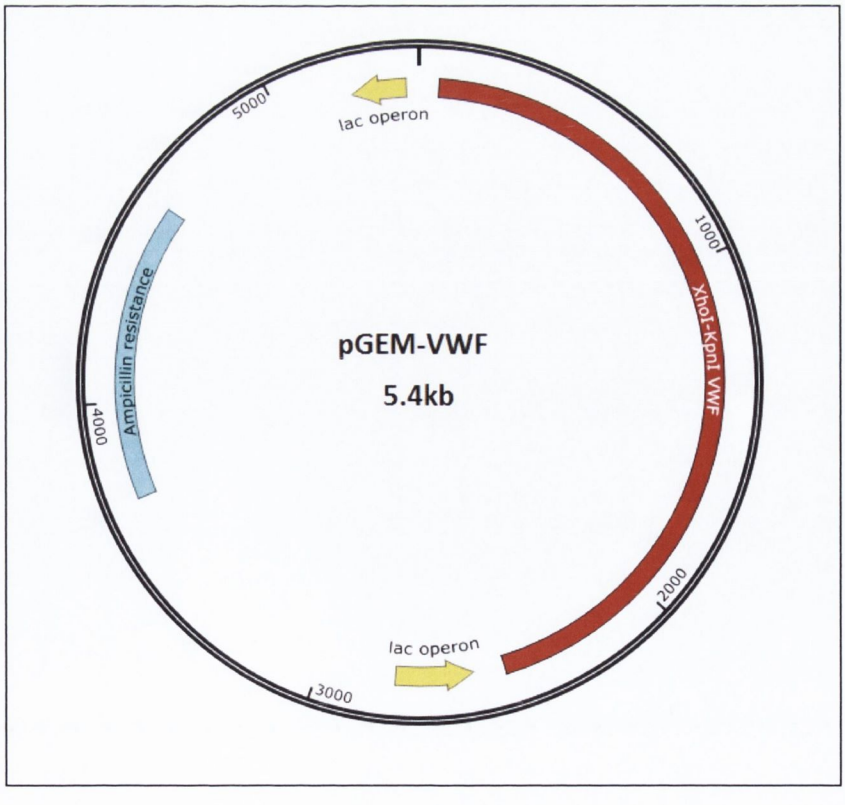


Figure 2.9 Shuttle vector for mutagenesis pGEM-VWF.

2.6 Site directed mutagenesis of VWF

A number of full-length and truncated VWF mutants were produced in this study. Point mutations were introduced via site directed-mutagenesis using mutagenesis primers (table 2.6) specifically designed using the freely available Primer X software. Primers were between 25 and 45 base pairs in length, had a GC content of 40-60% and had melting temperatures of 75-85^{0}C.

Primer ID	Primer sequence 5′ → 3′
Forward VWF R1204H	GTGTGAGGTGGCTGGC**CAT**CGTTTTGCCTCAGGAAAG
Reverse VWF R1204H	CTTTCCTGAGGCAAAACG**ATG**GCCAGCCACCTCACAC
Forward VWF R1205H	GTGGCTGGCCGG**CAT**TTTGCCTCAGGAAAG
Reverse VWF R1205H	CTTTCCTGAGGCAAA**ATG**CCGGCCAGCCAC
Forward VWF R1205C	TGGCTGGCCGG**TGT**TTTGCCTCAGGAA
Reverse VWF R1205C	TTCCTGAGGCAAA**ACA**CCGGCCAGCCA
Forward VWF R1205S	AGGTGGCTGGCCGG**AGT**TTTGCCTCAGGAA
Reverse VWF R1205S	TTCCTGAGGCAAA**ACT**CCGGCCAGCCACCT
Forward VWF R1450E	GAGCTGGAGCAGCAA**GAG**GACGAGATCGT
Reverse VWF R1450E	ACGATCTCGTC**CTC**TTGCTGCTCCAGCTC
Forward VWF N1515Q	ATTGGTGAAGCCGACTTC**CAA**AGGAGCAAGGAGTTC
Reverse VWF N1515Q	GAACTCCTTGCTCCT**TTG**GAAGTCGGCTTCACCAAT
Forward VWF N1574Q	AGATCCGCTACCAGGGCGGC**CAA**AGGACCAACACT
Reverse VWF N1574Q	AGTGTTGGTCCT**TTG**GCCGCCCTGGTAGCGGATCT

Table 2.6 Forward and reverse VWF mutagenesis primers

Various VWF plasmids were used as mutagenesis templates. Parent vectors and their associated mutagenesis templates are outlined in table 2.7.

Parent Vector	Mutagenesis Template	Mutant Vector
pcDNA VWF	pGEM-VWF	pcDNA VWF R1204H
	pGEM-VWF	pcDNA VWF R1205H
	pGEM-VWF	pcDNA VWF R1205C
	pGEM-VWF	pcDNA VWF R1205S
	pGEM-VWF	pcDNA VWF R1450E
	pGEM-VWF	pcDNA VWF N1515Q
	pGEM-VWF	pcDNA VWF N1574Q
pcDNA VWF ΔA1	pcDNA VWF ΔA1	pcDNA VWF ΔA1 R1205H
pcDNA D'A3 VWF	pcDNA D'A3 VWF	pcDNA VWF R1205H
	pcDNA D'A3 VWF	pcDNA VWF R1205C
	pcDNA D'A3 VWF	pcDNA VWF R1205S

Table 2.7 Template vectors for VWF mutagenesis.

2.6.1 Site directed mutagenesis utilizing pGEM-VWF as a template

To enable efficient mutagenesis of full-length pcDNA VWF a shuttle vector strategy was employed (figure 2.9). Site directed mutagenesis was carried out using shuttle vector pGEM-VWF as a template. A truncated fragment incorporating the desired mutation was then excised from the pGEM-VWF shuttle vector sub-cloned into the full-length VWF construct via *Bam*HI/*Kpn*I restriction digest.

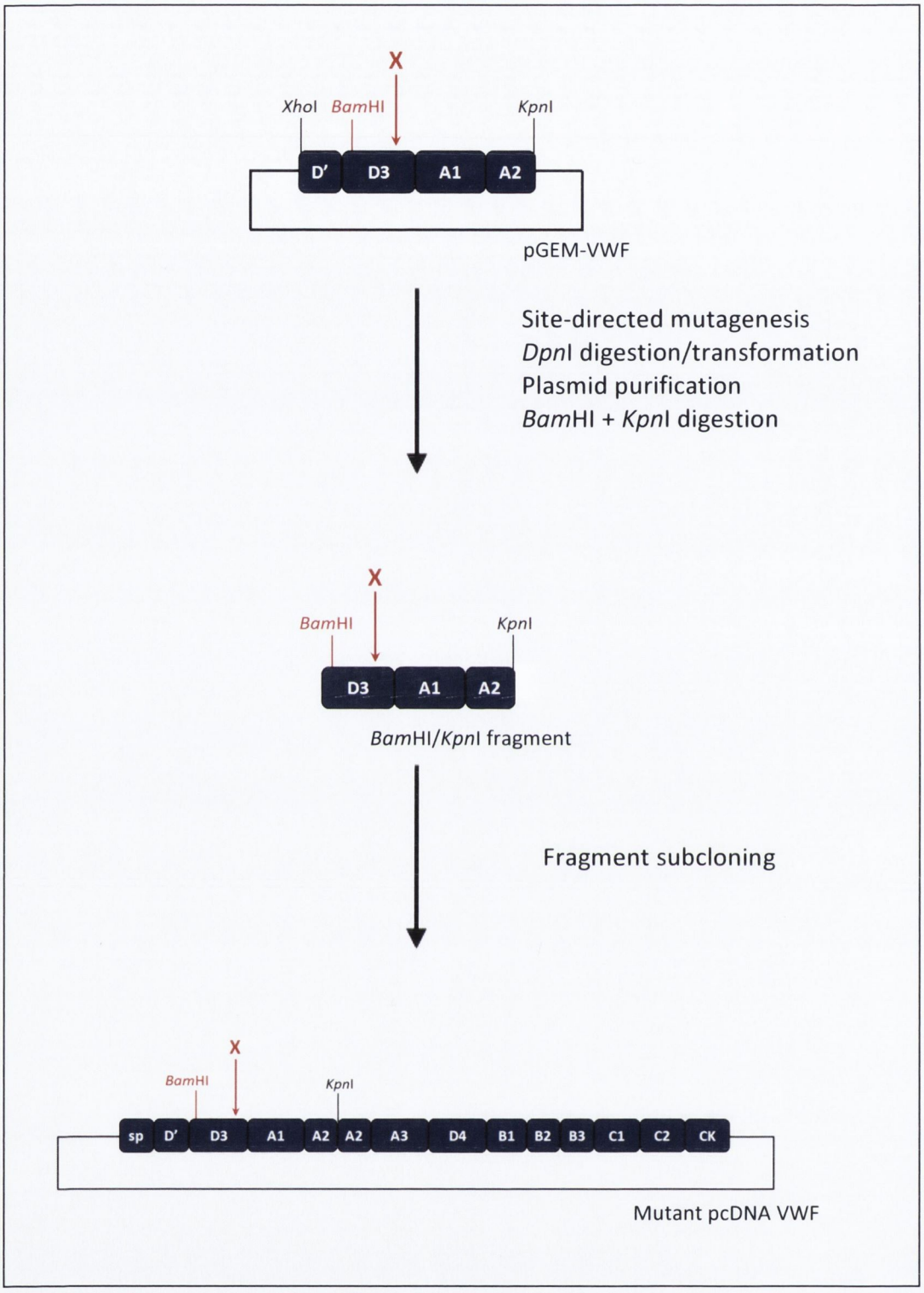


Figure 2.10 Mutagenesis strategy involving pGEM-VWF shuttle vector.

2.6.1.1 Mutagenesis PCR

Site-directed mutagenesis was carried out using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, UK) according to the manufacturer's instructions using pGEM-VWF as a template. Mutagenesis reactions were set up in thin-walled dome-capped PCR tubes (Fisher Scientific, Ireland). Reactions contained 10ng plasmid DNA (pGEM-VWF or pcDNA D'A3), 125ng of each of the appropriate forward and reverse mutagenesis primers, 1µL dNTP mix, 5µL 10X reaction buffer, 3µl QuikSolution and nuclease free water to a final volume of 50µL. 1µL *PfuUltra* HF DNA polymerase (2.5 U) was then added. Mutagenesis PCR cycling was performed using the MJ Research PTC 200 Thermo Cycler (GMI, MN, USA). Cycling parameters are outlined in table 2.8.

Step	Temperature	Time	Cycles
Initial Denaturation	95°C	1 minute	1
Denaturation	95°C	50 seconds	18
Annealing	60°C	50 seconds	
Extension	68°C	1 minute/kb	
Final Extension	68°C	7 minutes	1

Table 2.8 PCR cycling parameters for mutagenesis with QuikChange II XL Site-Directed Mutagenesis Kit.

Following cycling, reactions were cooled to 4°C. 1µL of the *Dpn*I restriction enzyme (10 U) was added to each reaction and incubated at 37°C for 1 hour. *Dpn*I digests the methylated and hemimethylated DNA of the template plasmid DNA but not newly synthesized mutant DNA. *Dpn*I (New

England Biolabs, UK) digested DNA was then used to transform competent *E.coli* as described in section 2.5.2.6. Mutant plasmid DNA was isolated (section 2.5.2.7) and the presence of the desired mutation was confirmed by sequencing.

2.6.1.2 Subcloning the *BamH*I-*Kpn*I fragment of pGEM-VWF

In order to introduce the desired mutation into the full length VWF expression vector pcDNA VWF, subcloning of the *BamH*I-*Kpn*I fragment (2035bp) of mutant pGEM-VWF was carried out as described in figure 2.9. The *BamH*I-*Kpn*I fragment was released via sequential *Bam*HI and *Kpn*I digestion. Approximately 1µg of mutant pGEM-VWF was digested with 10U *Bam*HI in NEBuffer 4 for 1 hour at 37°C. 10U of *Kpn*I were then added and the sample was incubated with NEBuffer 1 for 1 hour at 37°C. The *BamH*I-*Kpn*I fragment was purified via agarose gel electrophoresis and gel extraction was carried out (section 2.4.2.3 and 2.4.2.4). pcDNA VWF was also sequentially digested with *Bam*HI and *Kpn*I to facilitate insertion of the mutant fragment. In addition, pcDNA VWF was treated with Heat Inactivated Alkaline Phosphatase (Invitrogen, UK) to prevent vector self-ligation and recircularization. The 14kb fragment *Bam*HI-*Kpn*I fragment of pcDNA VWF was then gel purified as before. Purified DNA was quantified using the Nanodrop 2000 microvolume spectrophotometer (Thermo Scientific, UK) and visualised on 0.7% agarose gels to assess purity (figure 2.10).

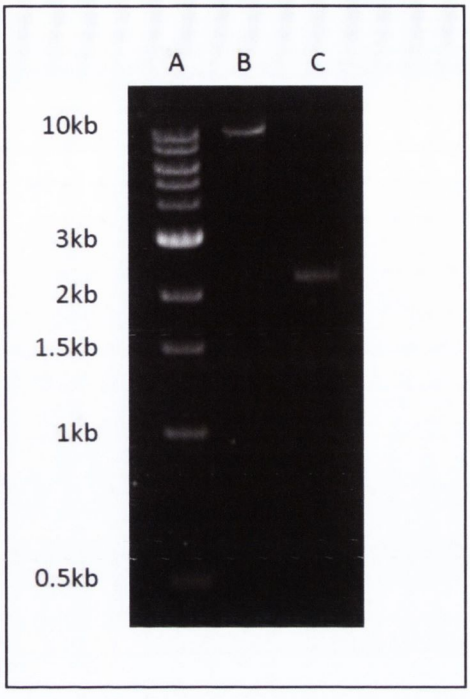


Figure 2.11 Agarose gel electrophoresis.
Lane A) 1kb ladder (NEB, UK), lane B) Purified 14kb *Bam*HI-*Kpn*I fragment of pcDNA VWF, lane C) Purified 2035bp *Bam*HI-*Kpn*I fragment of pGEM-VWF.

2.6.1.3 Ligation of mutant *BamH*I-*Kpn*I fragment into pcDNA VWF

Ligation of the 2035bp *BamH*I-*Kpn*I fragment of mutant pGEM-VWF into the pcDNA VWF vector was carried out using the LigaFast™ Rapid DNA Ligation System (Promega, WI, USA). Ligation reactions were used to transform competent *E.coli* and propagate mutant DNA as before (section 2.4.2.6). Transformed *E.coli* were grown on LB agar with ampicillin overnight at 37°C. The resulting colonies were used to inoculate 5mL cultures in LB broth with ampicillin. Cultures were grown overnight at 37°C with shaking. Plasmid DNA was isolated (section 2.4.2.7) and screened for the presence of the *BamH*I-*Kpn*I fragment on the basis of size. Insertion of the *BamH*I-*Kpn*I fragment was also confirmed by sequencing.

2.6.2 Site directed mutagenesis using pcDNA VWF ΔA1 as a template.

pcDNA VWF ΔA1 encodes a VWF protein lacking the A1 domain. Since the *Bam*HI-*Kpn*I fragment of pGEM-VWF spans the D'-A2 domains the pGEM-VWF strategy is not suitable for mutagenesis of this construct. Site-directed mutagenesis of pcDNA VWF ΔA1 was carried out using the KAPA HiFi HotStart DNA polymerase (Kapa Biosystems, MA, USA) as this polymerase is capable of amplifying larger plasmid targets up to 18 kb in size. Reactions were set up in thin-walled, dome-capped PCR tubes. 10ng pcDNA VWF ΔA1 was used as a template and forward and reverse mutagenesis primers were added to a final concentration of 0.3μM. PCR cycling was performed using the MJ Research PTC 200 Thermo Cycler with heated lid (GMI, MN, USA). Cycling parameters are outlined in table 2.9.

Step	Temperature	Time	Cycles
Initial Denaturation	95°C	3 minutes	1
Denaturation	98°C	20 seconds	
Annealing	65°C	15 seconds	18
Extension	72°C	1 minute/kb	
Final Extension	72°C	1 minute/kb	1

Table 2.9 PCR cycling parameters for site-directed mutagenesis using KAPA HiFi Hotstart DNA polymerase.

Following amplification, template plasmid DNA was digested with *Dpn*I and newly synthesized DNA was propagated in *E.coli* (section 2.4.2.6). The presence of the mutation was verified by sequencing.

2.6.3 Site-directed mutagenesis using pcDNA D'A3 VWF as a template

Mutagenesis of pcDNA D'A3 VWF was carried out using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, UK) using pcDNA D'A3 VWF as a template. Reaction cycling conditions are outlined in section 2.6.1.1.

2.7 Expression and purification of recombinant VWF

2.7.1 Cell culture

Cell culture was performed in a class II laminar flow cabinet. Cells were grown in humidified incubators at 37°C in an atmosphere containing 5% CO_2. Human Embryonic Kidney (HEK 293T) cells (ATCC, LGC Standards UK) were utilized for expression of recombinant VWF variants. Several previous studies have demonstrated the effectiveness of the HEK 293T expression system in the context of VWF production. HEK 293T is an adherent cell line that also expresses the SV40 large T-antigen. This allows for episomal replication of transfected plasmids containing the SV40 origin of replication such as pcDNA VWF. HEK 293T were grown in Minimal Essential Media alpha (MEMα; Bio-Sciences, Ireland) supplemented with 1U/mL penicillin, 0.1mg/mL streptomycin (Bio-Sciences, Ireland), 2mM L-glutamine (Bio-Sciences, Ireland), and 10% Foetal Calf Serum (FCS; Bio-Sciences, Ireland). Cells were routinely cultured in T-175 flasks and passaged at ~80% confluence.

2.7.1.1 Transient transfection of HEK 293T cells

Recombinant VWF was produced via transient transfection of HEK 293T cells in serum free medium. 20 x T-175 flasks were seeded with HEK 293T and grown in supplemented MEMα until confluent. Cells were washed with sterile PBS and 18mL serum free Opti-MEM (Bio-Sciences, Ireland) was added to each flask. Branched polyethylenimine (bPEI; Sigma-Aldrich, Ireland) was utilized as a transfection reagent. bPEI is a cationic polymer with a high positive charge density that condenses anionic DNA into positively charged nano-complexes. Interaction of these positively charged nano-complexes with anionic cell surface residues then results in endocytic uptake of the nano-complex (Eikenboom et al. 2013). Once inside the cell protonation of bPEI amines results in osmotic swelling causing the endocytic vesicle to burst, releasing the nano-complex into the cytoplasm (Turecek et al. 2000). Subsequent unpacking of the complex facilitates diffusion of DNA to the nucleus. For transient transfection of recombinant VWF a bPEI to DNA ratio of 2:1 was used. Plasmid DNA was diluted to a final DNA concentration of 2μg/mL in 150mM NaCl. bPEI diluted to a final concentration of 1μg/mL in 150mM NaCl was added to the diluted plasmid DNA drop-wise. The mixture was pulse vortexed for 15 seconds and complex formation was allowed to proceed for 30 minutes at room temperature. Following incubation, 2mL of the bPEI:DNA complex was added to each flask drop-wise. Flasks were returned to the incubator and conditioned medium was harvested after 72 hours. Cellular debris was cleared via centrifugation at 4,000rpm for 30 minutes.

2.7.2 Concentration of conditioned medium via anion exchange chromatography

Condition medium containing recombinant VWF protein was concentrated via anion-exchange chromatography. Anion exchange chromatography utilizes a positively charged binding resin to bind proteins with a net negative charge. Negatively charged proteins are then eluted from the resin by increasing the ionic strength (salt concentration) of the buffer. Competition for binding to the immobilised matrix releases proteins from the resin.

Conditioned medium was loaded onto a HiTrap Q HP column (pre-packed with Q Sepharose™ High Performance; GE Healthcare, UK) in 20mM Tris, pH 7.4 at a flow rate of 1.5mL/min. The column was washed with 20mM Tris, 100mM NaCl, pH7.4 at a flow rate of 2mL/min to contaminants. VWF was then eluted in 20mM Tris, 500mM NaCl, pH7.4. The VWF containing fraction (~5mL) was dialyzed against 20mM Tris, pH7.4 at $4^{o}C$ using Snakeskin Dialysis Tubing with a molecular weight cut off of 10kDa (Thermo Scientific, UK) to remove salt.

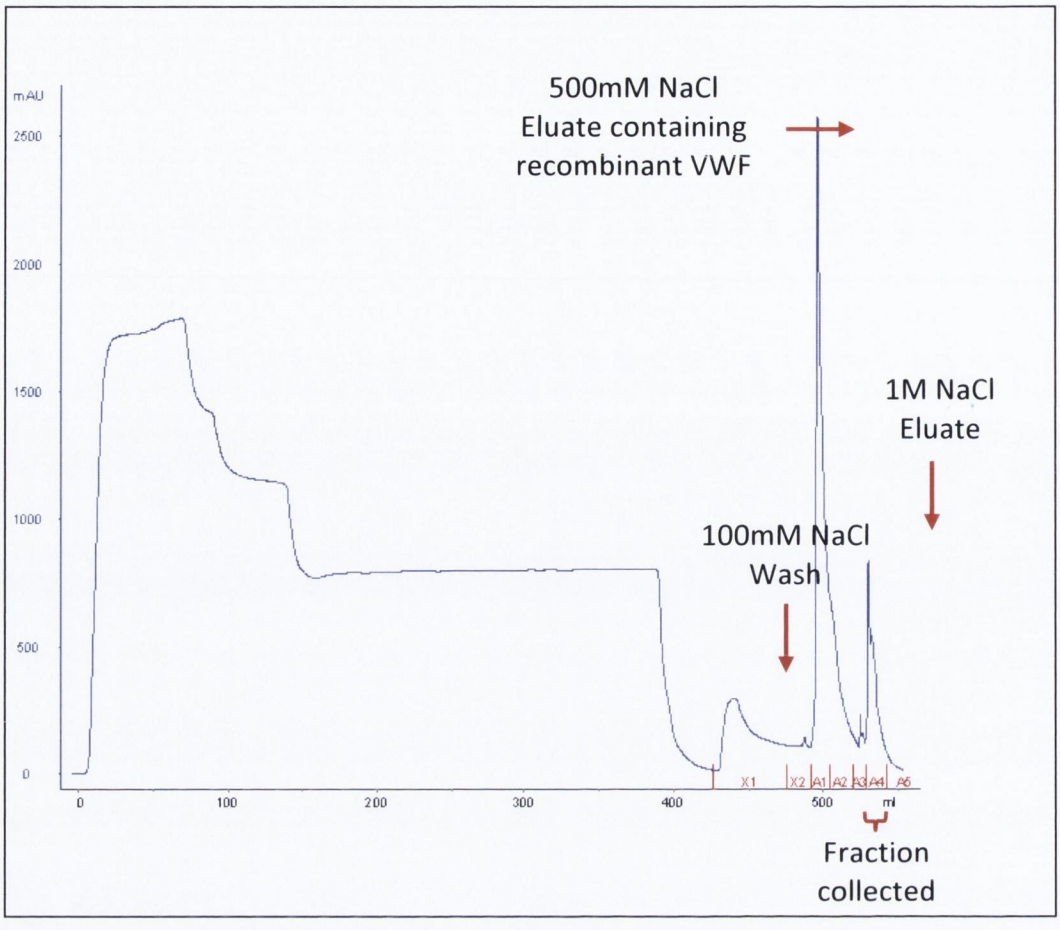


Figure 2.12 Anion exchange chromatography.
Concentration of conditioned medium containing recombinant VWF. VWF was eluted using 0.5M NaCl.

2.7.3 Spin column concentration

Full-length recombinant VWF variants were further concentrated using Amicon Ultra-15 100K MWCO devices (Millipore, Ireland). 5mL anion exchange elutes were concentrated to a volume of ~1mL via centrifugation at 4,000g. VWF was then quantified by ELISA (section 2.8.1) and analyzed by western blotting.

2.7.4 Purification via metal affinity chromatography

Truncated D'A3-VWF and A1A2A3-VWF constructs were expressed with polyhistidine tags for purification purposes. Following concentration via anion exchange chromatography, these proteins were purified via metal ion affinity chromatography. Affinity chromatography separates proteins on the basis of a reversible interaction between a protein and a specific ligand coupled to a chromatography matrix. A 1mL HiTrap Chelating Column (GE Healthcare, UK) was charged with 0.1M $NiCl_2$. The dialyzed anion exchange elute (~5mL) was then loaded onto the column at a flow rate of 0.5mL/min in 20mM Tris, 150mM NaCl, 5mM Imidazole, pH7.4. The column was washed with approximately 20 column volumes of loading buffer at a flow rate of 1mL/min before recombinant truncated VWF was eluted with 20mM Tris, 150mM NaCl, 300mM Imidazole, pH7.4. The ~1mL eluate fraction was dialyzed against 20mM Tris, pH7.4 at $4^{o}C$ overnight and VWF concentration was then quantified by BCA (section2.8.3). VWF purity was analyzed by SimplyBlue™ staining and western blotting (section 2.8.5 and 2.8.6).

2.8 Analysis of VWF

2.8.1 VWF:Ag ELISA

VWF antigen (VWF:Ag) was measured by sandwich enzyme-linked immunosorbent assay (ELISA). Maxisorp plates (Nunc, Denmark) were coated with rabbit anti-human VWF antibody (Dako, Denmark) at a final concentration of 6.2µg/mL in 50mM carbonate buffer (pH 9.6), overnight at 4°C. Wells were washed three times with PBS-T (PBS, 0.05% v/v Tween-20) and then blocked for non-specific binding with PBS-T containing 3% bovine serum albumin (BSA; Sigma, Ireland) for 1 hour at room temperature. Wells were again washed three times with PBS-T before test samples at appropriate dilutions were added. Reference plasma (Technoclone, Austria) diluted 1:80-1:2560 (final concentration 0.125-0.0039µg/mL) was used to generate a standard curve. After 2 hour incubation at 37°C wells were washed three times with PBS-T and 100µl of anti-VWF-HRP (DAKO, Denmark) diluted 1:500 in PBST (final concentration 2.2µg/mL) was added and incubated for 1 hour at 37°C. Following another 3 PBS-T washes, 100µl of HRP substrate 3,3',5,5'-Tetramethylbenzidine (TMB; Substrate Reagent Pack, R&D Systems, UK) was added to the wells. The reaction was subsequently stopped with 50µL 1M H_2SO_4. Absorbance was read at 492nM using a VERSAmax microplate reader (Molecular Devices, UK).

ELISA samples were tested in triplicate, using two replicate wells for each set of test conditions. The intra-assay and inter-assay coefficients of variation were both less than 10%. A standard curve was generated by plotting VWF concentration (µg/mL) on the x-axis against optical density

(absorbance @ 450nm) on the y-axis. The curve was linearized by performing a logarithmic transformation and the equation of the line and R^2 values were calculated using Microsoft Excel.

2.8.2 VWF collagen binding assay

Collagen binding activity (VWF:CB) is a sensitive measure of VWF multimeric composition. High molecular weight VWF multimers bind to collagen with significantly greater affinity than low molecular weight forms (Sporn et al. 1987). VWF:CB of VWF was determined using an ELISA based collagen binding assay. Maxisorp plates (Nunc, Denmark) were coated with recombinant human collagen III (BioVision, CA, USA) at a final concentration of 5µg/mL in carbonate buffer (50mM Na_2CO_3, pH 9.6) overnight at 4°C. Wells were washed three times in PBS-T and blocked with 3% BSA in PBS-T for 1 hour at room temperature. After washing three times with PBS-T, test samples were added to the plate. Reference plasma (Technoclone, Austria) diluted 1:80-1:2560 (final concentration 0.125-0.0039µg/mL) was used to generate a standard curve. Samples and reference plasma were incubated on the plate for 2 hours at 37°C. Following washing, polyclonal rabbit anti-human VWF-HRP (Dako, Denmark) diluted 1/500 in PBS-T (final concentration 2.2µg/mL) was added to the wells and incubated for 1 hour at 37°C. Wells were washed and colour was developed as described before (section).

A standard curve and log-log plot were constructed as for VWF:Ag ELISA and VWF:CB for each of the test samples was determined in µg/mL. Collagen binding activity was then expressed as a ratio relative to VWF:Ag levels.

2.8.3 BCA Protein Assay

For truncated VWF variants, total protein was quantified following purification using the Thermo Scientific™ Pierce™ BCA Protein Assay Kit. The BCA (bicinchoninic acid) assay is a colourimetric means by which to quantify total protein concentration and is based on the reduction of Cu^{2+} ions to Cu^{+} by the peptides bonds of proteins. The amount of Cu^{2+} reduced is proportional to the amount of protein present in the solution. Two molecules of BCA then chelate with each Cu^{+} ion, forming a purple-coloured product that absorbs at 562 nm.

Each protein sample was serially diluted and 25µL of each dilution was added to a 96 well plate in duplicate. To construct a standard curve, BSA was diluted over a range of 20-2000µg/mL and 25µL of each BSA dilution was also added to the plate in duplicate. The working reagent (WR) was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). 200µL of WR was then added to each well and mixed thoroughly. The plate was incubated for 30 minutes at 37°C and absorbance at 562nm was read. A standard curve was prepared by plotting the average blank-corrected 562nm measurement for each BSA standard against concentration (µg/mL). Sample concentrations were extrapolated from the curve via linear regression analysis using GraphPad Prism software (GraphPad Prism version 5.0 for Windows; GraphPad Software, CA, USA).

2.8.4 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Pre-cast gradient gels (4-12% NuPAGE® Bis-Tris Gels; Life Technologies, UK) were used for analysis of VWF following purification. Samples containing ~50ng VWF (for western blot) or ~1-2ng (for Coomassie Blue staining) were mixed with NuPAGE® LDS Sample Buffer (4X; Life Technologies, UK) and incubated at 70°C for 5 minutes before loading onto the pre-cast gels. For reducing conditions, samples were mixed with NuPAGE® LDS Sample Buffer as well as NuPAGE® Sample Reducing Agent (10X; Life Technologies, UK) and were heated to 70°C for 5 minutes. A pre-stained high molecular weight marker (PageRuler Prestained Protein Ladder; ThermoScientific, UK) was run in parallel with all test samples for protein identification based on size. Electrophoresis was carried out at 185V for approximately 45 minutes in NuPAGE® MOPS SDS running buffer (Life Technologies, UK). Detection of proteins was performed by SimplyBlue™ staining and western blotting.

2.8.5 SimplyBlue™ Staining

SimplyBlue™ SafeStain (Life Technologies, UK) is a technique used to visualise non-specific protein bands following SDS-PAGE. Gels were washed with dH_2O 3 times for 5 minutes following electrophoresis and were incubated with SimplyBlue™ SafeStain for one hour at room temperature with gentle agitation. Gels then were de-stained with dH_2O for 1 hour.

2.8.6 Western blotting

Following electrophoresis, proteins were transferred to a PVDF membrane (Immobilon-P; Millipore, Ireland) using an electroblot system (Mini-Protean®II; BIO-RAD, Fannin Ltd, Ireland). The PVDF membrane was pre-activated by immersion in methanol. Transfer was performed at 100V for 1 hour in transfer buffer (25mM Tris, 192mM glycine and 20% (v/v) methanol). Following protein transfer, the membrane was blocked for non-specific binding with 3% solution of fat-free milk in PBS-T at room temperature. The membrane was washed with PBS-T, and then incubated with rabbit polyclonal anti-human VWF-HRP (Dako, Denmark) diluted 1:20,000 in PBS-T for 1 hour at room temperature with gentle agitation. After thorough washing, bound antibody was detected using the SuperSignal® West Pico Chemiluminescent Substrate kit (Thermo Scientific, Ireland) according to the manufacturer's instructions. The membrane was then exposed to autoradiography (x-ray) film (Fujifilm; Fisher Scientific, Ireland) and the films were developed using the AGFA CP1000 automatic film-developing system (AGFA, Germany).

2.9 Expression and purification of recombinant galectin-3

2.9.1 Bacterial expression of recombinant galectin-3

BL21(DE3) *E. coli* (Invitrogen, UK) were transformed with pET 151 Gal-3 and grown overnight at 37°C on LB agar with ampicillin (section2.4.2.6). Colonies were picked and used inoculate 2 x 5mL LB ampicillin starter cultures. The starter cultures were grown overnight at 37°C overnight with shaking and were subsequently used to inoculate 2 x 200mL expression cultures in Overnight Express™ Instant TB Medium (Novagen, UK). The expression cultures were incubated overnight at 37°C with vigorous shaking and bacterial cells were harvested via centrifugation at 4000rpm for 20 minutes. The cell pellet was resuspended by vortexing in approximately 20mL 20mM Tris, 500mM NaCl, 20mM Imidazole, 0.5mM DTT, pH 7.4. The resulting cell suspension was then aliquoted for sonication. Cell suspensions were sonicated (Soniprep 150, MSE, UK) on ice at an amplitude of 10 microns for 30 seconds followed by 30 seconds resting. The sonication/resting cycle was repeated 3-4 times. The solution was then centrifuged for 30min at 20,000g and the resulting supernatant was filtered through syringe driven filter units (0.22µm pore, Millipore, Ireland). Recombinant galectin-3 was purified from the filtrate via nickel affinity chromatography.

2.9.2 Purification of recombinant galectin-3

The filtrate was loaded onto a 1mL HiTrap Chelating Column (GE Healthcare, UK) that was previously charged with 0.1M $NiCl_2$. The filtrate was loaded at a flow rate of 0.5mL/min in binding buffer (20mM Tris, 500mM

NaCl, 20mM Imidazole, 0.5mM DTT, pH 7.4). After loading, the column was washed with approximately 20 column volumes of binding buffer. Galectin-3 was eluted with 20mM Tris, 500mM NaCl, 500mM Imidazole, 0.5mM DTT, pH 7.4). The galectin-3 containing fraction was then dialyzed against 20mM Tris containing 0.5mM DTT overnight at 4°C. Purity was assessed by SimplyBlue ™ staining and galectin-3 was quantified by BCA (section 2.8.3).

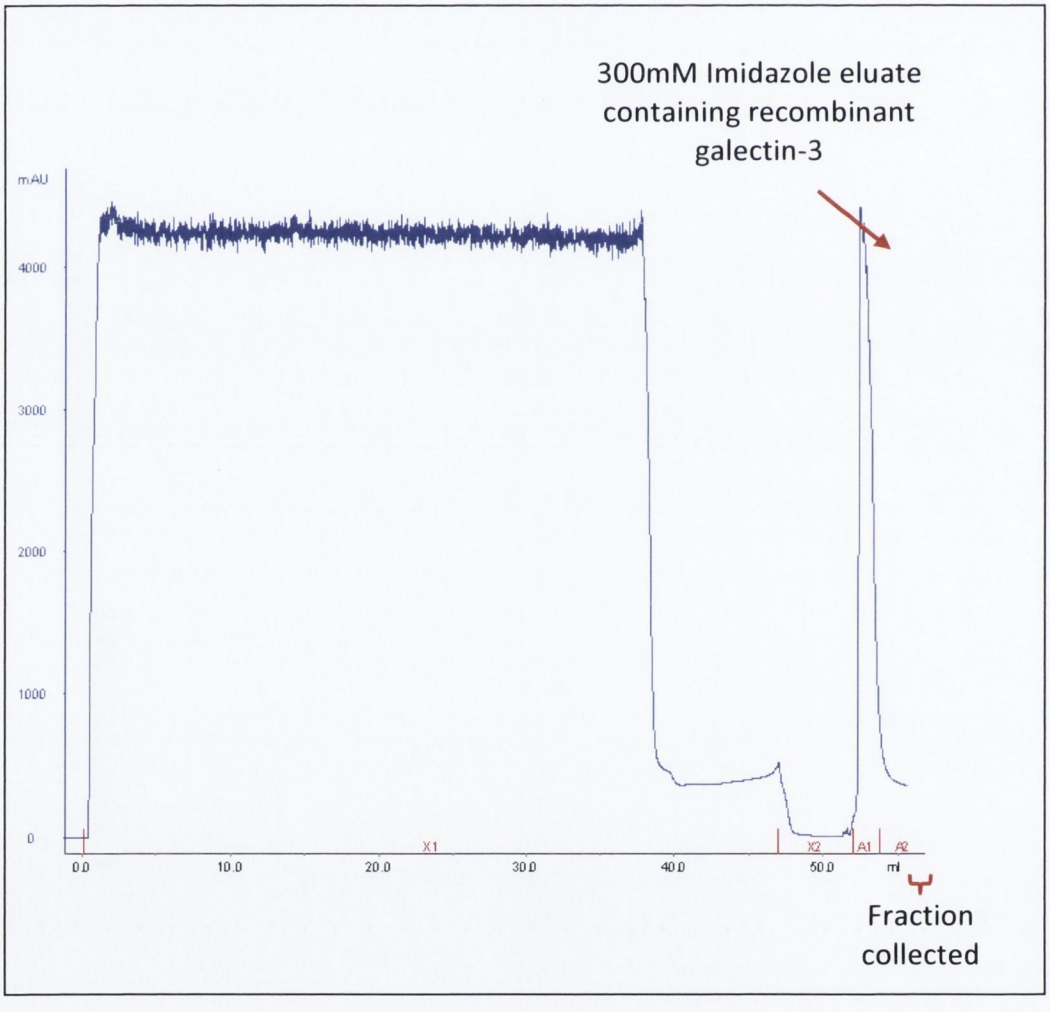


Figure 2.13 Purification of recombinant galectin-3 via nickel affinity chromatography.

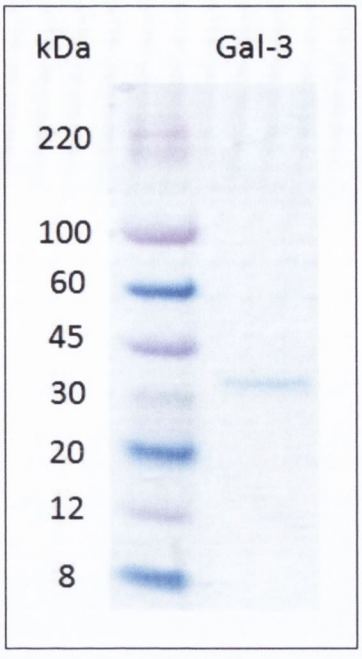


Figure 2.14 SDS-PAGE assessment of galectin-3 purity following nickel affinity chromatography.
Galectin-3 purity was assessed by SDS-PAGE followed by SimplyBlue™ staining.

2.10 *In vitro* binding studies

2.10.1 Galectin-3 binding studies

In order to assess potential VWF/galectin-3 binding interactions a modified ELISA was used. Maxisorp plates (Nunc, Denmark) were coated with 33nm galectin-3 in carbonate buffer (50mM Na_2CO_3, pH 9.6) overnight at 4°C. Wells were washed three times with PBS-T and blocked with 3% BSA in PBS-T for 1 hour at room temperature. After washing, 100µL of VWF (plasma-derived or recombinant) diluted in PBS-T was added to the galectin-3 coated wells and incubated for 2 hours at 37°C. After further washing, 100µL of anti-VWF-HRP (DAKO, Denmark) diluted 1:500 in PBST (final concentration 2.2µg/mL) was added and incubated for 1 hour at 37°C. Following another 3 PBS-T washes, 100µl of TMB (R&D Systems, UK) was added to the wells for colour development. The reaction was stopped with 50µL 1M H_2SO_4 and absorbance was read at 492nM. Galectin-3 binding of glycan-modified VWF was expressed as a percentage of untreated pd-VWF. Binding of recombinant VWF variants was expressed as a percentage of recombinant wild-type VWF.

2.10.2 THP-1 binding studies

THP-1 is a human monocytic cell-line derived from a patient with acute monocytic leukaemia (Tsuchiya et al. 1980). THP-1 grow in suspension as single round cells that express distinct monocyte markers. *In vitro,* THP-1 cells can be differentiated into macrophage-like cells using phorbol 12-myristate 13-acetate (PMA) (Tsuchiya et al. 1982). Differentiation induces significant morphological change such that THP-1 cells become flat and amoeboid in

shape and also become adherent. Importantly, characterization of THP-1 macrophages via flow cytometry has demonstrated that PMA stimulation followed by a resting period in culture produces THP-1 macrophages that closely resemble the phenotype of primary human monocyte-derived macrophages (Daigneault et al. 2010). A major advantage of using THP-1 cells over primary monocytes is their homogeneous genetic background which minimizes the variability in gene expression profiles.

2.10.2.1 THP-1 cell culture

THP-1 cells were seeded on Nunc™ microwell plates with Nunclon™ Delta surface (Fisher Scientific, Ireland) at a density of 20,000cells/well in growth medium (RPMI supplemented with 5% FCS, 100U/mL penicillin, 100U/mL streptomycin, 0.05mM β-mercaptoethanol; Biosciences, Ireland), and were differentiated for 3 days with 20ng/μL PMA (Sigma-Aldrich, Ireland). After 3 days, fresh growth medium was added to the cells, which were then rested for an additional 4 days.

2.10.2.2 THP-1 binding assay

THP-1 macrophages were serum starved in serum free growth medium for 30 minutes at 37°C. Cells were then cooled on ice for a further ~10 minutes to prevent endocytosis. Recombinant VWF or variants thereof were diluted to a final concentration of 2.5μg/mL in 100μl ice-cold serum free growth medium in the presence of either 1mM $MnCl_2$ or 1mg/mL ristocetin and incubated with the cells for 1 hour on ice. THP-1 macrophages were then washed with 100μL ice cold PBS twice and fixed with 4% paraformaldehyde

(Sigma-Aldrich, Ireland) in PBS. After further washing, nuclei were stained with Hoechst 33342 (Thermo Fisher, Ireland) and bound VWF was detected using polyclonal rabbit anti- human VWF (Dako, Denmark) followed by Alexa Fluor® 488 donkey anti-rabbit IgG (Life Technologies, UK). THP-1 binding studies were carried out in triplicate, using three replicate wells for each VWF variant.

2.10.2.3 THP-1 inhibition studies

For THP-1 inhibition studies 5mM mannose-6-phosphate, 1μM NIF or 1μM RAP were added to VWF in growth medium supplemented with 1mM $MnCl_2$ and incubated with the cells for one hour on ice. Binding was detected as described in section 2.10.2.2.

2.11 High Content Analysis

High content analysis (HCA) is an automated platform for performing fluorescent microscopy and quantitative image analysis. HCA has several advantages over manual cell-based assays including standardization, speed and accuracy. High-throughput image acquisition minimises the impact of experimental error and biologic variation while image-processing algorithms enhance signal-to-noise ratios for more precise and accurate analysis. In the context of THP-1 macrophage binding, HCA permits rapid, simultaneous analysis and comparison of multiple VWF variants on a single microwell plate.

2.11.1 Image acquisition

Binding of VWF to the surface of THP-1 cells was imaged using the IN Cell Analyzer 1000 (GE Healthcare, UK) with appropriate filter selection (D/F/TR). Seven fields of view were analyzed per well at a magnification of 20x, equating to the analysis of approximately 800 cells/well.

2.11.2 Image analysis

Following image acquisition, image analysis was carried out using IN Cell 1000 Image Analysis Software (GE Healthcare, UK). The Multi-Target Analysis protocol was used for quantification of FITC-labelled VWF. Using the Hoechst stained nucleus as a reference, this protocol defines a collar around the nuclear region to identify individual cells. Fluorescent inclusions within the collar are attributed to that cell and then quantified. Therefore, mean area (mean area of inclusions; averaged by all inclusions within the cell), intensity (average intensity of pixels within inclusions) and total area (total area of inclusions attributed to the cell) can be calculated.

Nuclei were identified on the basis of Hoechst staining using the Top Hat segmentation method with a minimum area of 60μM^2. Cells were segmented using the Collar method with a radius of 10μM. Finally, FITC-labelled VWF was identified as an "organelle" and was segmented using the Multi-scale top hat algorithm set to identify granules ranging from 1.61μM to 8μM in size. FITC-labelled VWF was quantified and reported as total area. Data were graphed as percentage fluorescent area/cell relative to WT-VWF binding (mean ± SEM) using GraphPad Prism software (GraphPad Prism version 5.0 for Windows; GraphPad Software, CA, USA).

2.12 Mouse clearance studies

2.12.1 Animal husbandry

Male and female wild-type C57Bl, $VWF^{-/-}$ and $ASGPR^{-/-}$ mice were used between 6-10 weeks of age. Mice were housed in individually ventilated and filtered cages under positive pressure (Techniplast, UK). Food and water were supplied ad libitum. All animal experiments were performed in compliance with Irish Medicines Board regulations and with ethical approval from Trinity College Dublin ethics board.

2.12.2 $VWF^{-/-}$ mouse model

$VWF^{-/-}$ mice were developed by targeted disruption of exons 4 and 5 of the *VWF* gene and were bred on a C57Bl background (Denis et al. 1998). Homozygous $VWF^{-/-}$ mice are viable, fertile and appear normal at birth though they have no detectable plasma, platelet, endothelial VWF or VWF propeptide. These mice are also characterized by significantly reduced levels of circulating FVIII (~20% of wild type). $VWF^{-/-}$ have prolonged bleeding times and approximately 10% of neonates exhibit spontaneous bleeding episodes. The haemostatic deficiencies of $VWF^{-/-}$ mice make them suitable for use as a model of type 3 VWD.

2.12.3 $Asgr1^{-/-}$ mouse model

$Asgr1^{-/-}$ mice were produced via deletion of the MHL-1 major subunit coding region in exon 2 of the Asgr1 locus and were bred on a C57Bl background (Fernandez et al. 1982). Homozygous $Asgr1^{-/-}$ mice are viable and

fertile and exhibit impaired hepatic clearance of exogenous asialoglycoproteins. These mice may also exhibit elevated plasma VWF antigen levels (Grewal et al. 2008). Asgr1 deficiency is also associated with reduced bleeding time and enhanced platelet survival. Asgr1$^{-/-}$ mice may be particularly informative in the context of VWF clearance given the critical role of VWF glycan structures in regulating plasma clearance.

2.12.4 VWF clearance studies

In vivo VWF clearance studies were carried out by Ms Jamie O'Sullivan (Haemostasis Research Group, Trinity College Dublin). VWF$^{-/-}$, ASGPR$^{-/-}$ or wild-type C57Bl mice were intravenously injected with 7.5µg/100µL PBS of recombinant WT-VWF or variants thereof. At appropriate timepoints, mice were anaesthetised with an intraperitoneal injection of 10mL/kg Hypnorm/Hypnovel (0.2133 mg/kg fentanyl citrate, 6.75 mg/kg fluanisone, 3.375 mg/kg midazolam)and blood from a subclavicle incision (~500µL) was sampled into heparin-coated microcontainers (BD Unitech, Ireland) and mixed well. Three to five mice were used per timepoint and each mouse was bled only once. Blood samples were centrifuged at 2,500g for 15 minutes at room temperature and plasma was removed. VWF:Ag levels in mouse plasma was subsequently analysed by ELISA. Clearance values were expressed and graphed as residual VWF (%) relative to the amount injected against time (minutes). Data were fitted to monoexponential equations, based on analysis of the Akaike Information Criterion (AIC). The slope and intercept of the equation of the line were used to solve for the pharmacokinetic parameters Mean Residence Time (MRT), and half-life ($t_{1/2}$).

Monoexponential clearance data were fitted to the equation:

$$Y=(Y0-Plateau)*e(-K*X)+ Plateau$$

Where Y refers to the amount of residual VWF antigen in plasma relative to the amount injected, X=time, K=rate constant and Plateau=0.

The equation for monoexponential MRT was given as:

$$MRT= 1/K$$

Monoexponential half-life was given as:

$$t_{1/2} = \ln 2/K$$

Data are presented as mean ± SEM. To assess statistical differences, the data were analyzed using one way ANOVA with post-hoc Dunnett's test for multiple comparisons using GraphPad Prism software (GraphPad Prism version 5.0 for Windows; GraphPad Software, CA, USA).

2.12.5 Clodronate-mediated macrophage depletion

Clodronate-liposomes were used to selectively deplete macrophage populations exposed to circulating blood, including liver Kupffer cells and red pulp macrophages in the spleen. Liposome-encapsulated clodronate was a kind gift from Dr. Nico van Rooijen (Clodronate Liposome Foundation, Vrije University, Amsterdam). Phosphatidylcholine and cholesterol liposomes were

prepared as previously described and clodronate was encapsulated at a final concentration of approximately 7mg/mL solution. (Van Rooijen and Sanders 1994) Mice were intravenously injected with clodronate liposomes (100µL/10g body weight) 24 hours prior to the injection of VWF.

2.13 Analysis of "Low VWF" patient data

2.13.1 Subjects

The VWD database of the National Centre for Hereditary Coagulation Disorders (NCHCD) was retrospectively reviewed (Prof. James O'Donnell and Dr. Michelle Lavin, National Centre for Hereditary Coagulation Disorders, St. James's Hospital, Dublin 8) for patients with mild to moderate reductions in plasma VWF levels ('low VWF levels' 0.3 - 0.5U/mL). Patient VWF:Ag, VWF:RCo and FVIII:C values previously measured using standard laboratory assays were then recorded. In addition, ABO blood group was determined via reverse grouping in the Blood Transfusion laboratory, St. James's Hospital, Dublin 8.

2.13.2 DDAVP infusion

DDAVP was infused intravenously over 15 minutes after the patients had rested for a minimum of 20 minutes. The dose given was 0.3µg/kg. Venous blood samples were taken just minutes prior to the start of the DDAVP infusion and then at various points post-DDAVP infusion. Venous blood was collected from patients with by sterile venepuncture directly into

Vacuette® tubes containing 0.109M sodium citrate (blood:citrate 9:1). Platelet-poor plasma was prepared by centrifuging whole blood at 2000g for 15 minutes at room temperature. Plasma supernatant was removed, aliquoted and stored at -80^{0}C.

2.13.3 Calculation of post-DDAVP VWF:Ag and FVIII:C $t_{1/2}$

The VWF and FVIII response to DDAVP were plotted as concentration against time and data were fitted to a monoexponential curve according to the equation:

$$Y=(Y0-Plateau)*e(-K*X)+ Plateau$$

Where Y refers to the amount of VWF antigen in plasma, X=time, K=rate constant and Plateau=0. Peak VWF:Ag and FVIII:C levels were obtained at 1 hour post-DDAVP so the 1 hour timepoint was taken as the beginning of the elimination phase. T_0 values were subtracted from the post-DDAVP values to graph post-DDAVP response.

The VWF and FVIII half-lives were calculated from the first-order rate constant (k) for the elimination phase of the DDAVP response curve according to the equation:

$$t_{1/2} = \ln 2/k$$

2.13.4 VWF propeptide ELISA

VWF propeptide levels (VWFpp) were measured in citrated patient plasma using a commercial ELISA according to the manufacturer's instructions. Maxisorp plates (Nunc, Denmark) were coated with CLB-Pro35 monoclonal mouse anti-propeptide antibody (Sanquin Reagents, The Netherlands) diluted 1:100 in carbonate buffer (50mM Na_2CO_3, pH 9.6) overnight at 4°C. Wells were washed three times with PBS-T and blocked with

3% bovine serum albumin in PBS-T for 1 hour at room temperature. After washing, wells were incubated with 100µl of test samples diluted 1:40-1:80 in PBS-T. Reference plasma (Technoclone, Austria) diluted 1:10-1:320 (final concentration 1-0.03125µg/mL) was used as a standard. After 2 hours incubation at 37°C, wells were washed three times in PBS-T and 100µl HRP conjugated CLB-Pro-14.3 antibody (Sanquin reagents; Mast Group LTD) diluted 1:100 in PBST was added and incubated for 2 hours at 37°C. TMB substrate was added to the wells following 3 additional PBS-T washes. The reaction was stopped using 1M H_2SO_4 and VWFpp concentration determined as described for VWF ELISA in section 2.8.1. VWF propeptide was then expressed as a ratio relative to VWF:Ag levels (units:units).

2.14 Data and statistical analysis

Experiments were performed in triplicate and data are presented as mean ± SEM. For galectin-3 binding studies, THP-1 binding studies and mouse clearance studies, data were analyzed using one way ANOVA with post-hoc Dunnett's test for multiple comparisons using GraphPad Prism software (GraphPad Prism version 5.0 for Windows; GraphPad Software, CA, USA).

3.0 Characterizing the biochemical basis through which VWF interacts with galectin-3 in normal plasma.

The galectins are a family of soluble lectins characterised by their affinity for β-galactosides. Thus far, 15 mammalian galectins have been identified, all containing a conserved carbohydrate recognition domain (CRD) of approximately 130 amino acids. Members of the galectin family have been classified into three subtypes based on structural homology: the prototype galectins; tandem repeat galectins; and chimera galectins. Prototype galectins have a single CRD through which they can dimerise (e.g. galectin-1). Tandem-repeat galectins contain two homologous but significantly distinct CRDs on a single polypeptide chain (e.g. galectin-8). Galectin-3, the sole chimera type galectin, contains a single CRD connected to an unusual collagen-like, non-lectin N-terminal domain. The CRDs of each member of the galectin family have been found to share an affinity for the minimum saccharide ligand Galβ1-4GlcNAc, [N-acetyllactosamine, (LacNAc)]. Interestingly however, it has been demonstrated that individual galectins recognise various modifications of the basic unit and the sugar binding-specificities of several galectins have been characterised in detail (Hirabayashi et al. 2002; Stowell et al. 2008) For example, it was demonstrated via frontal affinity chromatography that galectin-1 displays dramatically enhanced affinity for complex N-glycans, but poorly enhanced affinity for repeating LacNAc units. Conversely, galectin-3 demonstrates enhanced affinity for repeating LacNAc units but not for branched oligosaccharides (Hirabayashi et al. 2002). Differential recognition of

sialylated glycans and blood group antigens was also demonstrated (Hirabayashi et al. 2002; Stowell et al. 2008). Interestingly, of all the galectins, galectin-3 was found to display the highest affinity for blood group A or blood group B containing glycans.

The biological importance of galectins has been investigated in depth. Consequently, galectins have been proposed to contribute to a vast array of diverse biological processes including cell adhesion, inflammation and immunity (Hughes 2001; Almkvist and Karlsson 2004; Rabinovich and Toscano 2009). For example, several studies identified galectin-1 as a key regulator of T-cell activation and function (Perillo et al. 1995; Chung et al. 2000). More recently however, a role has emerged for specific members of the galectin family in mediating thrombosis and haemostasis (Romaniuk et al. 2010; Romaniuk et al. 2012; Saint-Lu et al. 2012; Zappelli et al. 2012). For example, both galectin-1 and galectin-8 were identified as novel mediators of platelet activation. Galectin-1 triggered platelet activation in a carbohydrate-dependent manner, acting via the α_{IIb} subunit of $\alpha_{IIb}\beta_3$ (Romaniuk et al. 2012). Consequently, galectin-1 deficient mice demonstrated prolonged bleeding times as well as impaired platelet $\alpha_{IIb}\beta_3$ signalling demonstrating a critical role for galectin-1 in normal primary haemostasis (Romaniuk et al. 2012). Galectin-8 also triggered platelet activation in a carbohydrate-dependent manner. GPIbα represented the major functional galectin-8 counter-receptor and was essential for galectin-8 dependent signal transduction (Romaniuk et al. 2010).

A recent report identified both galectins-1 and -3 as novel binding partners for VWF (Saint-Lu et al. 2012). Proximity ligation analysis revealed colocalization of both galectin-1 and galectin-3 with VWF within endothelial

cells. Furthermore, galectin-1 and galectin-3 were coprecipitated with VWF, demonstrating that VWF and galectins -1 and -3 remain in complex once secreted into normal plasma. Importantly, this interaction was physiologically relevant as galectin-binding directly modulated VWF function. More specifically, galectin-1 and galectin-3 acted as negative regulators of platelet string formation at the endothelial cell surface *in vitro*. Platelet perfusion over HUVEC monolayers revealed increased platelet string formation in the presence of galectin inhibitor lactose. Furthermore, galectin inhibition was associated with altered thrombus formation following ferric chloride-induced injury in dual galectin-1/galectin-3 knockout mice, suggesting galectins -1 and -3 may modulate VWF-mediated thrombus formation *in vivo.*

Though it is clear that VWF glycan structures are critical modulators of VWF function, the molecular mechanisms through which the glycans exert their effects remain poorly understood. To investigate how VWF glycan structures modulate its biology we sought to characterize the biochemical basis through which VWF interacts with galectins in normal plasma. In particular, we sought to characterize the recently documented novel interaction between VWF and galectin-3, since galectin-3 binds VWF with greater affinity *in vitro* (Saint-Lu et al. 2012). To this end, pd-VWF was purified from the VWF/FVIII concentrate Haemate P® via gel filtration. VWF glycosylation was then modified using specific exoglycosidases and quantified using lectin ELISAs. The effects of altered glycan expression on galectin-3 binding were then assessed using modified immunosorbant assays.

3.1 VWF binds galectin-3 in a dose-dependent and glycan-dependent manner.

Immobilized galectin-3 was incubated with varying concentrations of pd-VWF to investigate potential binding interactions. Bound VWF was subsequently detected using HRP-conjugated polyclonal rabbit anti-human VWF. Purified plasma-derived VWF (pd-VWF) bound galectin-3 in a dose-dependent and saturable manner (Kd_{app} 0.57 ± 0.12nM). This is in keeping with the recent report of Saint-Lu *et al* who demonstrated that both galectins -1 and -3 bound pd-VWF *in vitro* (Saint-Lu et al. 2012).

To investigate the glycan determinants responsible for mediating the VWF/galectin-3 interaction a number of VWF glycan variants were generated via specific glycosidase digestion. Modification of the VWF glycosylation profile via treatment with PNGase F to completely remove N-linked carbohydrate structures (PNG-VWF) markedly attenuated binding to galectin-3 (56.8 ± 2.2%, $p<0.001$). Removal of both the N- and O-linked glycan structures via combined PNGase F and O-glycosidase digestions further attenuated binding (21 ± 1.0%, $p<0.0001$). Consequently, these data establish a role for the both the N- and O-linked VWF glycans in modulating its interaction with galectin-3. This is not surprising given that both the N-linked and O-linked VWF glycans express preferential saccharide ligands for galectin-3 binding. For example, over 80% of the VWF N-linked glycans are complex type glycans that abundantly express the minimal saccharide ligand for galectin recognition (LacNAc; Galβ1-4GlcNAc) (Canis et al. 2012). In addition, galectin-3 is known to show significant affinity for the sialylated T-antigen

which comprises 70% of the VWF O-linked glycan structures (Samor et al. 1989; Bian et al. 2011).

Interestingly, the apparent affinity of the VWF/galectin-3 interaction was in the nanomolar range. This seems surprising given that lectin/glycan interactions tend to constitute relatively weak interactions. For example, the average affinity of galectin-3 for a range of serum glycoproteins was previously estimated to correspond to Kd ~1–5 μM (Saenko et al. 1999). Importantly however, high-affinity VWF/galectin binding was also observed by Saint-Lu and colleagues (Kd_{app} 20-80nmol/L) (Saint-Lu et al. 2012). High-affinity binding may be explained by the multimeric/multivalent nature of both VWF and galectin-3. VWF exists in plasma as a series of multimers ranging in size from 2-40 subunits, with each individual subunit containing 12 N-linked and 10 O-linked glycan sites. Importantly, galectin-3 binds multi-glycosylated proteins such a VWF with positive cooperativity. Upon ligand binding, galectin-3 forms higher order multimers (up to pentamers) via its unique collagen-like N-terminal domain to produce high-avidity cross-linked lectin-glycan lattices. Moreover, recent studies have demonstrated that ligand binding also promotes galectin-3 oligomerization via its CRD (Lepur et al. 2012). Therefore, the multimeric nature of both VWF and galectin-3 facilitates interaction between multiple galectin-3 CRDs and multiple VWF glycans to promote high-affinity binding.

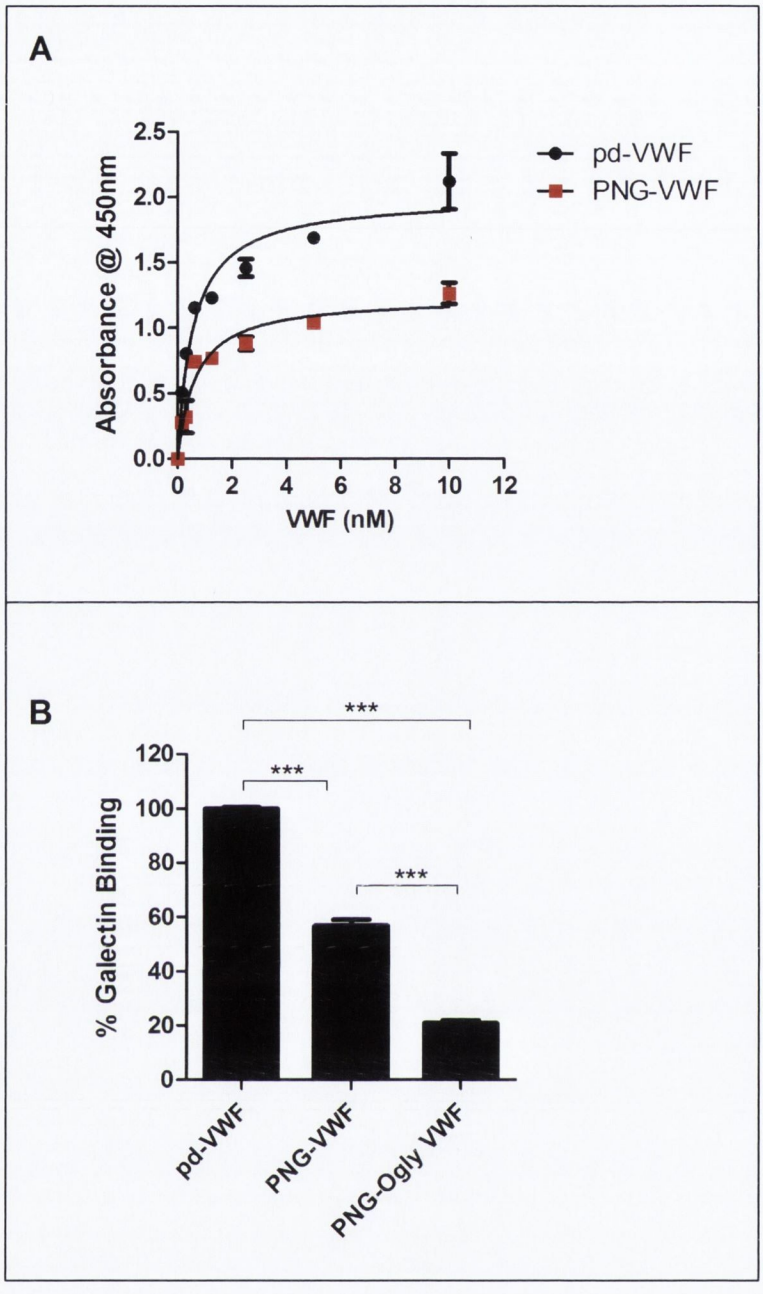


Figure 3.0 Pd-VWF binds galectin-3.
Pd-VWF binds galectin-3 in a dose-dependent (A) and glycan-dependent manner (B). SEM is not visible at certain points due to its small size.

3.2 Terminal glycan determinants critically regulate the VWF/galectin-3 interaction.

To investigate a role for specific terminal VWF glycan determinants in mediating the VWF/galectin-3 interaction, terminal sialic acid was removed via α2-3,6,8,9 neuraminidase digestion. The efficacy of α2-3,6,8,9 neuraminidase digestion was previously quantified by HPLC analysis (McGrath et al. 2010). Approximately 70% of terminal sialic acid was removed from both the N- and O-linked glycans of pd-VWF using this enzyme. Removal of terminal sialic acid via α2-3,6,8,9 neuraminidase (Neu-VWF) digestion with subsequent exposure of subterminal galactose significantly enhanced binding to galectin-3 (135.5 ± 5.7%, $p<0.05$). Approximately 80% of total sialic acid is present on the N-linked glycan chains, where it is predominantly α2-6 linked to penultimate galactose residues. This is of particular importance in the context of galectin binding, as the 6-OH group of the galactose ring constitutes a critical element for galectin recognition, forming hydrogen bonds with the galectin CRD (Hirabayashi et al. 2002). Substitution of this critical hydroxyl group with for example α2-6 linked sialic acid is detrimental to galectin binding as the bulky sialic acid moiety impedes access to the galectin CRD. This is consistent with the observation that enzymatic removal of terminal sialic acid enhanced galectin binding.

O-linked sialic acid constitutes approximately 20% of total sialic acid and may be α2-3 or α2-6 linked (McGrath et al. 2010). To investigate a specific role for O-linked sialic acid, pd-VWF was digested with α2-3 neuraminidase. Specific removal of O-linked sialic acid had no significant effect on galectin-3

binding (98.0 ± 6.4%, $p > 0.05$). Consequently, although terminal sialic acid expression on VWF N-linked glycans critically regulates VWF/galectin-3 binding, O-linked sialic acid is not important. This is in keeping with the observation that the 3-OH group of the galactose ring is not critical for galectin binding and may readily be substituted.

Based on the well defined affinity of members of the galectin family for the sugar galactose, pd-VWF was exposed to sequential neuraminidase and galactosidase digestions to remove both terminal sialic acid and sub-terminal galactose residues (NeuGal-VWF). In contrast to the enhanced binding of Neu-VWF, binding of NeuGal-VWF to galectin-3 was significantly reduced compared to pd-VWF (52.3 ± 5.7%, $p<0.005$) confirming that galactose constitutes an important ligand for galectin binding.

ABO(H) blood group antigens are terminally expressed on both the N-linked and O-linked glycans of pd-VWF (Canis et al. 2010). Moreover, ABO(H) determinants influence VWF plasma half-life (AB ≥ B ≥ A > O) and susceptibility to ADAMTS13 proteolysis (O ≥ B > A ≥ AB) (Bowen 2003; O'Donnell et al. 2005; Gallinaro et al. 2008). To investigate the impact of ABO(H) antigen expression on the VWF/galectin-3 interaction, VWF was purified from blood group specific (Groups AB and O) plasma via cryoprecipitation and galectin-3 binding was assessed. VWF from normal group AB individuals bound galectin-3 significantly better than group O VWF (482.5 ± 19.2% versus 116.2 ± 7.2%; $p<0.0001$). Conversely, no significant difference in binding was observed for Group O versus Bombay VWF (116.2 ± 7.2% versus 100.0 ± 0.6%, $p > 0.05$). Group O is characterised by expression of terminal fucose which is lacking in individuals who have the rare Bombay

phenotype. Therefore, terminal expression of group A (GalNAc) and group B (Gal) sugar moieties significantly enhances galectin-3 binding. In contrast, terminal α1-2 fucose expression has no such effect.

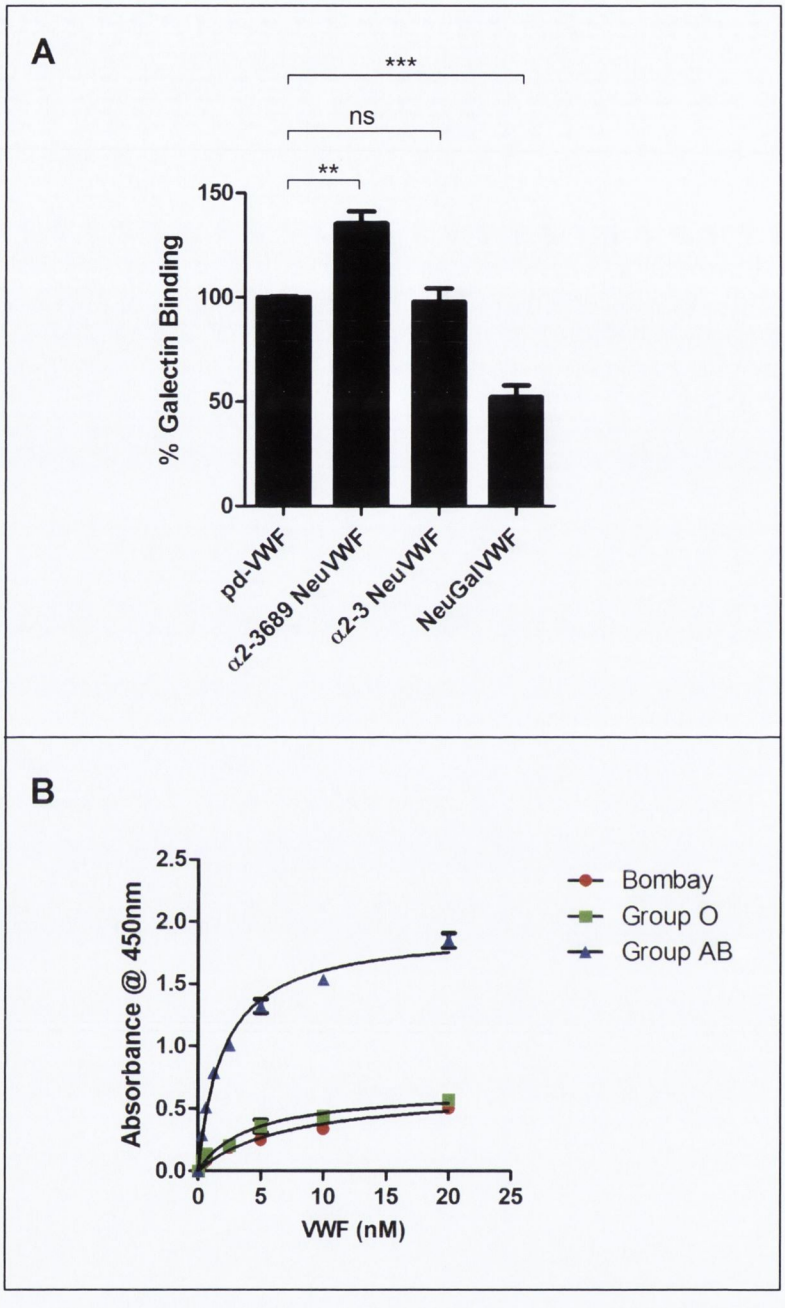


Figure 3.1 Terminal glycan determinants critically regulate the VWF/galectin-3 interaction.

A) Terminal expression of N-linked sialic acid negatively regulates galectin-3 binding. B) Galectin-3 displays enhanced affinity for group AB VWF. SEM is not visible at certain points due to its small size.

3.3 Cellular origin and differential glycosylation modulates VWF/galectin-3 binding.

The glycosylation profile of VWF synthesized within megakaryocytes differs significantly to that produced within endothelial cells (Williams et al. 1994; Kagami et al. 2000). In particular, total sialic acid and galactose expression are reduced twofold on platelet-vwf (plt-VWF) (Williams et al. 1994). In addition, plt-VWF fails to express terminal ABO(H) blood group antigens (Brown et al. 2002). Thus plt-VWF exists as a distinct natural glycoform. To characterize the effects of differential glycosylation on galectin-3 binding, plt-VWF was isolated and purified by platelet snap freeze-thawing followed by immuno-affinity chromatography. In keeping with the reduction in galactose and ABO(H) blood group antigen expression, plt-VWF bound significantly less well to galectin-3 than its plasma-derived counterpart (67.5 ± 7.1% $p<0.05$).

It is well established that recombinant proteins express glycan determinants that differ markedly to those expressed on native human proteins. For example, recombinant wild-type VWF (WT-VWF) fails to express the ABO(H) blood group determinants that are found on both the N-linked and O-linked glycans of plasma-derived (pd-VWF). To investigate the impact of this, binding of galectin-3 to WT-VWF was assessed. Unsurprisingly, WT-VWF bound significantly less well to galectin-3 than to pd-VWF (43.9 ± 6.8%, $p<0.0001$). Taken together, the above data demonstrate that cellular origin and differential glycosylation are key regulators of the VWF/galectin-3 interaction.

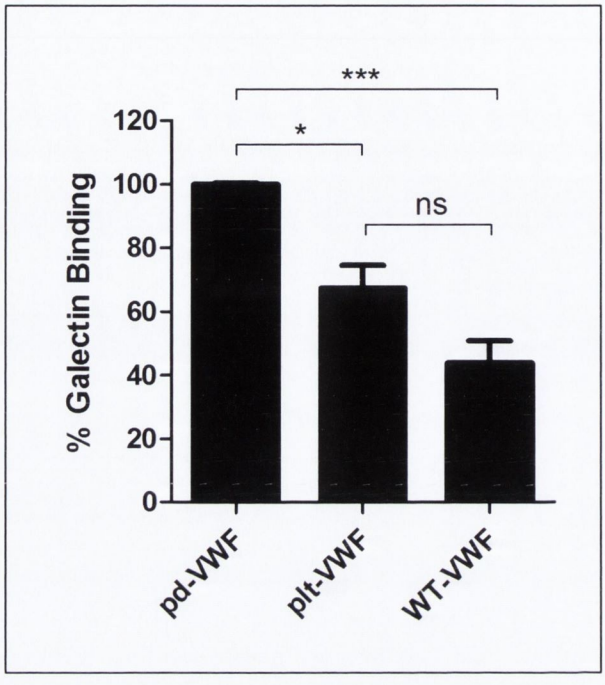


Figure 3.2 Differential glycosylation critically modulates galectin-3 binding.

3.4 VWF glycosylation and multimer composition critically regulate galectin-3 binding.

Both terminal glycosylation and multimer composition regulate VWF functional activity. HMWM (high molecular weight multimers) were previously shown to bind collagen and platelets with significantly higher affinity than LMWM (low molecular weight multimers) (Sporn et al. 1987; Arya et al. 2002). To investigate the relative contributions of multimer composition and terminal blood group antigen expression to galectin-3 binding, HMWM and LMWM were isolated from group AB and group O plasmas via cryoprecipitation. Binding studies were then carried out utilising size-fractionated, blood group specific VWF.

Galectin-3 preferentially bound HMWM compared to LMWM of the same blood group. Galectin-3 bound group AB HMWM with significantly enhanced affinity compared to group AB LMWM (103.1 ± 3.2% versus 56.5 ± 4.4% respectively, $p < 0.0001$). Similarly, galectin-3 displayed enhanced affinity for group O HMWM compared to group O LMWM (38.2 ± 2.2% versus 18.0 ± 2.6% respectively, $p < 0.0001$). Interestingly however, group AB LMWM bound galectin-3 with significantly enhanced affinity compared to group O HMWM (56.5 ± 4.4% vs 38.2 ± 2.2%, $p<0.05$) indicating that terminal ABO(H) expression is a more potent regulator of VWF/galectin-3 binding than multimer composition. This is not surprising given the dramatically enhanced affinity of galectin-3 for group AB versus group O sugars. Cumulatively, these novel data demonstrate that both glycosylation and multimer composition are crucial regulators of VWF/galectin-3 interaction in normal plasma.

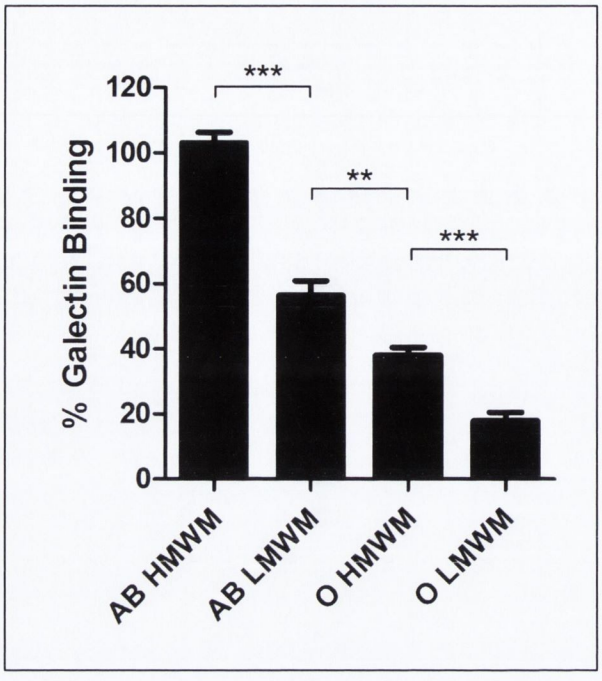


Figure 3.3 VWF multimer composition regulates the VWF/galectin-3 interaction.

3.5 Conformation of the VWF A domains critically regulates VWF/galectin-3 binding.

The haemostatic activities of the VWF are modulated by a number of shear-stress dependent interactions. Platelet recruitment at sites of vascular injury requires shear-dependent exposure of the GPIbα binding site in the VWF A1 domain (Savage et al. 1996). Similarly, proteolytic inactivation of VWF by ADAMTS13 requires shear-dependent exposure of the cryptic Tyr1605-Met1606 cleavage site within the VWF A2 domain (Yoshii et al. 2002). To investigate the impact of shear-stress on the VWF/galectin-3 interaction, artificial modulator ristocetin was employed to mimic shear stress *in vitro*. Ristocetin exposes the cryptic GPIbα and ADAMTS13 cleavage sites in the absence of shear stress by inducing conformational change in the VWF A domains. Incubation with increasing concentrations of ristocetin dramatically enhanced binding of pd-VWF to galectin-3 (205 ± 23%, $p<0.05$).

Further evidence for the critical contribution of the VWF A domains was gained from binding studies utilizing VWD type 2B mutation R1450E. Introduction of this "gain-of-function" mutation was previously shown to constitutively disrupt VWF A domain conformation (Yago et al. 2008). In keeping with this, galectin-3 bound to R1450E-VWF with significantly enhanced affinity compared to WT-VWF (117 ± 3%, $p<0.05$). Collectively, these data demonstrate the structural conformation of the VWF A domains is a key modulator of the VWF/galectin-3 interaction. Moreover, these findings also show that galectin-3 binds "active" VWF with significantly higher affinity.

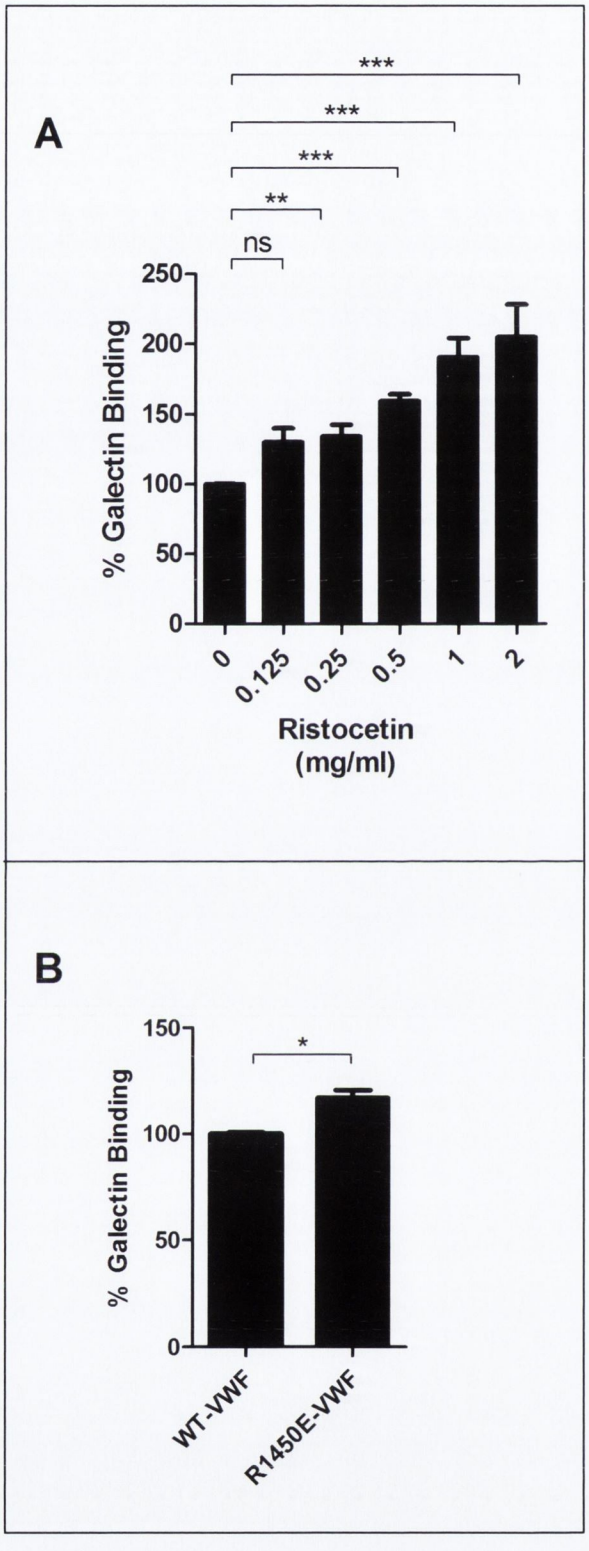


Figure 3.4 The structural conformation of the VWF A domains modulates galectin-3 binding.

3.6 The N-linked glycans of the VWF A2 domain are important regulators of the VWF/galectin-3 interaction.

Enhanced galectin-3 binding upon modulation of VWF A domain conformation most likely results from increased accessibility to the bulky N-linked glycan structures located in the VWF A2 domain adjacent to the ADAMTS13 cleavage site. To investigate the role of A2 domain N-linked glycans in mediating this interaction, A1A2A3-VWF was treated with PNGase F and residual galectin-3 binding was assessed. Complete removal of the N-linked glycan structures resulted in significantly decreased affinity for galectin-3 (66 ± 3.2%, $p<0.0001$).

To further define a role for the A2 domain N-linked glycans, targeted removal of individual glycans at Asn1515 and Asn1574 was carried out via site directed mutagenesis in a full length VWF construct. Targeted removal of Asn1574 significantly reduced binding to galectin-3 (44.2 ± 3.0%, $p<0.0001$). Moreover, specific removal of the glycan at Asn1515 abrogated binding to galectin-3 (23.9 ± 3.1%, $p<0.0001$). Recent characterization of the VWF N-linked glycans by mass spectrometry revealed that the glycan at Asn1515 expresses lactosaminic extensions, for which galectin-3 demonstrates enhanced affinity (Hirabayashi et al. 2002; Canis et al. 2012). Therefore it is not surprising that targeted removal of this particular glycan dramatically impacted galectin-3 binding. Taken together, these data suggest that the N-linked glycans of the VWF A2 domain are key mediators of the VWF/galectin-3 interaction.

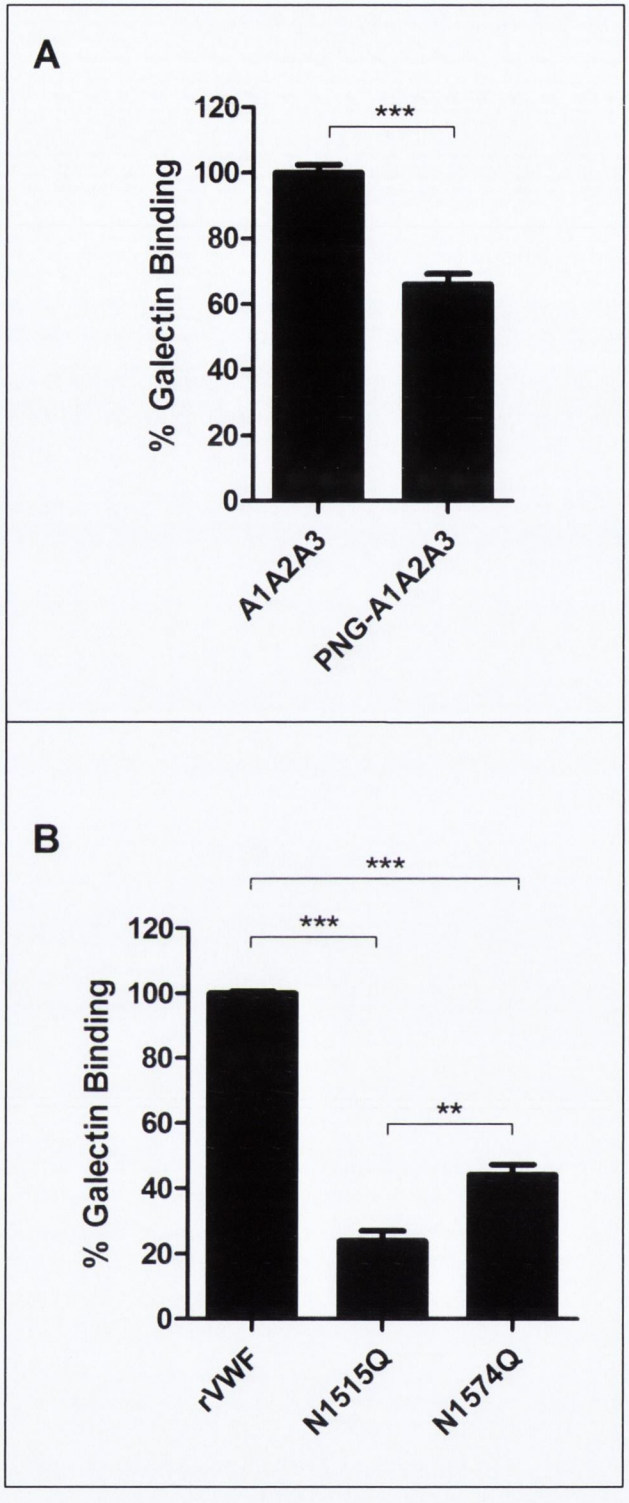


Figure 3.5 The N-linked glycans of the VWF A2 domain are key regulators of galectin-3 binding.

3.7 ADAMTS13 as a novel binding partner for galectin-3.

In normal plasma VWF multimer distribution is critically regulated by the action of metalloprotease ADAMTS13. ADAMTS13 specifically cleaves VWF at the Tyr1605-Met1606 peptide bond within the A2 domain to limit the amount of circulating haemostatically active UL-VWF. ADAMTS13 is abundantly glycosylated with 10 putative N-linked glycosylation sites described (Brinkman-Van der Linden et al. 2000). In addition, ADAMTS13 is O-fucosylated in at least 6 of its thrombospondin type 1 repeats (Bovenschen et al. 2003). Importantly, both the N-linked and O-linked glycans are of direct functional significance. Expression studies demonstrated that the N-linked and the O-linked glycans were essential for efficient ADAMTS13 secretion, while site directed mutagenesis identified specific N-linked glycan sites that appeared to regulate VWF cleaving activity (Brinkman-Van der Linden et al. 2000; Bovenschen et al. 2003). Given the putative role for galectin-3 in modulating platelet string formation at the endothelial cell surface it was of interest to investigate the potential interaction between galectin-3 and ADAMTS13.

Surface plasmon resonance analysis was performed to investigate potential interactions between recombinant ADAMTS13 and galectin-3. Galectin-3 was coupled to amine reactive biosensors and binding to partially purified recombinant ADAMTS13 was assessed. Interestingly, recombinant ADAMTS13 bound galectin-3 in a dose-dependent manner. Thus VWF cleaving protease ADAMTS13 constitutes a novel binding partner for galectin-3.

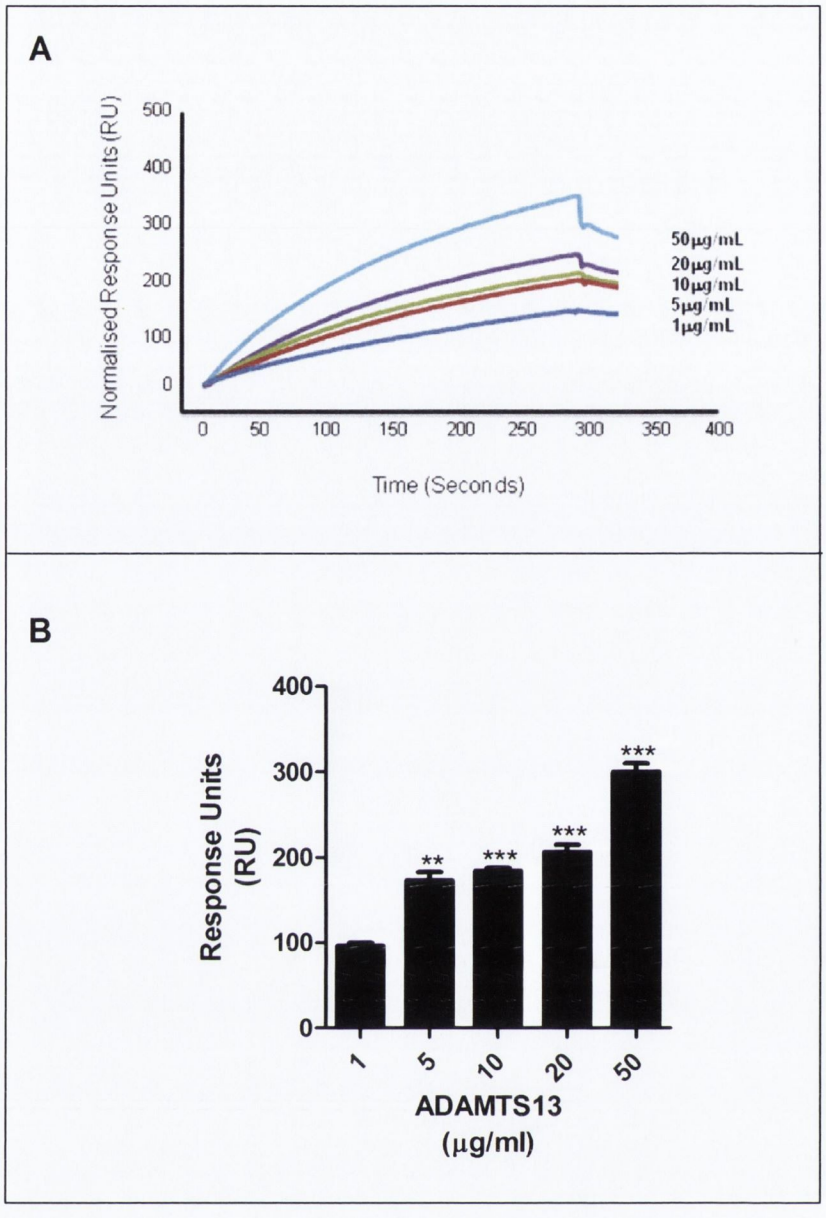


Figure 3.6 Recombinant ADAMTS13 as a novel binding partner for galectin-3.

3.8 FVIII as a novel binding partner for galectin-3.

VWF acts as a molecular chaperone for procoagulant FVIII prolonging its circulatory half-life and protecting it from premature proteolytic degradation. Once activated, FVIII plays a crucial role in the coagulation pathway acting as a cofactor for FXa generation by FIXa. Like VWF, FVIII is abundantly glycosylated with both N-linked and O-linked glycans. The N-linked glycans of human plasma-derived FVIII (pd-FVIII) are capped with negatively-charged sialic acid residues and terminal expression of ABO(H) blood group determinants has also been described (Sodetz et al. 1979; Hironaka et al. 1992). To date, several reports have highlighted the functional importance of the FVIII glycan structures. FVIII glycans were shown to influence folding and intracellular trafficking via LMAN-1 during biosynthesis and have also been shown to regulate FVIII uptake by dendritic cells and by the ASGPR (Pittman et al. 1994; Pipe et al. 1998; Spijkers et al. 2005; Pendu et al. 2006).

To assess whether recombinant FVIII also constitutes a novel binding partner for galectin-3, preliminary binding studies were carried out using surface plasmon resonance (Biacore X100, GE Healthcare, UK). In keeping with its abundant glycan content, recombinant FVIII bound galectin-3 in a dose-dependent manner.

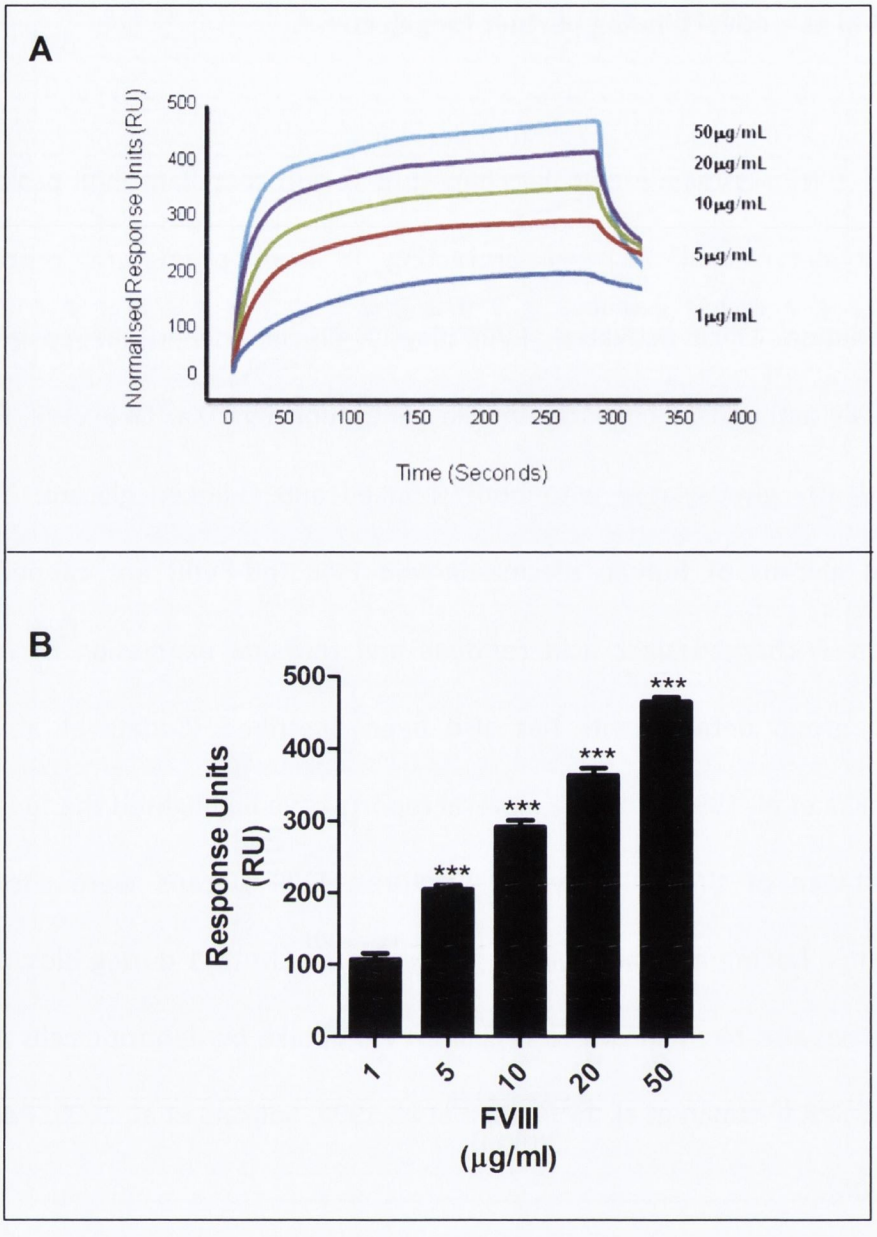


Figure 3.7 Recombinant FVIII as a novel binding partner for galectin-3.

3.9 Discussion

Glycan structures are attached to over half of all known proteins and play essential roles in determining circulatory half-life and biological activity (Lauc et al. 2010). In normal plasma, a vast array of lectins exists that recognize and bind discrete glycan structures thus mediating a host of physiological and pathological processes. Galectin-3 is a soluble and cell-surface expressed lectin of the galectin family that plays a role in multiple biological processes including inflammation, apoptosis, metastasis and angiogenesis (Akahani et al. 1997; Markowska et al. 2010; Reticker-Flynn et al. 2012). An additional novel role for galectin-3 was recently described in modulating primary haemostasis (Saint-Lu et al. 2012). Both galectins-1 and -3 were found to circulate in complex with sialoglycoprotein VWF and were also shown to negatively regulate VWF/platelet string formation at the endothelial cell surface. Though it has long since been established that the VWF glycan structures modulate its haemostatic activity, the molecular mechanisms involved remain undefined. Indeed a specific role for lectin/glycan interactions had not previously been described. The data described herein define for the first time the molecular basis underlying the physiological VWF/galectin-3 interaction. In particular, these data demonstrate that both N- and O-linked glycan determinants modulate VWF/galectin-3 binding through terminal sialic acid and ABO(H) blood group antigen expression with an additional role for specific N-linked glycans. Furthermore a critical role for the VWF A domains in modulating this interaction was also identified.

3.9.1 VWF binds galectin-3 with high-affinity.

In vitro binding studies demonstrated that VWF bound galectin-3 in a dose-dependent and glycan-dependent manner. Interestingly, high-affinity binding was observed as VWF bound galectin-3 with an apparent affinity in the nanomolar range (Kd_{app} 0.57 ± 0.12nM), a phenomenon also observed by Saint-Lu and colleagues (Saint-Lu et al. 2012). Lectin/glycan interactions typically occur with lower affinity than protein/protein interactions. However, these interactions are associated with significant avidity given that most lectins bind multiple glycan moieties. Indeed, galectin-3 binds multi-glycosylated proteins with positive cooperativity (Massa et al. 1993). Galectin-3 affinity increases with the number of glycan epitopes per molecule as it recruits additional lectin molecules to form higher order multimers for lectin/glycan lattice formation. Since VWF is multivalent in terms of the number of N- and O-linked sites, the clustered glycan surface provided by multimeric VWF likely facilitates this process. Thus the multimeric nature of both VWF and galectin-3 contributes to the observed high affinity binding.

3.9.2 Expression of terminal glycan determinants modulates VWF/galectin-3 binding.

Glycan modification via exoglycosidase digestion identified a specific role for N-linked sialic acid in mediating the VWF/galectin-3 interaction. N-linked sialic acid constitutes ~80% of total VWF sialic acid expression and is

predominantly attached to penultimate galactose residues via an α2-6 linkage (McGrath et al. 2010). N-linked sialic acid expression is of direct physiological relevance given that expression of α2-6 linked sialic acid on pd-VWF specifically enhances susceptibility to ADAMTS13 mediated proteolysis (McGrath et al. 2010). Importantly, quantitative N-linked VWF sialic acid expression has been reported to vary widely both in the normal population and in patients with suspected bleeding disorders (Ellies et al. 2002). Thus the ability of VWF to interact with various lectins such as galectin-3 within the plasma may be altered in some disease states. Though the physiologic and pathologic significance of quantitative variation in VWF sialic acid expression remain unclear, increased Gal/GalNAc exposure may lead to enhanced lectin binding which may in turn contribute to rapid clearance of VWF from plasma. Indeed galectin-3 has previously been shown to regulate a number of endocytic clearance pathways including clearance of advanced glycation end products (AGE) and acetylated-low density lipoproteins (LDLs) (Pugliese et al. 2001; Zhu et al. 2001).

Binding studies utilizing blood group specific VWF identified terminal ABO(H) blood group antigens as key regulators of the VWF/galectin-3 interaction. Galectin-3 bound group AB VWF with significantly enhanced affinity compared with group O or Bombay VWF. The significant effect of blood group on VWF functionality is surprising, given that ABO(H) determinants differ with respect to a single terminal sugar and are expressed only on a minority of the N- and O-linked glycan chains. However, since galectin-3 preferentially binds group AB versus group O expressing glycans,

the increased affinity of galectin-3 for group AB VWF may represent a mechanism by which glycan modification modulates VWF activity *in vivo*.

3.9.3 Cellular origin of VWF modulates its interaction with galectin-3.

The significance of terminal glycosylation is also evident when considering binding of galectin-3 to plt-VWF versus pd-VWF. Though both plt- and pd-VWF are extensively glycosylated, the glycosylation profile of plt-VWF differs significantly to that of its plasma-derived counterpart. Plt-VWF expresses ~50% less sialic acid and galactose than pd-VWF and also fails to express ABO(H) determinants (Williams et al. 1994; Matsui et al. 1999; Brown et al. 2002). Moreover, the overall reduction in sialic acid expression is almost entirely attributable to a specific reduction in N-linked sialic acid (McGrath et al. 2013). Consequently, plt-VWF exhibits functional properties distinct from those of pd-VWF. Plt-VWF displays markedly lower affinity for GPIbα compared with pd-VWF, but displays enhanced affinity for GpIIb/IIIa (Williams et al. 1994). Plt-VWF also exhibits significant resistance to ADAMTS13 mediated proteolysis compared with pd-VWF (McGrath et al. 2013). Additionally, in keeping with the reduced sialic acid and galactose content as well as the lack of ABO(H) expression, galectin-3 bound plt-VWF significantly less well than pd-VWF. Hence, the differential lectin binding activity of plt-VWF compared with pd-VWF may directly modulate functional activity of these naturally occurring glycoforms.

Galectin-3 also bound HEK293-T expressed WT-VWF significantly less well than pd-VWF. Therefore, the glycan structures expressed on the recombinant form differ significantly to those expressed on pd-VWF. In light of the major role played by glycan determinants in modulating functional activity, this may have significant implications for future administration of recombinant VWF concentrates to patients with VWD. Though no recombinant VWF concentrates are currently available for patient use, a phase-1 clinical trial recently demonstrated safety and efficacy of Chinese hamster ovary (CHO) expressed recombinant VWF in patients with type 3, severe type 1 and type 2A VWD, and phase-3 trials are on-going (Mannucci et al. 2013).

3.9.4 Conformation of the VWF A domains modulates VWF/galectin-3 binding.

Shear stress induced unfolding of VWF facilitates exposure of a number of cryptic functional sites within the VWF A domains. Exposure of the GPIbα binding site in the VWF A1 domain permits platelet capture at sites of vascular injury while exposure of the ADAMTS13 cleavage site in the VWF A2 domain facilitates proteolytic regulation of multimer distribution. These data now demonstrate that conformational change within the VWF A domains also regulates galectin-3 binding. Enhanced affinity following conformational change most likely results from increased accessibility to the complex N-glycans located at Asn1515 and Asn1574 in the VWF A2 domain. This is consistent with the observation that removal of either of these glycan

structures via targeted mutagenesis abrogates galectin-3 binding. Recent characterization of these glycan structures revealed that the glycan located at Asn1515 expresses lactosamine extensions, for which galectin-3 shows markedly enhanced affinity (Hirabayashi et al. 2002). However, the functional significance of this has yet to be determined.

3.9.5 Identification of ADAMTS13 and FVIII as novel binding partners for galectin-3.

Finally, preliminary binding studies have indicated that VWF cleaving protease ADAMTS13 and procoagulant molecule FVIII also constitute binding partners for galectin-3. Whether these proteins circulate in complex with galectin-3 remains to be established and further functional studies are required to elucidate the significance of these interactions.

In conclusion, these novel data define the molecular determinants responsible for mediating the physiological interaction between VWF and galectin-3 and provide insight into the mechanisms through which glycan expression may modulate VWF function. In light of the major role played by glycan determinants in modulating VWF functional activity, glycan heterogeneity and the subsequent ability to interact with various lectins within the circulation may have significant implications for VWF functionality. Terminal glycan determinants such as sialic acid and ABO(H) blood group antigens may be of particular importance in this regard.

4.0 Investigating the molecular mechanisms underlying enhanced clearance in VWD Vicenza.

To date, several amino acid substitutions have been described that are associated with enhanced VWF clearance. Of these, the best characterised is the "Vicenza" mutation. VWD Vicenza was originally described in 1988 in two Italian kindreds with lifelong bleeding tendencies inherited as an autosomal dominant trait (Mannucci et al. 1988). Subsequent investigations demonstrated that VWD Vicenza is characterised by significantly reduced plasma (but normal platelet) VWF levels with a proportionate reduction in VWF activity, as well as the presence of ultra-large VWF (UL-VWF) multimers and decreased satellite band intensity. Despite the relatively mild phenotype associated with VWD Vicenza, VWF antigen levels may be as low as 0.1U/ml due to rapid clearance of plasma VWF.

The causative mutation (G3864A) underlying VWD Vicenza was identified in exon 27 of the *VWF* gene, which results in the arginine to histidine substitution at position 1205 in the VWF D3 domain (Schneppenheim et al. 2000). Initial haplotype studies suggested a common genetic origin for R1205H as the mutation occurred in apparently unrelated families on a shared founder haplotype. However, subsequent analysis demonstrated that R1205H can arise as a *de novo* mutation (Cumming et al. 2006). An additional mutation in exon 17 (G2470A; M740I) was also described in the original Vicenza cohort. However, it has not been described in affected kindreds from other areas (Castaman et al. 2000). Although the M740I mutation has not

been identified in unaffected family members or randomly selected normal controls, it does not appear to contribute to the Vicenza phenotype and may represent a rare normal variant (Castaman et al. 2000; Casonato et al. 2006).

Several hypotheses were initially advanced to explain the dramatically decreased plasma VWF levels and circulating UL-VWF multimers associated with VWD Vicenza (Schneppenheim et al. 2000; Gezsi et al. 2010). However, a number of studies have demonstrated that enhanced plasma clearance of VWF constitutes the principle pathological mechanism in these patients (Casonato et al. 2002). Following DDAVP administration, the plasma half-life of both VWF:Ag and FVIII were significantly shorter in patients with VWD Vicenza compared to healthy controls. VWF:Ag was cleared with a half-life of just 1.24 hours versus 5.51 hours for healthy controls. The important role of enhanced clearance in the pathogenesis of VWD Vicenza has also been confirmed by *in vivo* clearance studies involving recombinant VWF infusion, and by hydrodynamic *VWF* gene expression in the $VWF^{-/-}$ mouse. Infused recombinant human or recombinant mouse VWF-R1205H demonstrated significantly faster plasma clearance than recombinant wild-type VWF (Lenting et al. 2004; Pruss et al. 2011). Similarly, lower VWF plasma levels were observed for VWF-R1205H versus WT-VWF following hydrodynamic expression and VWF:pp/VWF:Ag ratios were indicative of enhanced clearance (Pruss et al. 2011). Interestingly, *in vitro* expression studies have suggested that impaired synthesis may also contribute to the reduced VWF plasma levels in VWD Vicenza. Recombinant expression of both mouse and human VWF-R1205H in several cell lines demonstrated decreased protein production compared to WT-VWF (Pruss et al. 2011).

In addition to the R1205H mutation, two additional amino acid mutations have been identified at position 1205 in patients with VWD Vicenza (R1205C and R1205S respectively). These R1205C and R1205S mutations were also associated with significant reductions in plasma VWF survival following DDAVP administration (elimination half-life of 3.3 hours and 1.9 hours respectively compared to 11.4 hours in controls) (Millar et al. 2008). Interestingly however, the R1205H mutation was associated with the shortest post-DDAVP survival and the greatest increase in VWF:pp/VWF:Ag ratio suggesting faster clearance of this particular variant.

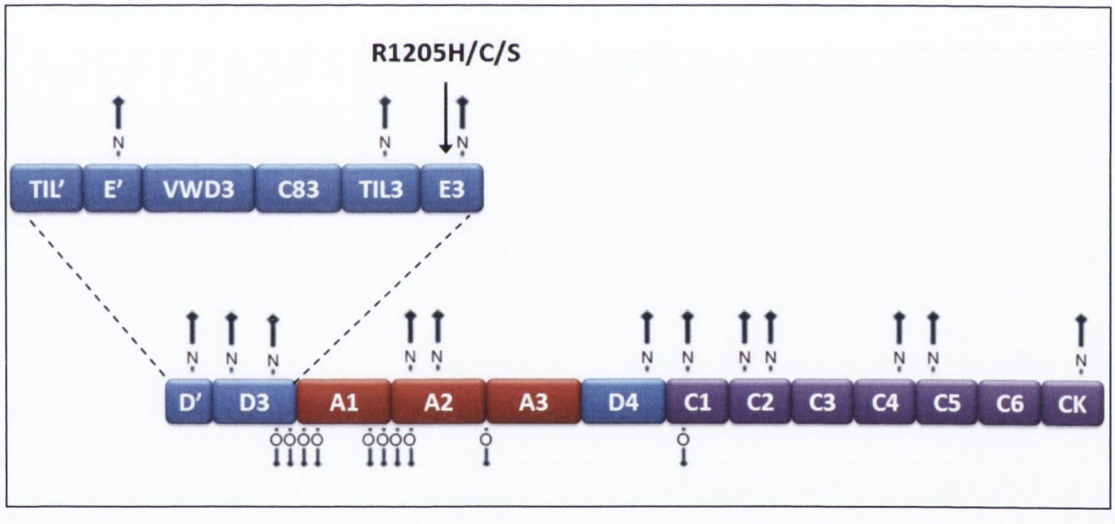


Figure 4.0. Domain diagram of VWF showing the location of R1205 variants. VWD Vicenza is caused by the R1205H mutation located in the E3 module of the VWF D3 domain.

Though it is well established that the R1205H mutation is responsible for enhanced VWF clearance, the molecular mechanisms underlying this process are not known. However, based on current knowledge of VWF

biology, two hypotheses may be advanced. First, enhanced VWF clearance in VWD Vicenza may be due to abrogation of the inhibitory effect of the D'D3 region upon the VWF A domains as accumulating evidence suggests that the VWF D'D3 domains can negatively modulate VWF A domain function. GPIbα binding studies and platelet aggregation studies have demonstrated apparent shielding of the VWF A1 domain by the D'D3 domains (Ulrichts et al. 2006). Deletion of the D'D3 domains resulted in significantly enhanced GPIbα binding, suggesting that the D'D3 domains directly impede access to the VWF A1 domain. Furthermore, *in vivo* clearance studies utilising a series of truncated VWF domain fragments have shown that shielding of the VWF A domains by D'D3 may also play an important inhibitory role in VWF clearance. Lenting *et al* demonstrated that truncated fragment A1A2A3-VWF was cleared at the same rate as full length WT-VWF However, N-terminal addition of the VWF D'D3 domains (D'A3-VWF) markedly prolonged plasma survival suggesting an inhibitory role for D'D3 in modulating VWF clearance (Lenting et al. 2004). Since the R1205 substitutions occur within the VWF D3 domain, it seems plausible that mutation of this residue disrupts the shielding effect thereby enhancing clearance through a clearance receptor site(s) within the A1A2A3 domains. This is also consistent with the observation that the majority of patient mutations associated with enhanced VWF clearance appear to cluster within the VWF D'D3 and A1 domains (Casari et al. 2013). The alternative hypothesis suggests that substitution of R1205 may serve to enhance VWF clearance by creating a novel receptor-binding site within the D'D3 region.

In order to investigate the above hypotheses, clearance of full-length VWF and truncated VWF domain fragments incorporating the VWD Vicenza mutations was studied in the $VWF^{-/-}$ mouse. Recombinant VWF was transiently expressed in HEK293-T cells and after 72 hours, conditioned medium was harvested and concentrated via anion exchange chromatography. Recombinant WT-VWF, D'A3-VWF or variants thereof, were then intravenously injected into $VWF^{-/-}$ mice. At appropriate time points blood from a sub-clavicle incision was sampled and VWF plasma survival was assessed by VWF:Ag ELISA (section 2.8.1).

4.1 Clearance of WT-VWF and VWF-R1205H in the $VWF^{-/-}$ mouse.

WT-VWF was cleared monophasically and clearance data was fitted to the equation:

$$Y = 4.8e(-0.09*X)$$

VWF-R1205H was also cleared in a monophasic fashion and clearance data was fitted to the equation:

$$Y = 5.8e(-0.13*X)$$

The clearance parameters percentage recovery (% recovery), plasma half-life ($t_{1/2}$) and mean residence time (MRT) for WT-VWF and VWF-R1205H are shown in table 4.0.

	% Recovery (3 mins)	$t_{1/2}$ (mins)	MRT (mins)
WT-VWF	**71.4** [± 6.0]	**7.9** [7.0-8.9]	**11.4** [10.1 - 12.8]
VWF-R1205H	**95.3** [± 4.6]	**5.4** [4.9-6.0]	**7.8** [7.1 - 8.7]

Table 4.0 Clearance parameters for WT-VWF and VWF-R1205H in the $VWF^{-/-}$ mouse.

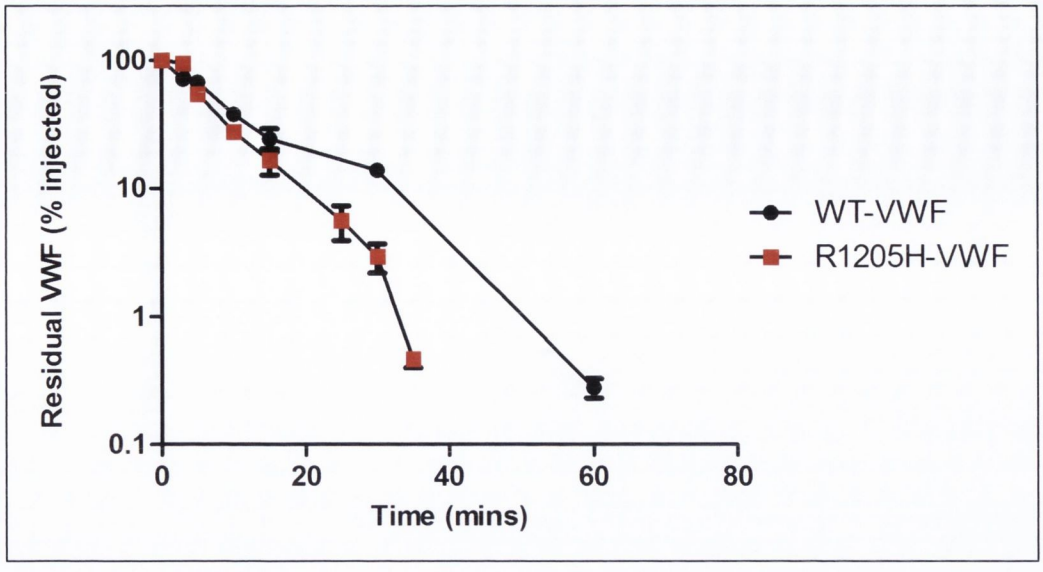


Figure 4.1 Enhanced clearance of VWF-R1205H in the VWF$^{-/-}$ mouse.
Data are graphed as mean ± SEM. In some cases SEM cannot be seen due to its small size.

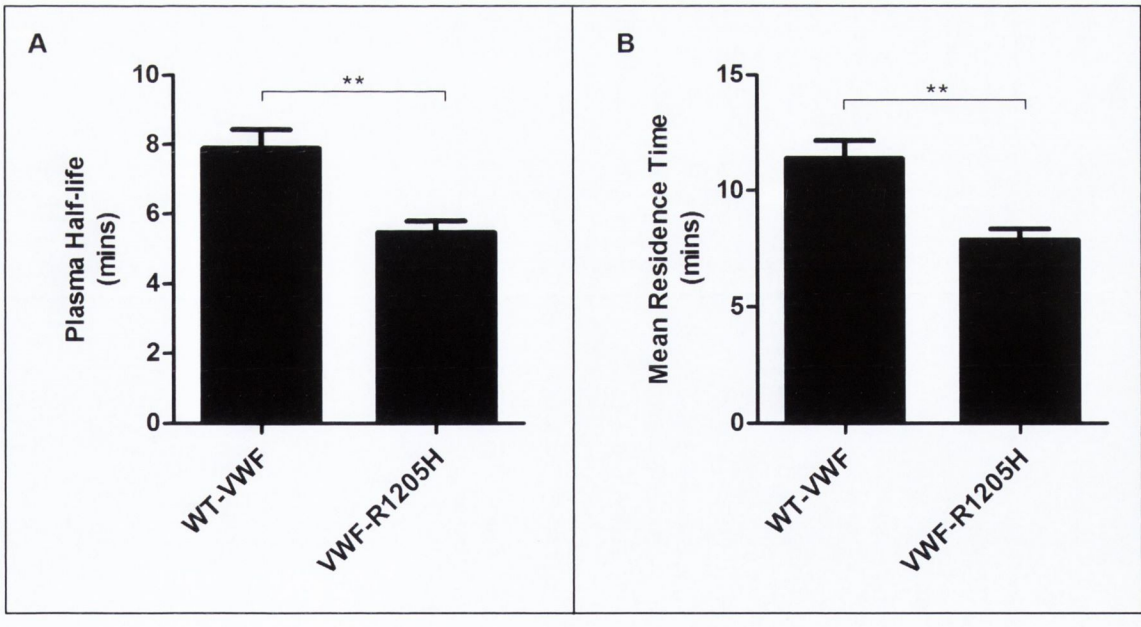


Figure 4.2 Clearance parameters for WT-VWF and VWF-R1205H in the VWF$^{-/-}$ mouse.
A) Plasma half-life values for WT-VWF vs VWF-R1205H. B) Mean residence time values for WT-VWF vs VWF-R1205H. Data are graphed as mean ± SEM.

These data confirm the critical role of the R1205H mutation in causing enhanced clearance. VWF-R1205H was cleared monophasically with a $t_{1/2}$ of 5.4 mins compared to 7.9 mins for WT-VWF ($p \leq 0.05$). Thus, human VWF-R1205H was cleared 1.5 fold faster than WT-VWF in $VWF^{-/-}$ mice. The role of R1205H in regulating VWF clearance has been investigated in two previous animal studies. Pruss *et al* examined clearance of recombinant mouse VWF-R1205H in $VWF^{-/-}$ mice. In keeping with the results outlined above, mouse VWF-R1205H was cleared in a monophasic manner, approximately 1.5 fold faster than mouse WT-VWF ($t_{1/2}$ 21.9 mins versus 35.1 mins respectively) (Pruss et al. 2011). In addition, Lenting *et al* studied clearance of recombinant human VWF-R1205H in $VWF^{-/-}$ mice. Interestingly, human VWF-R1205H was cleared 10 fold faster than human WT-VWF ($t_{1/2}$ 0.3 hours versus 3.0 hours respectively) and clearance of both WT-VWF and VWF-R1205H proceeded in a biphasic manner (Lenting et al. 2004).

It is noteworthy that the absolute rate of clearance varies widely in each of the studies outlined above. While sequence variation is a critical modulator of VWF clearance, other key regulators such as glycosylation also play critical roles in determining VWF plasma levels. Importantly, it is well established that glycosylation profiles vary significantly in a cell-line dependent manner. Recombinant VWF as described in this study was transiently produced in a HEK293-T expression system. Similarly, Pruss *et al* used HEK293-T cells to produce recombinant mouse VWF. In both of these studies HEK293-T expressed VWF-R1205H was cleared 1.5 fold faster than HEK293-T expressed WT-VWF (Pruss et al. 2011). In contrast, a BHK expression system was employed by Lenting and colleagues. Interestingly,

BHK expressed VWF-R1205H was cleared 10 fold faster than BHK expressed WT-VWF (Lenting et al. 2004). These discrepant results are likely due to glycosylation differences between HEK293-T derived verses BHK-derived recombinant VWF. Notwithstanding these issues, it is clear that the R1205H mutation is associated with significantly enhanced clearance in each of the studies described.

Significantly, the overall rate of VWF clearance in $VWF^{-/-}$ mice is markedly more rapid than the rate of VWF clearance in humans. The half-life of recombinant human WT-VWF varies from 7.9 mins to 3 hours in $VWF^{-/-}$ mice (Lenting et al. 2004). In humans, pd-VWF is cleared with a considerably longer half-life of approximately 12 hours (van Mourik et al. 1999). It has been proposed that this reflects the increased basal metabolic rate of mice (Lenting et al. 2004). However, glycan variation may also contribute to this process.

Though VWF-R1205H was cleared significantly faster than WT-VWF in the $VWF^{-/-}$ mouse the effect of the mutation on VWF survival was not as dramatic as observed in Vicenza patients. In $VWF^{-/-}$ mice, VWF-R1205H was cleared 1.5 fold faster than WT-VWF. In contrast, patients with VWD Vicenza clear endogenous VWF (VWF-R1205H) 10-fold faster than exogenous VWF (WT-VWF) (Gezsi et al. 2010). In addition to glycan variation, this discrepancy may reflect differences between the murine and human VWF clearance pathways. While several key clearance receptors have now been identified, the absolute contribution of particular receptors to VWF clearance in mice compared to humans is not yet known.

Following intravenous infusion of recombinant VWF, initial recovery has been determined in this study by measuring plasma VWF:Ag levels after 3

minutes. This methodology was also used in previous studies (Lenting et al. 2004; Schooten et al. 2005). Unexpectedly, significantly better recovery was observed for VWF-R1205H compared to WT-VWF (recovery 95.3% and 71.4% respectively, $p \leq 0.05$) despite the overall enhanced clearance of this variant. This phenomenon was also observed by Lenting and colleagues in a previous study of human R1205H-VWF clearance in $VWF^{-/-}$ mice (Lenting et al. 2004). At three minutes post-infusion 97 ± 8% of the total injected amount was recovered for VWF-R1205H compared to 79 ± 14% for WT-VWF. Intriguingly this phenomenon appears to be VWF-R1205H specific. Van Schooten *et al* previously characterized plasma clearance of a number of cysteine variants in the $VWF^{-/-}$ mouse (Schooten et al. 2005). VWF-C1130F, VWF-C1149R and VWF-C2671Y were rapidly cleared from the circulation with significantly shorter half-lives than WT-VWF. However, percentage recovery values were comparable to those of WT-VWF, suggesting that the enhanced recovery is specifically associated with the R1205H mutation. In contrast, enhanced VWF-R1205H recovery was not described by Pruss *et al* who studied clearance of mouse VWF-R1205H (Pruss et al. 2011). Therefore, enhanced recovery was only observed for human VWF-R1205H in $VWF^{-/-}$ mice. The significance of this, as well as the molecular mechanism responsible remains unclear. Importantly, the presence of the R1205H mutation does not affect ADAMTS13-mediated VWF proteolysis or its ability to bind platelet receptor GPIbα (Lenting et al. 2004; Pruss et al. 2011). Thus the enhanced recovery phenomenon is independent of these processes.

4.2 Clearance of VWF-R1205C and VWF-R1205S in the $VWF^{-/-}$ mouse.

In addition to the R1205H mutation, a number of other VWD causing mutations have been described at position 1205 (Millar et al. 2008). Both R1205C and R1205S have been associated with enhanced clearance in patients with Type 1 VWD but to date, no *in vivo* clearance studies involving these variants have been carried out. Moreover, it is not known if the mechanism by which these variants are rapidly cleared is the same as that by which VWF-R1205H is cleared. To investigate this, VWF-R1205C and VWF-R1205S were recombinantly expressed and plasma clearance was studied in the $VWF^{-/-}$ mouse.

VWF-R1205C was cleared in a monophasic manner and clearance data was fitted to the equation:

$$Y = 5.03e(-0.25*X)$$

VWF-R1205S was also cleared in a monophasic manner and clearance data was fitted to the equation:

$$Y = 4.98e(s-0.46*X)$$

The clearance parameters percentage recovery (% recovery), plasma half-life ($t_{1/2}$) and mean residence time (MRT) for VWF-R1205C and VWF-R1205S are shown in table 4.1.

	% Recovery (3 mins)	$t_{1/2}$ (mins)	MRT (mins)
WT-VWF	**71.4** [± 6.0]	**7.9** [7.0-8.9]	**11.4** [10.1 - 12.8]
VWF-R1205H	**95.33** [± 4.6]	**5.4** [4.9-6.0]	**7.8** [7.1 - 8.7]
VWF-R1205C	**48.67** [± 2.4]	**2.9** [2.7-3.0]	**4.1** [3.9 - 4.4]
VWF-R1205S	**24.09** [± 5.8]	**1.5** [1.3-1.7]	**2.2** [1.9 - 2.4]

Table 4.1 Clearance parameters for VWF-R1205C and VWF-R1205S in the $VWF^{-/-}$ mouse.

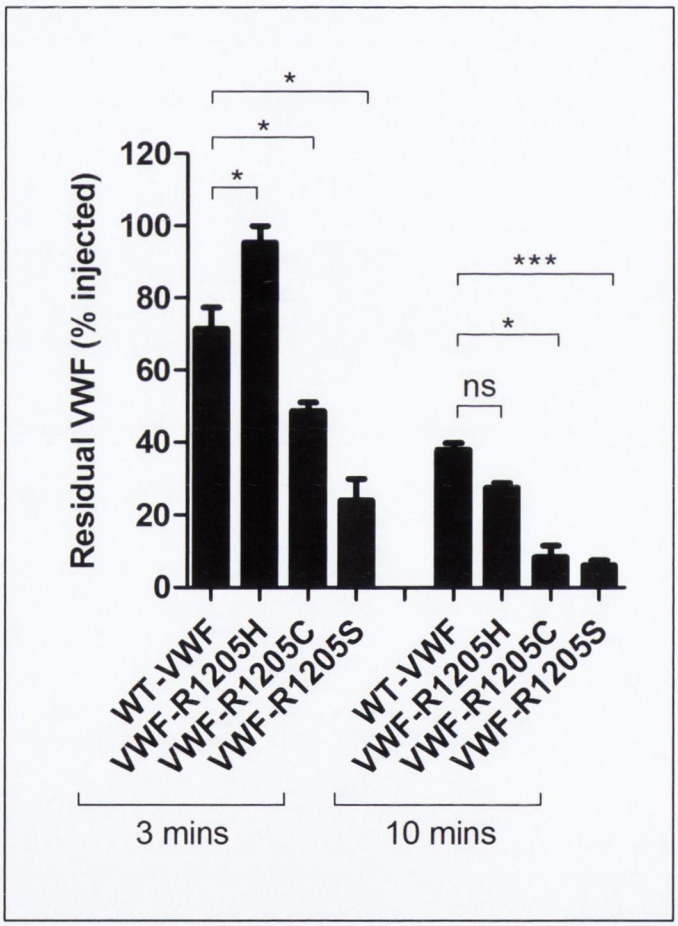


Figure 4.3 Enhanced clearance of VWF-R1205C and VWF-R1205S in the $VWF^{-/-}$ mouse.

Data are graphed as mean ± SEM.

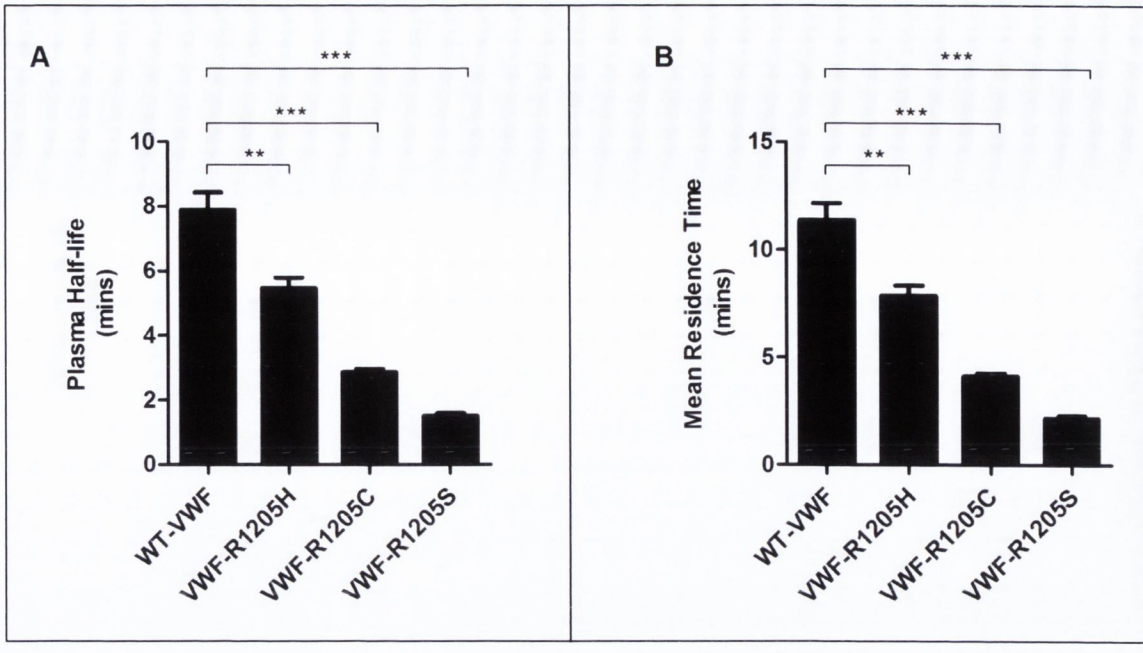


Figure 4.4 Clearance parameters for VWF-R1205C and VWF-R1205S in the VWF$^{-/-}$ mouse.

A) Plasma half-life values for VWF-R1205C and VWF-R1205S. B) Mean residence time values for VWF-R1205C and VWF-R1205S. Data are graphed as mean ± SEM.

The results of this *in vivo* study confirm that both the R1205C and R1205S mutations are also associated with enhanced VWF clearance in $VWF^{-/-}$ mice. VWF-R1205C was cleared with a $t_{1/2}$ of 2.9 mins ($p \leq 0.001$) and VWF-R1205S was cleared with a $t_{1/2}$ of 1.5 mins ($p \leq 0.001$) compared to a $t_{1/2}$ of 7.9 mins for WT-VWF. MRT values for both VWF-R1205C and VWF-R1205S were also significantly shorter than for WT-VWF. These data demonstrate that conservation of the arginine residue at position 1205 is of critical importance for normal VWF clearance. Interestingly, clearance of both VWF-R1205C and VWF-R1205S was significantly faster than clearance of VWF-R1205H in $VWF^{-/-}$ mice. Though a series of mutations have been associated with enhanced VWF clearance in type 1 VWD, the R1205H mutation has the shortest described plasma half-life in patients. However limited pharmacokinetic information regarding clearance of VWF-R1205C and VWF-R1205S is available, as each of these mutations has only been described in a single patient (Millar et al. 2008). Differential clearance of the R1205 variants may provide insight into the molecular mechanisms underlying enhanced clearance in VWD Vicenza based on differential amino acid side-chain properties.

4.3 Clearance of VWF-R1204H in the VWF-/- mouse.

The arginine residue at position 1205 in the VWF D3 domain is highly conserved suggesting a critical functional role for this residue. The neighbouring arginine residue at position 1204 is also conserved among mammals.

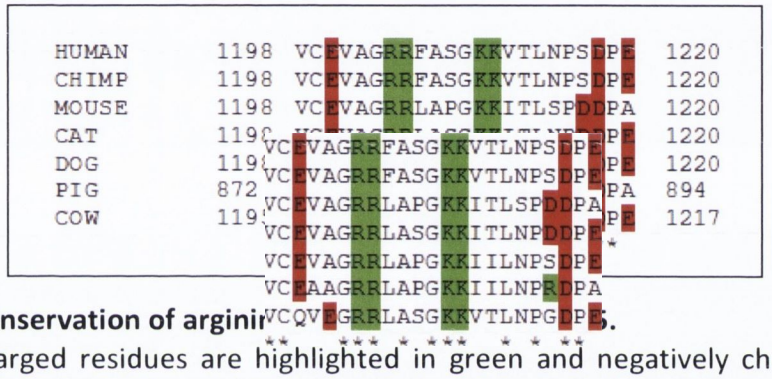


Figure 4.5 Conservation of arginir

Positively charged residues are highlighted in green and negatively charged residues are highlighted in red. An asterisk (*) indicates positions which have a single, fully conserved residue.

To investigate a potential role for this conserved residue in mediating VWF clearance, clearance of VWF-R1204H was studied in the $VWF^{-/-}$ mouse. VWF-R1204H was cleared in a monophasic fashion and clearance data was fitted to the equation:

$$Y = 4.79e(-0.07*X)$$

The clearance parameters percentage recovery (% recovery), plasma half-life ($t_{1/2}$) and mean residence time (MRT) for VWF-R1204H are shown in table 4.2.

	% Recovery (3 mins)	$t_{1/2}$ (mins)	MRT (mins)
WT-VWF	**71.4** [± 6.0]	**7.9** [7.0-8.9]	**11.4** [10.1 - 12.8]
VWF-R1204H	**72.1** [± 16.9]	**9.9** [7.6-14.1]	**15.2** [11.1 - 20.3]

Table 4.2 Clearance parameters for VWF-R1204H in the VWF-/- mouse.

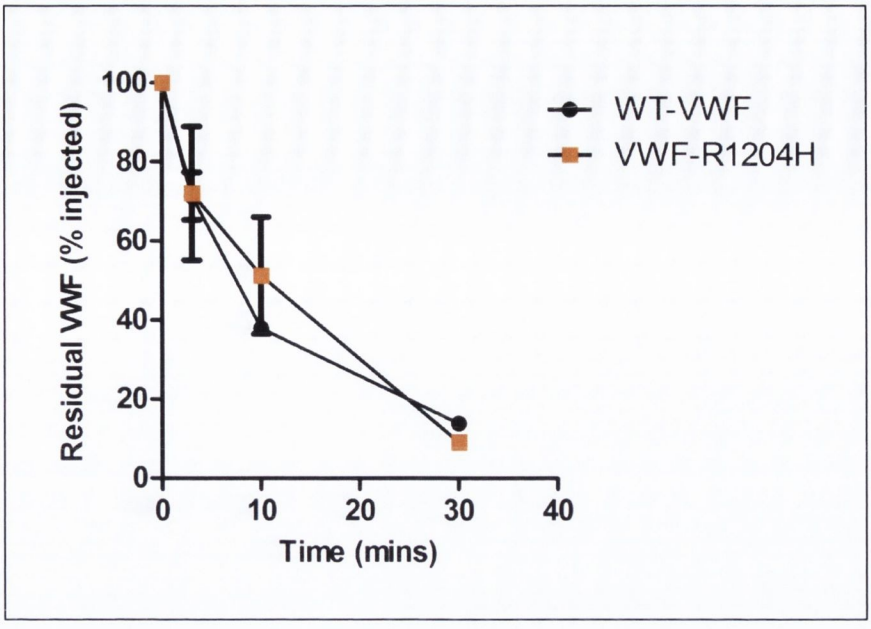


Figure 4.6 VWF-R1204H is cleared at the same rate as WT-VWF in the VWF$^{-/-}$ mouse.
Data are graphed as mean ± SEM. In some cases SEM cannot be seen due to its small size.

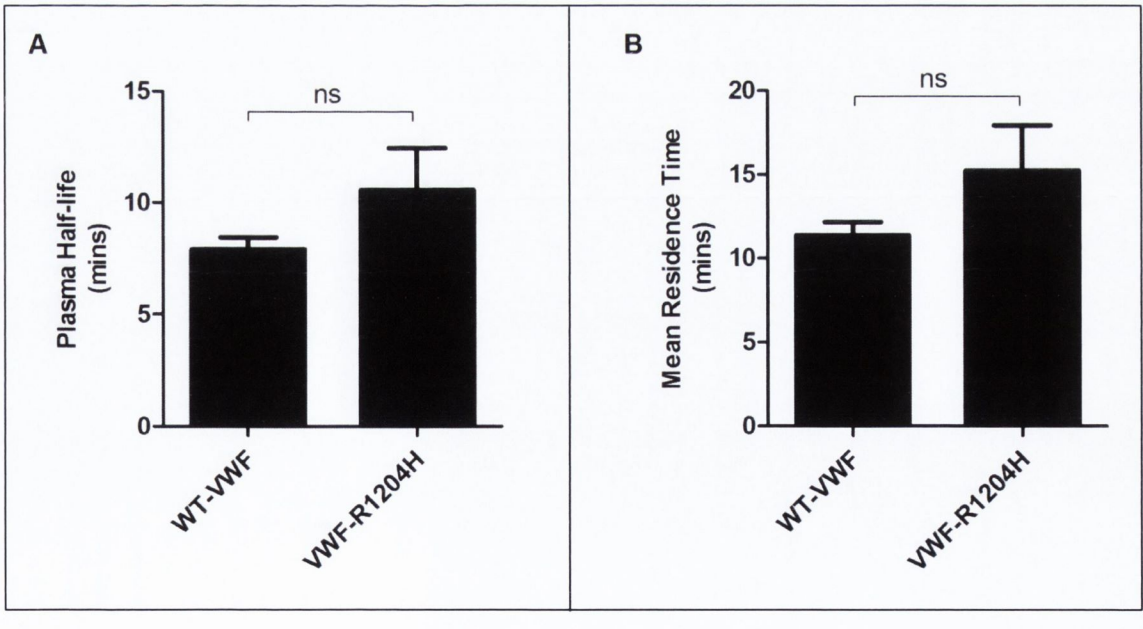


Figure 4.7 Clearance parameters for VWF-R1204H in the VWF$^{-/-}$ mouse.
A) Plasma half-life values for WT-VWF vs VWF-R1204H. B) Mean residence time values for WT-VWF vs VWF-R1204H. Data are graphed as mean ± SEM.

There was no significant difference between the rate of clearance of VWF-R1204H and WT-VWF in the $VWF^{-/-}$ mouse. VWF-R1204H was cleared with a $t_{1/2}$ of 9.9 mins compared to 7.9 mins for WT-VWF (p = 0.24). Consequently, in marked contrast to R1205, R1204 is not an important modulator of VWF clearance. Moreover, H1204 exists as a natural sequence variant in reptiles suggesting that an important role for arginine at position 1204 is unlikely. Cumulatively, these data demonstrate that normal VWF clearance is not simply determined by the presence of net positive charge in the vicinity of position 1205, but depends upon the presence of the positively charge arginine residue at position 1205. R1205 may mediate direct binding interactions with an as yet unidentified clearance receptor. Consequently, substitution of this residue may result in enhanced binding affinity for the receptor. Alternatively, R1205H may play a role in stabilizing local D3 domain conformation. Indeed, arginine residues are frequently involved in protein stabilization via salt bridge formation. Thus, replacement of R1205 with histidine, cysteine or serine residues may disrupt such stabilizing interactions, thereby inducing conformational changes which ultimately expose clearance receptor recognition site(s).

4.4 Clearance of D'A3 Vicenza variants in the VWF-/- mouse.

In light of the complex multi-domain structure of VWF, previous clearance studies have sought to assess the relevant contribution of individual domains to VWF plasma clearance (Lenting et al. 2004). Utilising a series of VWF domain truncations these studies identified an inhibitory role for the VWF D'D3 domains. A truncated fragment comprising the VWF A domains (A1A2A3-VWF) was cleared at a similar rate to WT-VWF. However, N-terminal addition of the D'D3 domains (D'A3-VWF) served to significantly prolong plasma half-life suggesting a protective or inhibitory role for these D domains.

To investigate the protective effective apparently conferred by the VWF D'D3 domains, the Vicenza mutations (R1205H, R1205C and R12505S) were introduced into truncated VWF fragment D'A3 comprising the D'-D3-A1-A2-A3 domains, and clearance was studied in the $VWF^{-/-}$ mouse.

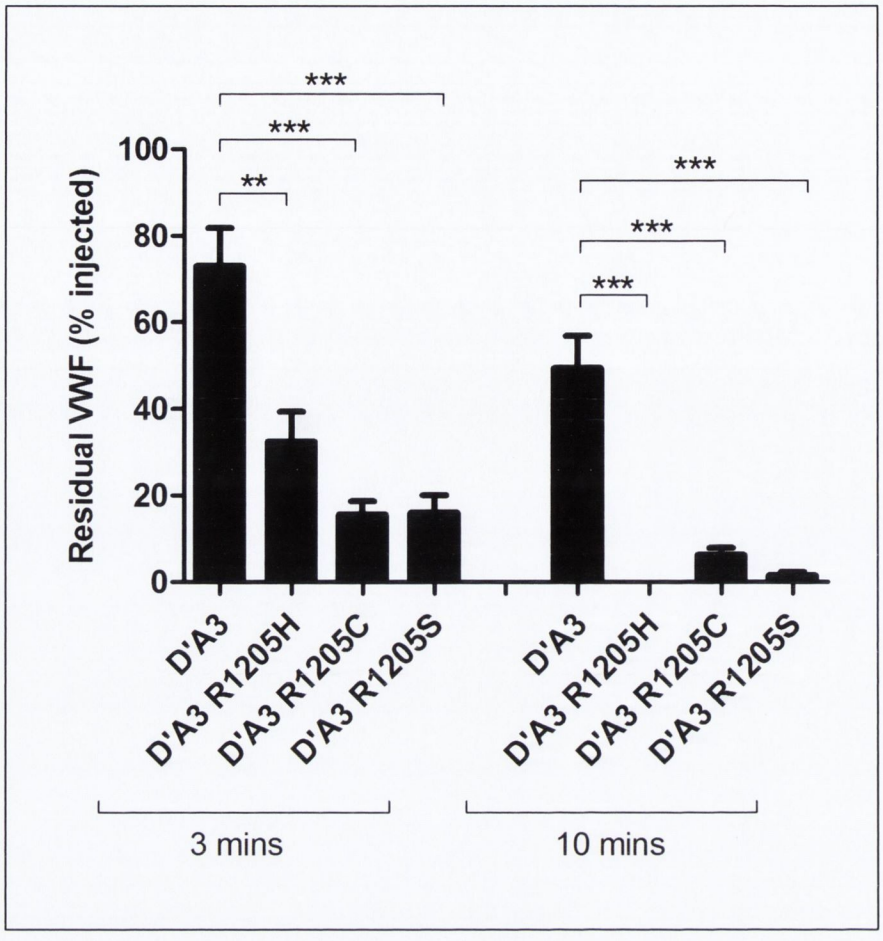


Figure 4.8 Enhanced clearance of D'A3-VWF Vicenza variants in the VWF$^{-/-}$ mouse.

Data are graphed as mean ± SEM.

In keeping with its effect in enhancing clearance of full length VWF, introduction of the R1205H mutation also served to significantly enhance clearance of the D'A3 truncation. At 10 mins post-injection D'A3-R1205H was not detectable in the plasma of $VWF^{-/-}$ mice while 49.5 ± 7.4% of the initial amount injected for D'A3. Both D'A3-R1205C and D'A3-R1205S were also rapidly cleared from the plasma of $VWF^{-/-}$ mice. At 10 mins post injection, plasma levels of D'A3 R1205C-VWF and D'A3 R1205S-VWF were only 6.3 ± 1.6% ($p \leq 0.001$) and 1.6 ± 0.8% ($p \leq 0.001$) respectively. In direct contrast to full length VWF-R1205H, no enhanced recovery was observed for D'A3 R1205H compared to D'A3. Cumulatively, these data demonstrate that the enhanced clearance associated with loss of R1205 is modulated through local effects within the D'A3 region of VWF. These data also demonstrate that D'A3 represents a VWF truncation in which the critical clearance receptor interactive sites are preserved.

4.5 Clearance of ΔA1 and ΔA1 R1205H in $VWF^{-/-}$ mice.

To date, elevated VWF:pp/VWF:Ag ratios have identified more than 20 mutations associated with enhanced plasma clearance of VWF (Haberichter et al. 2008; Casonato et al. 2010; Casari et al. 2013; Eikenboom et al. 2013). Remarkably, the majority of these mutations appear to cluster within the VWF D'D3 and A1 domains. These findings support the hypothesis that the D'D3 domains may shield the VWF A1 domain to modulate its function as a clearance receptor interactive site. Disruption of this shielding effect by mutations present in either the D'D3 of A1 domains may subsequently result in enhanced clearance. To investigate shielding of the VWF A1 domain by the D'D3 domains, clearance of A1 domain deleted VWF (VWF-ΔA1) and A1 domain deleted VWF incorporating the R1205H mutation (VWF ΔA1 R1205H) were studied in the $VWF^{-/-}$ mouse.

VWF-ΔA1 was cleared in a monophasic fashion and clearance data was fitted to the equation:

$$Y = 5.06e(-0.07*X)$$

VWF-ΔA1 R1205H was cleared in a monophasic fashion and clearance data was fitted to the equation:

$$Y = 4.88e(-0.19*X)$$

	% Recovery (3 mins)	$t_{1/2}$ (mins)	MRT (mins)
WT-VWF	**71.4** [± 6.0]	**7.9** [7.0-8.9]	**11.4** [10.1 - 12.8]
VWF-ΔA1	**81.5** [± 6.8]	**8.4** [7.4-9.7]	**12.3** [10.7 - 14.0]
VWF-ΔA1 R1205H	**53.6** [± 7.0]	**3.7** [3.2-4.2]	**5.4** [4.7 - 6.1]

Table 4.3 Clearance parameters for VWF-ΔA1 and VWF-ΔA1 R1205H in the $VWF^{-/-}$ mouse.

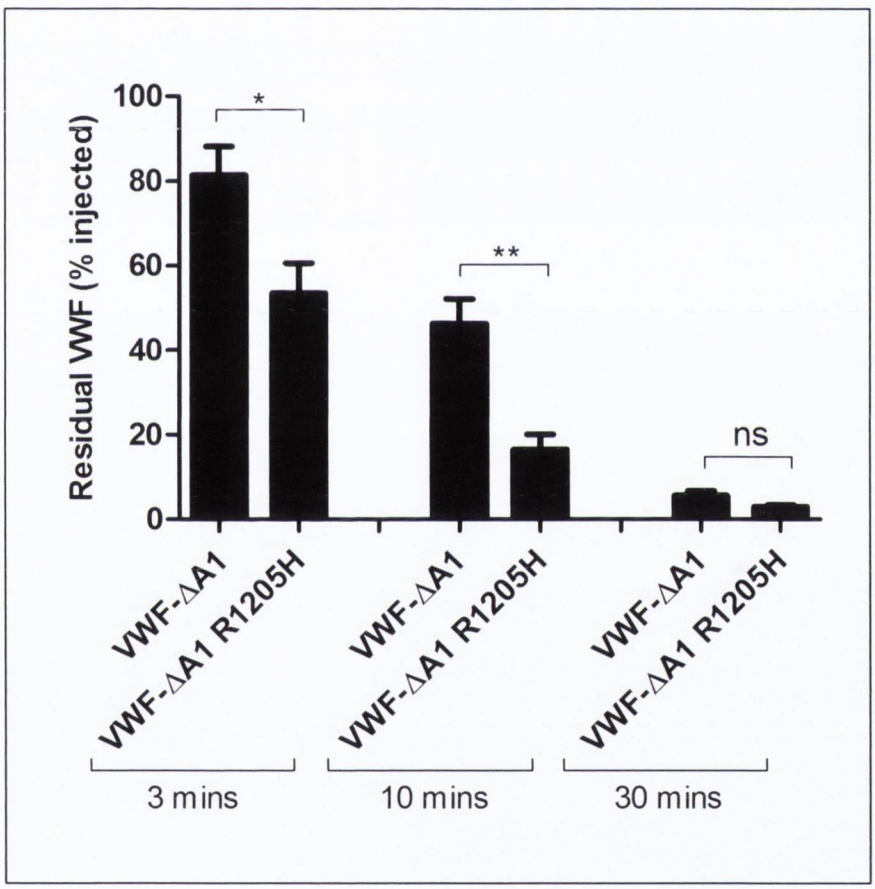


Figure 4.9 Clearance of VWF-ΔA1 and VWF-ΔA1 R1205H in the $VWF^{-/-}$ mouse. Data are graphed as mean ± SEM.

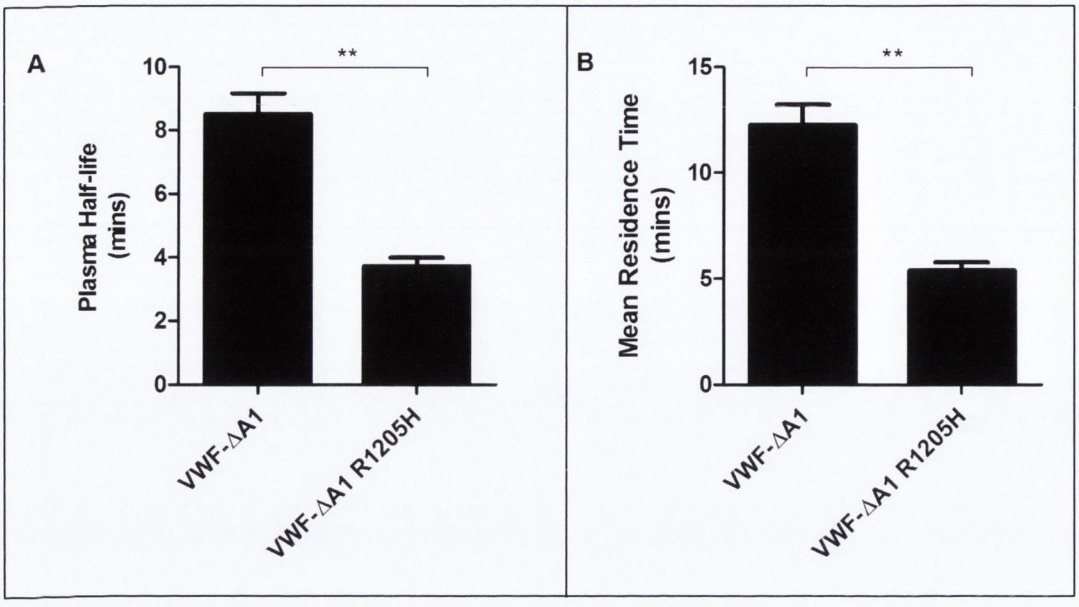


Figure 4.10 Clearance parameters for VWF-ΔA1 and VWF-ΔA1 R1205H in the VWF$^{-/-}$ mouse.

A) Plasma half-life values for VWF-ΔA1 and VWF-ΔA1 R1205H. B) Mean residence time values for VWF-ΔA1 and VWF-ΔA1 R1205H. Data are graphed as mean ± SEM.

VWF-ΔA1 was cleared with a $t_{1/2}$ of 8.4 mins compared to 7.9 mins for WT-VWF. Thus, VWF-ΔA1 was cleared at the same rate as WT-VWF in $VWF^{-/-}$ mice. Importantly, this novel finding demonstrates that despite clustering of patient mutations that cause enhanced clearance within this domain, the VWF A1 domain is not important in the context of clearance. In addition, these data contradict the suggestion that a clearance receptor recognition site is located in the VWF A1 domain. Logically, deletion of this domain should thus serve to prolong VWF half-life. Indeed, abolition of the A1 domain which contains the GPIbα binding site ablates VWF-mediated platelet adhesion (Lankhof et al. 1995). Similarly, A1 domain deletion with consequent deletion of the major heparin binding site abolishes heparin binding activity (Sixma et al. 1991).

Clearance of VWF-ΔA1 R1205H was 2-fold faster than clearance of VWF-ΔA1. VWF-ΔA1 R1205H was cleared with a $t_{1/2}$ of 3.7 mins compared with 8.4 mins for VWF-ΔA1 ($p \leq 0.01$). Therefore, the R1205H mutation critically regulates VWF clearance in the absence of the VWF A1 domain. These data demonstrate that enhanced VWF clearance associated with the R1205H mutation is not due to disruption of D'D3 shielding effects on the neighbouring A1 domain. Further evidence for this comes from patient data. Ristocetin-induced platelet aggregation is normal in Vicenza patients suggesting that R1205H has no effect on VWF A1 domain function (Casonato et al. 2002). *In vitro* surface plasmon resonance studies also demonstrated that the R1205H mutation had no effect on GPIbα binding to the VWF A1 domain (Lenting et al. 2004). In summation, these experimental observations demonstrate a critical role for the VWF D'D3 domains in regulating normal

VWF clearance. Conversely, the VWF A1 domain is not an important modulator of VWF clearance.

4.6 Exploring a role for the ASGPR in mediating VWF-R1205H clearance.

The ASGPR has been shown to participate in VWF clearance mediating removal of both sialylated and desialylated VWF from plasma (Grewal et al. 2008). To examine a role for ASGPR in mediating enhanced clearance of VWF-R1205H, clearance of VWF-R1205H was studied in ASGPR$^{-/-}$ mice and wild-type (WT) C57/Bl6 mice. VWF-R1205H plasma levels were analyzed at 3 minutes and 15 minutes post-injection. Baseline endogenous VWF levels were established using a pool of ASGPR$^{-/-}$ or WT C57/Bl6 plasma and were used to normalize VWF-R1205H plasma levels.

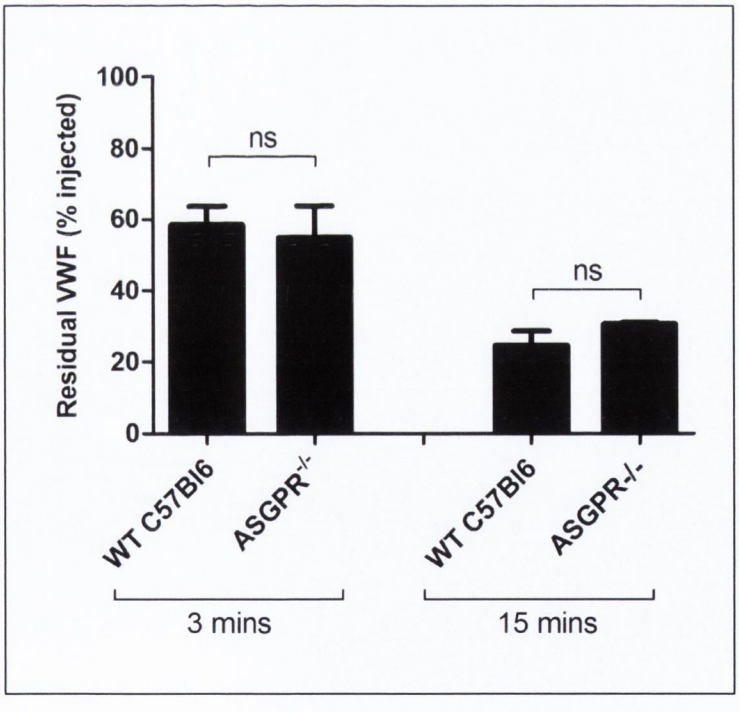


Figure 4.11 Clearance of VWF-R1205H in WT-C57Bl6 and ASGPR$^{-/-}$ mice.
Data are graphed as mean ± SEM.

Initial studies demonstrated that ASGPR$^{-/-}$ mice had higher endogenous VWF plasma levels compared with WT C57/Bl6 mice. Baseline VWF plasma levels were 1.5 fold higher in ASGPR$^{-/-}$mice than wild-type C57/Bl6 mice (1.8µg/ml versus 1.2µg/ml respectively). This is consistent with previous reports that ASGPR$^{-/-}$ mice have elevated VWF plasma levels due to an increased VWF half-life (Grewal et al. 2008).

Once corrected for endogenous VWF plasma levels, no significant difference in VWF-R1205H plasma levels was observed for ASGPR$^{-/-}$ mice compared with WT C57/Bl mice at 3 minutes (54.9 ± 8.8% versus 58.5 ± 5.2% respectively, $p > 0.05$) or 15 minutes post-infusion (30.7 ± 0.4% versus 24.6 ± 4.0% respectively, $p > 0.05$). Therefore, VWF-R1205H was cleared at the same rate in ASGPR$^{-/-}$ mice and WT C57/Bl6 mice. Consequently, these data exclude a role for ASGPR in mediating enhanced clearance in VWD Vicenza.

ASGPR has previously been shown to mediate normal homeostatic clearance of VWF in a glycan-dependent manner (Grewal et al. 2008). Additionally, a role for ASGPR in modulating clearance of sialylated proteins has also been described (Park et al. 2005). Despite this however, ASGPR does not mediate enhanced clearance of VWF-R1205H. Given the lack of evidence to suggest that the glycosylation status of VWF-R1205H differs to that of WT-VWF, this is not surprising. The arginine to histidine substitution does not result in the introduction of a novel consensus sequence for N-linked glycosylation or lead to the destruction of an existing one. However, whether the presence of the R1205H mutation could indirectly affect glycosylation status remains unclear. Attachment of glycan chains is dependent upon efficient transit through the endoplasmic reticulum and exposure to the

necessary glycosyltranfersases. Polypeptide folding and conformation are also important in this regard. Consequently, missense mutations such as the R1205H substitution, within the polypeptide sequence may potentially influence this intracellular process, ultimately affecting glycan attachment.

Interestingly, enhanced recovery (section 4.1) of VWF-R1205H was not observed in WT C57/Bl6 or ASGPR$^{-/-}$ mice. Though clearance of a number of VWF variants has now been studied (Lenting et al. 2004; Schooten et al. 2005; Pruss et al. 2011), enhanced recovery has only been documented for VWF-R1205H (Lenting et al. 2004). Furthermore, it would appear that this phenomenon is associated only with human VWF-R1205H as enhanced recovery was not observed in clearance studies utilizing mouse VWF-R1205H (Pruss et al. 2011). Comparative data from various inbred mouse strains now demonstrate that this phenomenon is also specific to VWF$^{-/-}$ mice as there is no evidence of enhanced VWF-R1205H recovery in either WT C57/Bl6 or in ASGPR$^{-/-}$ mice. VWF$^{-/-}$ mice differ to WT C57/Bl6 and ASGPR$^{-/-}$ mice in a number of respects. As well as lacking detectable plasma, platelet or endothelial VWF, VWF$^{-/-}$ mice also have significantly reduced plasma FVIII levels (Denis et al. 1998). Consequently, VWF$^{-/-}$ mice exhibit delayed platelet adhesion and thrombus formation (Denis and Wagner 2007). VWF$^{-/-}$ mice also lack Weibel Palade bodies (WPBs) which are present in ASGPR$^{-/-}$ and C57/Bl6 mice. In addition to VWF, WPBs store a number of other proteins such as P-selectin, angiopoietin-2, and osteoprotegerin. Importantly, in the absence of WPBs these proteins are mistargeted. Indeed in VWF$^{-/-}$ mice, the absence of WPBs is associated with P-selectin mistargeting which leads to impaired leukocyte recruitment during acute inflammation (Denis et al. 2001).

However, how these differences might relate to quantitative effects on VWF clearance is not clear.

4.7 Role for macrophages in mediating enhanced clearance of VWF-R1205H.

Hepatic and splenic macrophages were previously identified as key regulators of VWF clearance. *In vitro* binding studies demonstrated rapid uptake and degradation of VWF by THP-1 macrophages as well as primary monocyte-derived macrophages (van Schooten et al. 2008; Castro-Nunez et al. 2012). Moreover, macrophage inhibition and/or depletion was shown to significantly prolong VWF survival in several animal models (Sodetz et al. 1977; van Schooten et al. 2008; Casari et al. 2013). In addition, macrophage LRP1 was recently identified as a candidate VWF clearance receptor (Castro-Nunez et al. 2012; Rastegarlari et al. 2012). To examine whether macrophages contribute to the enhanced clearance phenotype in VWD Vicenza, clearance of VWF-R1205H was studied in $VWF^{-/-}$ mice following clodronate-mediated macrophage depletion.

$VWF^{-/-}$ mice were intravenously injected with clodronate liposomes or liposomal controls 24 hours prior to VWF injection. WT-VWF and VWF-R1205H plasma levels were analyzed 10 minutes post-injection.

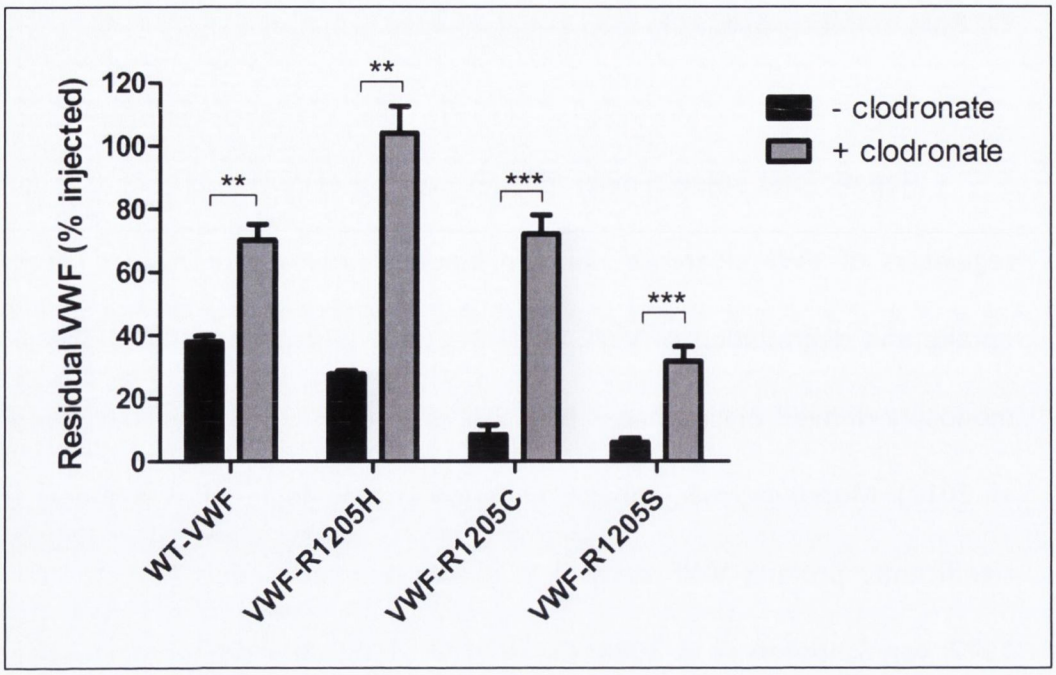


Figure 4.12 Effect of clodronate-mediated macrophage depletion on VWF survival in VWF$^{-/-}$ mice.
Data are graphed as mean ± SEM.

These data confirm a role for macrophages in mediating VWF clearance *in vivo*. For WT-VWF, 37.9 ± 1.9% of the initial amount injected was detectable in the plasma of $VWF^{-/-}$ mice 10 minutes post-infusion. Following treatment with clodronate liposomes, WT-VWF plasma levels were significantly increased to 70 ± 5.0% ($p \leq 0.01$). Thus macrophage depletion led to increased WT-VWF plasma levels confirming that macrophages contribute to the clearance of WT-VWF in $VWF^{-/-}$ mice. Macrophage depletion and/or inhibition were previously shown to prolong VWF plasma half-life in a number of animal models. In New Zealand white rabbits, inhibition of the reticulo-endothelial system using macro-aggregated albumin or zymosan significantly prolonged the plasma half-life of the VWF/FVIII complex (Sodetz et al. 1977). Similarly, gadolinium-chloride ($GdCl_3$) induced macrophage-depletion prolonged survival of recombinant VWF 2-fold in $VWF^{-/-}$ mice (van Schooten et al. 2008). More recently, rapid clearance of VWF/platelet complexes was also attenuated by clodronate-mediated macrophage-depletion in a mouse model of type 2B VWD (Casari et al. 2013).

VWF-R1205H was cleared significantly faster than WT-VWF such that at 10 minutes post-infusion, 27.67 ± 1.2% of the initial amount injected was detectable in the plasma of $VWF^{-/-}$ mice. Following clodronate-mediated macrophage depletion, 104.1 ± 8.5% of the initial amount injected was detected in the plasma ($p \leq 0.001$). Importantly, clodronate-liposome treatment also served to prolong survival of VWF-R1205C and VWF-R1205S. Following macrophage depletion, plasma levels of VWF-R1205C were significantly increased compared with untreated mice (60.4 ± 6.1% compared

with 8.4 ± 3.1%, $p \leq 0.01$). Similarly, plasma levels of VWF-R1205S were also increased (32.1 ± 7.2% compared with 6.02 ± 1.5%, $p \leq 0.05$).

The effect of macrophage depletion on VWF-R1205H clearance was more pronounced than that observed for WT-VWF, VWF-R1205C and VWF-R1205S. In the case of WT-VWF this is not surprising given that several cell types are known to contribute to VWF clearance. While it is clear that macrophages also contribute at least in part to the clearance of VWF-R1205C and VWF-R1205S in $VWF^{-/-}$ mice, the absolute contribution appears to be less significant compared with VWF-R1205H. Taken together, these data suggest that macrophages are the principal mediators of VWF-R1205H clearance. These data also suggest a role for additional cell types in modulating clearance of VWF-R1205C and VWF-R1205S. Alternatively, clearance of VWF-R1205C and VWF-R1205S may involve macrophage sub-populations that are not susceptible to clodronate treatment (Kinoshita et al. 2010). Cumulatively, these data demonstrate that the molecular mechanisms responsible for mediating enhanced clearance of VWF-R1205H may differ to those mediating enhanced clearance of VWF-R1205C and VWF-R1205S.

4.8 Discussion

Clinical data has identified enhanced VWF clearance as an important pathogenic mechanism in Type 1 VWD. In addition, recent studies also suggest that enhanced VWF clearance contributes to the etiology of Type 2B VWD. To date, more than 20 different VWF missense mutations have been associated with enhanced clearance. Of these, the most common is the VWD-Vicenza variant, which is characterized by the R1205H mutation in the VWF D3 domain. Reduced VWF survival in VWD Vicenza leads to marked reductions in plasma VWF levels and elevated steady-state VWF propeptide to VWF antigen ratios.

The work presented in this thesis highlights the essential role of R1205 in the VWF clearance process. Moreover, these data demonstrate that enhanced VWF clearance associated with substitution of R1205 is mediated through local effects within the D'D3 domains. Finally, macrophages were identified as major contributors to the enhanced clearance phenotype in VWD Vicenza.

4.8.1 R1205 is a critical regulator of VWF clearance.

R1205 was identified as a specific and critical regulator of VWF clearance. Substitution of this highly conserved arginine residue led to markedly reduced VWF survival in $VWF^{-/-}$ mice, while in contrast mutation of neighbouring residue R1204 had no such effect. Importantly, enhanced VWF clearance following mutation of R1205 appears to be due to loss of the arginine residue rather than the gain of histidine, serine or cysteine residues an observation that serves to highlight the specific importance of the positive charge at residue 1205. The importance of the positively charged arginine residue may be explained by the fact that arginine residues are often involved in salt bridge formation. Arginine residues form salt bridges with oppositely charged residues to stabilize local conformation. Thus substitution of arginine for uncharged cysteine or serine residues may impact VWF conformation ultimately causing enhanced VWF clearance. Importantly, histidine residues have partial positive character depending on the environment. At physiological pH, the imidazole side chain of histidine (pKa ~6.0) has 10% probability of becoming positively charged. Thus, the more conservative substitution for a histidine residue may partially preserve the positive charge at position 1205 and may not have as dramatic an effect on VWF conformation. This may explain the less compromised the plasma half-life of VWF-R1205H compared with that of VWF-R1205C and VWF-R1205S observed in this study.

4.8.2 Reduced survival associated with loss of R1205 is modulated through local effects in the D'D3 domains.

We previously hypothesized that substitution of R1205 may abrogate the inhibitory effects of D'D3 on clearance A1A2A3 mediated clearance or alternatively may result in creation of a novel receptor binding site(s) in the VWF D'D3 domains. Collective results from clearance experiments utilizing truncated D'A3 domain fragments incorporating the Vicenza mutations as well as A1 domain deleted VWF (ΔA1-VWF) variants now demonstrate that the enhanced clearance associated with substitution of R1205 is modulated through local effects within the D'D3 domains. Moreover, the VWF A1 domain does not appear to be important in the context of VWF clearance.

The D'D3 domains have been suggested to contain a clearance receptor-interactive site, which may explain why several mutations in this region are associated with enhanced VWF clearance. These include the R1205 variants, W1144G and W1120S, as well as cysteine mutations C1130F/G/R, C1149R, and C1190R. While cysteine mutations presumably play a role in altering VWF conformation leading to exposure of this receptor interactive site, the effects of other amino acid substitutions in the D'D3 region are less clear. While mutation of R1205, W1120 or W1144 may alter local D'D3 conformation in a manner similar to the cysteine mutations, it is also plausible that mutation of R1205, W1120 or W1144 may result in creation of an additional novel clearance receptor binding site or modify existing binding site(s) to enhance binding affinity. Importantly, the mechanisms underlying enhanced clearance of W1120S and W1144G may differ from those mediating

enhanced clearance of the R1205 variants. Indeed the unique sensitivity of the R1205 variants to macrophage clearance rather than other proposed clearance routes, illustrates the presence of multiple, distinct VWF clearance mechanisms that can be selectively accelerated by specific amino acid substitutions. Therefore, clearance studies utilizing additional variants W1120 and W1144 may provide further insight into pathological VWF clearance pathways.

5.0 Elucidating the mechanisms responsible for enhanced macrophage-mediated VWF clearance in VWD Vicenza.

Hepatic and splenic macrophages are well recognised as key modulators of VWF clearance. Van Schooten *et al* initially demonstrated that VWF bound to primary monocyte-derived macrophages and THP-1 macrophages *in vitro* (van Schooten et al. 2008). Subsequently, this binding was shown to result in rapid internalization and degradation of VWF (Castro-Nunez et al. 2012). Additional *in vivo* studies showed targeting of infused radiolabelled VWF to the liver and spleen of $VWF^{-/-}$ mice while immunohistochemical analysis revealed colocalization of VWF with cells of macrophage lineage (van Schooten et al. 2008). In particular, VWF colocalized with CD68+ Kupffer cells in the liver and with F4/80 macrophages in the red pulp of the spleen. Chemical inactivation of macrophages using gadolinium chloride ($GdCl_3$) was then shown to prolong survival of infused VWF approximately 2-fold in $VWF^{-/-}$ mice, thereby confirming a role for macrophages in mediating VWF clearance (van Schooten et al. 2008).

Importantly, recent studies have demonstrated a role for shear-stress in modulating VWF uptake by macrophages. VWF was bound and internalized by monocyte-derived macrophages only under conditions of shear stress, or in the presence of ristocetin (Castro-Nunez et al. 2012). Shear-dependent activation has previously been shown to critically regulate the haemostatic activity of VWF. Platelet recruitment at sites of vascular injury requires shear-dependent exposure of the GPIbα binding site in the VWF A1 domain (Savage

et al. 1996). Similarly, proteolytic inactivation of VWF by ADAMTS13 requires shear-dependent exposure of the cryptic Tyr1605-Met1606 cleavage site within the VWF A2 domain (Dong 2005).

In spite of these cumulative data suggesting that macrophages are important modulators of VWF clearance, the specific mechanisms involved have yet to be elucidated. However, macrophage LRP1 was recently identified as the first putative macrophage VWF clearance receptor (Castro-Nunez et al. 2012; Rastegarlari et al. 2012). LRP1 is a ubiquitously expressed scavenger receptor that participates in lipid metabolism and cellular signalling. LRP1 was also previously identified as a clearance receptor for free FVIII (Lenting et al. 1999; Saenko et al. 1999; Bovenschen et al. 2003). An additional role for LRP1 as a clearance receptor for VWF was proposed when genetic inactivation of macrophage LRP1 (macLRP$^-$) in mice resulted in significantly increased VWF plasma levels compared to controls (1.6U/ml versus 1.0U/ml respectively). Moreover, VWF plasma half-life was also prolonged 2-fold (Rastegarlari et al. 2012). This is in keeping with earlier reports that VWF plasma levels were increased in mice with conditional liver-specific LRP1 deficiency (Bovenschen et al. 2003) suggesting that LRP1 participates in VWF clearance. Additionally, perfusion studies demonstrated shear-stress dependent adhesion of VWF to LRP1 (Rastegarlari et al. 2012). Consequently, LRP1 may represent the shear-stress dependent VWF clearance receptor expressed on macrophages.

A putative role for macrophage β_2 integrins in mediating VWF clearance has also been suggested. VWF was previously shown to bind THP-1 macrophages in a β2 integrin-dependent manner (Koivunen et al. 2001). Consequently, macrophage expressed LRP1 and β_2 integrins can directly

interact with VWF. Interestingly, hydrodynamic expression of β_2 integrin inhibitor NIF led to increased VWF plasma levels in macLRP$^+$ but not macLRP1$^-$ mice suggesting that β_2 integrins may modulate VWF clearance, but do so in an LRP1 dependent manner (Rastegarlari et al. 2012). Indeed, LRP1 was previously identified as a regulator of β_2 integrin function, forming heterologous receptor complexes with β_2 integrins at the cell surface to facilitate integrin clustering (Spijkers et al. 2005). Whether macrophage expressed LRP1 and β_2 integrins independently mediate VWF clearance, or do so as part of a functional receptor complex, has not yet been determined.

Though it is clear that macrophages play an important role in VWF clearance, the contribution of macrophages to pathological VWF clearance has not been investigated. In particular, the contribution of macrophages to enhanced clearance in VWD Vicenza has not been assessed. We have now shown for the first time that macrophages are key mediators of pathological VWF clearance in VWD Vicenza and that clodronate-mediated macrophage depletion significantly prolongs survival of VWF-R1205H in VWF$^{-/-}$ mice. However, the macrophage receptors responsible remain unknown. Importantly, macrophage LRP1 is the only non-glycan VWF clearance receptor described to date. Given the contribution of sequence variation to the regulation of VWF plasma levels and the increasing number of amino acid mutations that are known to cause enhanced VWF clearance, this is a significant observation. Indeed enhanced clearance via macrophage LRP1 may represent an important pathogenic mechanism in VWD Vicenza. To further investigate the mechanisms underlying enhanced macrophage-mediated clearance in VWD Vicenza, THP-1 macrophage binding was studied *in vitro*.

5.1 Binding of WT-VWF to THP-1 macrophages *in vitro*.

Since macrophages bind VWF only under conditions of shear stress, binding of WT-VWF to THP-1 macrophages was analyzed in the presence and absence of ristocetin *in vitro*. WT-VWF was incubated with THP-1 macrophages on ice (to prevent endocytosis) and binding was detected using rabbit polyclonal anti-human VWF followed by a fluorescently tagged goat anti-rabbit antibody. Fluorescence was then quantified using the IN Cell Analyzer 1000 (GE Healthcare, UK).

In the presence of ristocetin WT-VWF bound to THP-1 macrophages *in vitro* (mean % THP-1 binding 100.0 ± 7.6%). In contrast, minimal binding was detected in the absence of ristocetin (mean % THP-1 binding 7.1 ± 3.3%, $p \leq 0.001$). This is in-line with recent reports that macrophage-mediated uptake of the VWF/FVIII complex is shear stress dependent (Castro-Nunez et al. 2012).

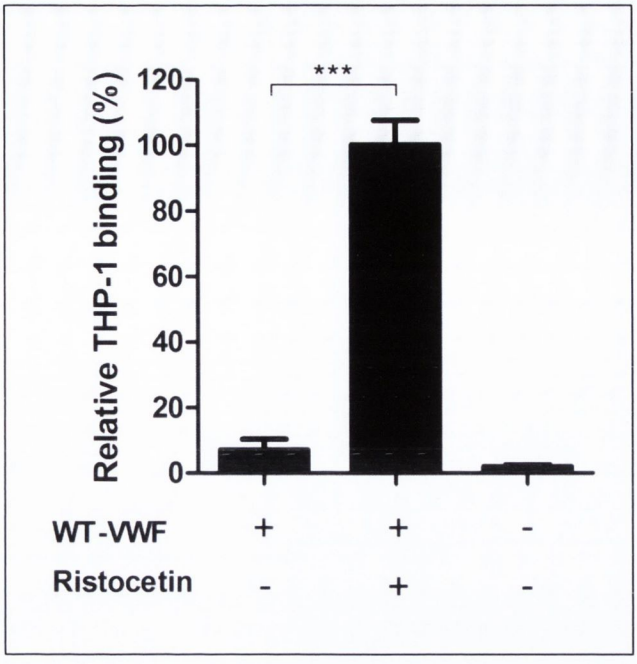


Figure 5.0 WT-VWF binds to THP-1 macrophages in the presence of ristocetin.
Data are expressed as a percentage of maximum binding (mean ± SEM).

5.2 A role for divalent cations in mediating WT-VWF/THP-1 binding.

Macrophages express a diverse receptor repertoire that mediates a range of functions including differentiation, activation, adhesion, and transendothelial migration. Importantly, the functional activity of several macrophage receptor subsets is directly dependent on the availability of divalent cations. For example, the glycan binding activity of macrophage C-type lectins is strictly Ca^{2+} dependent. In addition, the availability of Ca^{2+}, Mg^{2+}, and Mn^{2+} ions regulates macrophage integrin activation (Zhang and Chen 2012). To investigate a role for cation-dependent receptor subsets in mediating VWF/THP-1 binding, binding was studied in the presence of Ca^{2+}, Mg^{2+}, and Mn^{2+} ions.

In the presence of Ca^{2+} or Mg^{2+} ions no WT-VWF binding was observed (mean % THP-1 binding 19.5 ± 6.6% and 6.7 ± 2.5% respectively). However, in the presence of Mn^{2+} ions, VWF efficiently bound to the surface of THP-1 macrophages (mean % THP-1 binding 100.0 ± 4.9%, $p \leq 0.001$). Importantly, Mn^{2+} can facilitate VWF binding even in the absence of ristocetin. Whether Mn^{2+} promotes conformational changes similar to those induced by ristocetin is not known. However, it is clear that both ristocetin and Mn^{2+} can independently efficiently promote binding of WT-VWF to THP-1 macrophages.

Since both ristocetin and Mn^{2+} efficiently induce binding of WT-VWF to THP-1 macrophages, comparative binding studies were carried out. Binding of WT-VWF to THP-1 macrophages was studied in the presence of ristocetin or in the presence Mn^{2+}. Binding in the presence of both ristocetin and Mn^{2+} was also assessed. No significant difference in WT-VWF THP-1 binding was

observed in the presence of ristocetin compared to Mn^{2+} (mean % THP-1 binding 100.0 ± 7.6% and 96.6 ± 6.5% respectively, $p > 0.05$). Furthermore, addition of both ristocetin and Mn^{2+} did not significantly enhance binding compared to ristocetin or Mn^{2+} alone (mean % THP-1 binding 116.0 ± 1.0, $p > 0.05$).

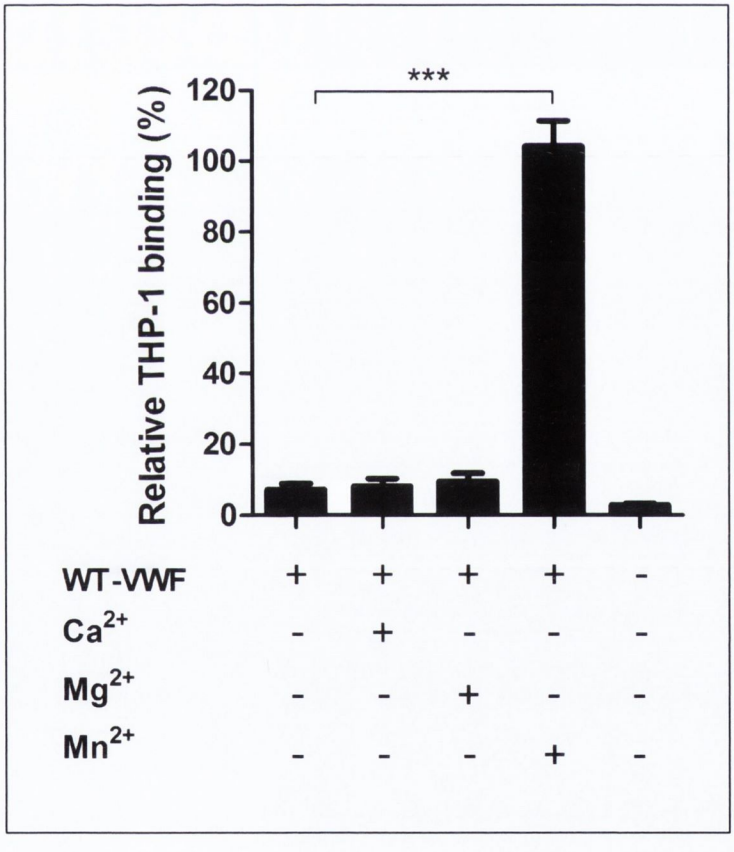


Figure 5.1 WT-VWF binds THP-1 macrophages in the presence of manganese. Data are expressed as a percentage of maximum binding (mean ± SEM).

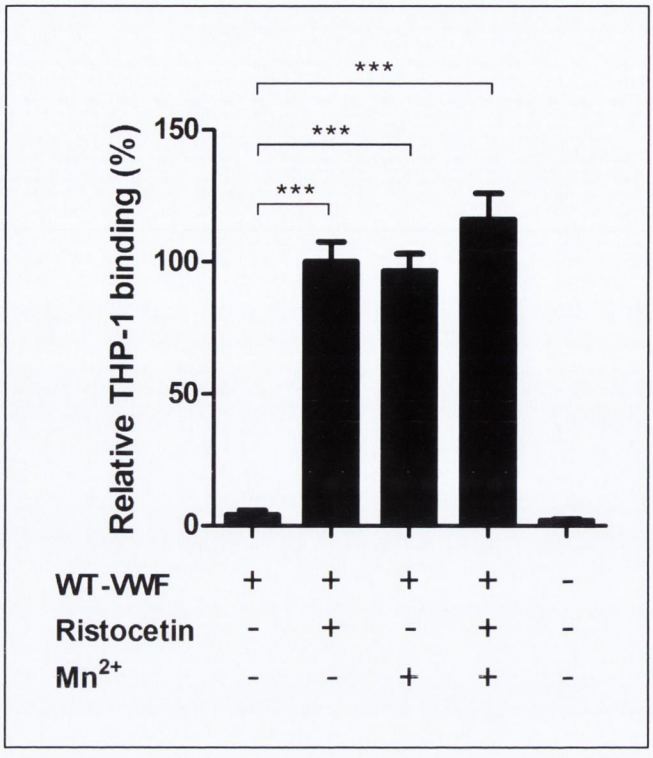


Figure 5.2 Comparison of WT-VWF THP-1 binding in the presence of ristocetin versus manganese.

Data are expressed as a percentage of WT-VWF binding in the presence of ristocetin (mean ± SEM).

5.3 Enhanced binding of VWF-R1205H to THP-1 macrophages.

The R1205H substitution is associated with rapid clearance of plasma VWF in patients with VWD Vicenza. Surprisingly however, the specific cells types and clearance receptors that contribute to enhanced clearance in VWD Vicenza are not unknown. However, i*n vivo* macrophage-depletion studies have now demonstrated that enhanced macrophage-mediated VWF clearance represents a key pathogenic mechanism in VWD Vicenza. To further assess the molecular mechanisms involved, binding of VWF-R1205H to THP-1 macrophages was studied.

VWF-R1205H bound THP-1 macrophages with significantly enhanced affinity compared with WT-VWF in the presence of Mn^{2+} (mean % THP-1 binding 123.2 ± 6.0% versus 93.7 ± 6.3% respectively, $p \leq 0.05$). Furthermore, VWF-R1205H also bound THP-1 macrophages significantly better than WT-VWF in the presence of ristocetin (mean % THP-1 binding 127.6 ± 8.4% versus 97.0 ± 7.3 %, $p \leq 0.05$). No VWF-R1205H binding was observed in the absence of ristocetin and Mn^{2+} (mean % THP-1 binding 0.3 ± 0.1%).

The above data provide insight into the molecular mechanisms through which the R1205H mutation causes enhanced macrophage-mediated clearance. VWF-R1205H bound THP-1 macrophages significantly better than WT-VWF. Thus enhanced macrophage binding and uptake may contribute to rapid VWF clearance in VWD Vicenza. Importantly, VWF-R1205H absolutely requires ristocetin or Mn^{2+} for macrophage binding. No spontaneous binding of VWF-R1205H was observed. Consequently, although both WT-VWF and VWF-R1205H require conformational change to interact with THP-1 macrophages, VWF-R1205H binds with enhanced affinity.

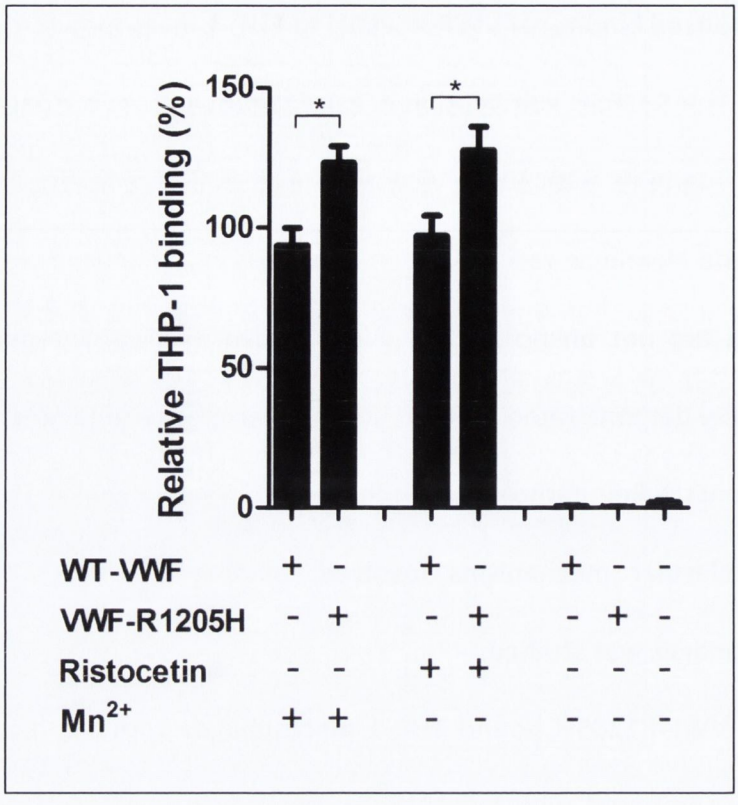


Figure 5.3 Enhanced binding of VWF-R1205H to THP-1 macrophages.
Data are expressed as a percentage of WT-VWF binding in the presence of ristocetin (mean ± SEM).

5.4 Enhanced binding of VWF-R1205C and VWF-R1205S to THP-1 macrophages.

In addition to the R1205H substitution, two other amino acid mutations have also been identified at position 1205 (R1205C and R1205S respectively), both of which are associated with rapid VWF clearance (Millar et al. 2008). In patients, differential clearance of these variants has been reported with R1205H > R1205S > R1205C. Differential clearance was also observed in $VWF^{-/-}$ mice (section 4.2). Remarkably, though macrophages appear to be the sole mediators of VWF-R1205H clearance, the contribution of macrophages to clearance of additional Vicenza variants VWF-R1205C and VWF-R1205S is considerably less significant. To further investigate this, comparative THP-1 binding was studied.

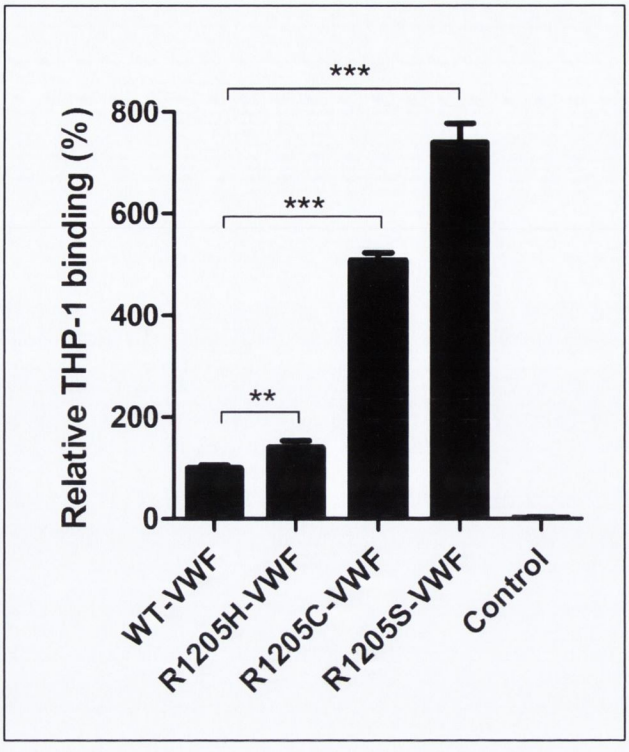


Figure 5.4 Enhanced binding of VWF-R1205C and VWF-R1205S to THP-1 macrophages.

Data are expressed as a percentage of WT-VWF binding (mean ± SEM).

VWF-R1205C bound THP-1 macrophages significantly better than WT-VWF (mean % THP-1 binding 509.3 ± 13.4% compared with 100.4 ± 5.6%, $p < 0.001$). In addition, VWF-R1205C also bound THP-1 macrophages significantly better than VWF-R1205H (mean % THP-1 binding 509.3 ± 13.4% compared with 141.3 ± 12.3%, $p < 0.001$). Similarly, enhanced THP-1 binding was observed for VWF-R1205S. VWF-R1205S displayed significantly higher affinity for THP-1 macrophages compared with both WT-VWF (mean % THP-1 binding: 740 ± 36.9% versus 100.4 ± 5.6%, $p < 0.001$) and VWF-R1205H (mean % THP-1 binding: 740 ± 36.9% versus 141.3 ± 12.3%).

These data appear to conflict with the results of VWF clearance studies in which macrophages were depleted in $VWF^{-/-}$ mice (section 4.2, section 4.7). Though VWF-R1205S and VWF-R1205C bound THP-1 macrophages with enhanced affinity compared to VWF-R1205H and were cleared significantly faster than VWF-R1205H *in vivo*, the contribution of macrophages to the clearance of these variants was less significant. Clodronate-mediated macrophage depletion only partially restored VWF-R1205C and VWF-R1205S plasma levels. Taken together, the above data suggest that macrophage subpopulations other than those depleted by clodronate liposome treatment may contribute to the enhanced clearance of VWF-R1205C and VWF-R1205S in $VWF^{-/-}$ mice.

5.5 Binding of VWF-R1204H to THP-1 macrophages.

Highly conserved amino acid sequences commonly constitute critical sites for protein function. For example, the arginine residue at VWF position 1205 is absolutely conserved among vertebrates and appears to regulate normal VWF clearance. An arginine residue also occupies neighbouring position 1204 and is also conserved. Interestingly however, mutation of R1204 to histidine had no effect on VWF clearance (section 4.3) suggesting that R1204 is not critical to this process. To further explore the molecular mechanisms mediating VWF clearance, binding of VWF-R1204H to THP-1 macrophages was assessed.

VWF-R1204H bound THP-1 macrophages in the presence of Mn^{2+}. In contrast to the enhanced THP-1 binding observed for VWF-R1205H, no significant difference in binding was observed for VWF-R1204H compared to WT-VWF (mean % THP-1 binding 75.9 ± 5.2% versus 100.0 ± 11.1%, $p > 0.05$). These data demonstrate that R1204 is not important for modulating macrophage-mediated VWF clearance.

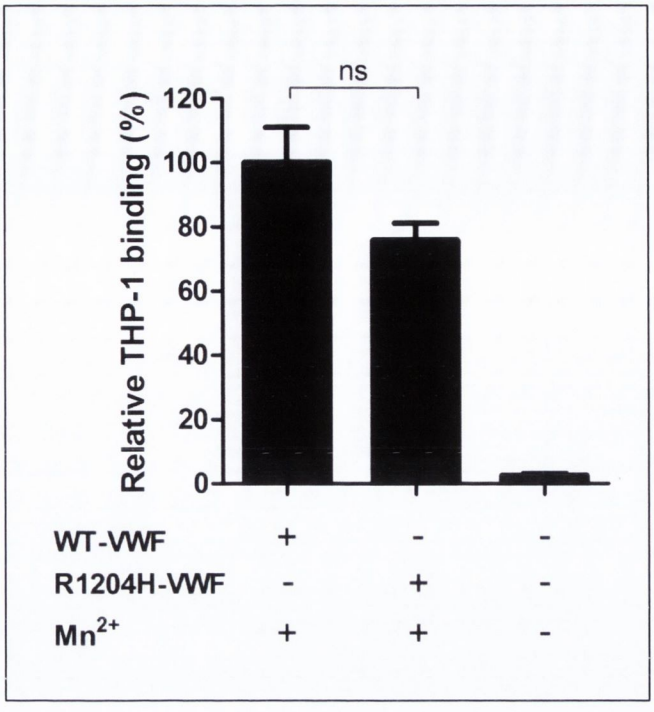


Figure 5.5 WT-VWF and VWF-R1204H bind THP-1 macrophages to the same extent.

Data are expressed as percentage of WT-VWF binding (mean ± SEM).

5.6 Investigating a role for the cation-dependent mannose-6-phosphate receptor in mediating enhanced VWF-R1205H binding.

The mannose-6-phosphate receptors are members of the P-type lectin family involved in the transport of newly synthesized lysosomal enzymes from the Golgi to endosomal–lysosomal system (Hille-Rehfeld 1995). Recognition of mannose-6-phosphate modified N-linked glycans results in the removal of the enzymes from the secretory pathway and facilitates delivery to the lysosomes. Both the cation-independent mannose-6-phosphate receptor (CI-MPR) and the cation-dependent mannose-6-phosphate receptor (CD-MPR) cycle between the cell surface and the intracellular compartments. Cell surface CI-MPRs internalize a variety of mannose-6-phosphate containing ligands as well as non-glycosylated ligands for degradation (Tong et al. 1988; Koster et al. 1994). Though the CD-MPR is also cycled to the cell surface no equivalent endocytic role has been described to date. Interestingly however, the CD-MPR exhibits enhanced ligand binding in the presence of Mn^{2+} compared with Ca^{2+} or Mg^{2+} ions (Ma et al. 1991). Given the significant Mn^{2+} effect in regulating VWF/THP-1 binding, it was of interest to explore whether CD-MPR might be involved in mediating VWF-R1205H binding to THP-1 macrophages. THP-1 binding of WT-VWF and VWF-R1205H was examined in the presence of competitive inhibitor mannose-6-phosphate (M6P).

In the presence of the specific inhibitor M6P, binding of WT-VWF to THP-1 macrophages was unaffected (WT-VWF versus WT-VWF + M6P, mean % THP-1 binding 100 ± 11.4% versus 92.8 ± 6.8%, $p > 0.05$). Similarly, R1205H-VWF binding was not affected (VWF-R1205H versus VWF-R1205H + M6P,

mean % THP-1 binding 126.2 ± 7.6% versus 118.7 ± 6.2%, $p > 0.05$). Collectively, these findings suggest that despite the observed Mn^{2+} effect, the CD-MPR does not play a major role in modulating enhanced macrophage-mediated clearance in VWD Vicenza.

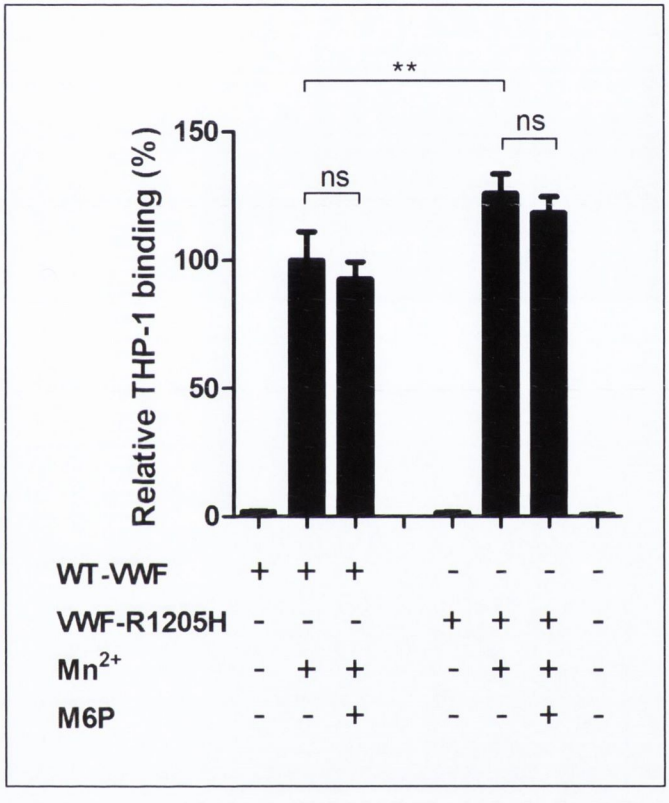


Figure 5.6 Enhanced binding of VWF-R1205H to THP-1 macrophages is not mediated by the cation-dependent mannose-6-phosphate receptor.
Data are expressed as percentage of WT-VWF binding (in the presence of Mn^{2+}; mean ± SEM).

5.7 Investigating a role for $\alpha_M\beta_2$ integrins in mediating enhanced R1205H-VWF binding.

Divalent cations are also essential for integrin function. For many integrins, Mn^{2+} is a potent activator, and the availability of Mn^{2+} ions induces conformational change that correlates with higher ligand binding affinities (Smith et al. 1994; Labadia et al. 1998). Indeed the I domain of the integrin α subunit, which shares homology with the VWF A domains, contains a unique metal binding site that is specific for Mn^{2+} and Mg^{2+}. In addition, it was previously shown that VWF binds THP-1 macrophages and polymorphonuclear leukocytes in a β_2 integrin dependent manner (Koivunen et al. 2001; Pendu et al. 2006).

To investigate a role for $\alpha_M\beta_2$ integrins in mediating interaction between VWF and THP-1 macrophages, binding was studied in the presence of the $\alpha_M\beta_2$ integrin antagonist, neutrophil inhibitory factor (NIF). NIF efficiently blocks $\alpha_M\beta_2$ integrin-mediated macrophage adhesion by interacting with the I-domain of the integrin α subunit (Muchowski et al. 1994; Rieu et al. 1994). In the presence of NIF, binding of WT-VWF to THP-1 macrophages was significantly impaired (WT-VWF versus WT-VWF + NIF, mean % THP-1 binding 100.0 ± 7.5% versus 34.4 ± 8.1%, $p \leq 0.001$). Thus inclusion of NIF lead to a ~75% reduction in binding of WT-VWF. In addition, binding of VWF-R1205H was also significantly reduced in the presence of NIF (VWF-R1205H versus VWF-R1205H + NIF, mean % THP-1 binding 130.6 ± 8.6% versus 33.4 ± 4.3%, $p \leq 0.001$). Cumulatively therefore, these novel findings demonstrate a role for

$\alpha_M\beta_2$ integrins in modulating binding of both WT-VWF and VWF-R1205H to THP-1 macrophages.

VWF contains two classical leucine-leucine-glycine (LLG) tripeptide motifs for β_2 integrin binding. Interestingly, one of these motifs is located in the VWF D3 domain. Given the proximity of this LLG motif to R1205, it seemed possible that mutation of R1205 may impact β2 integrin binding. Remarkably however, mutagenesis of both LLG motifs sites has been reported not to affect binding of monocytes to VWF suggesting that these motifs play a minor role in modulating the interaction (Pendu et al. 2006).

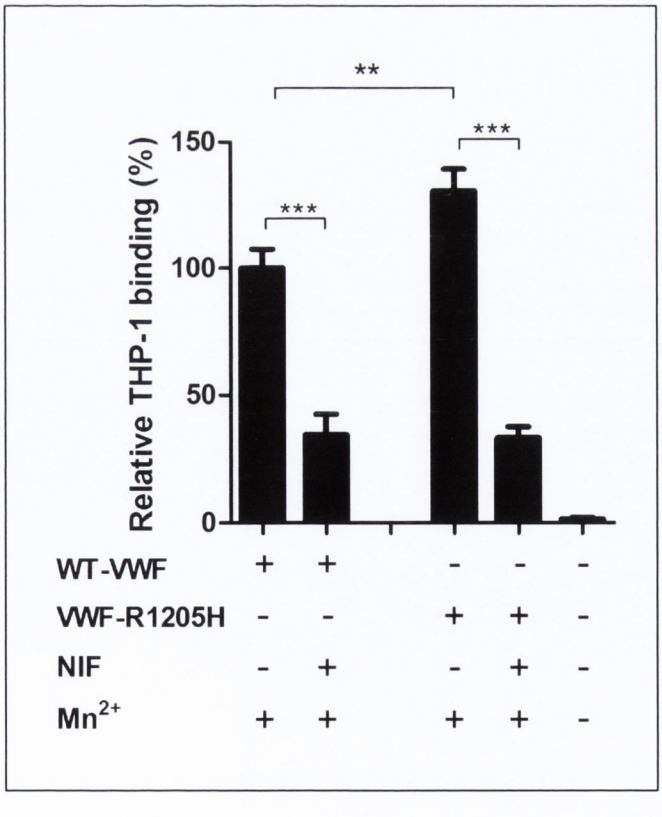


Figure 5.7 $\alpha_M\beta_2$ integrins mediate enhanced binding of VWF-R1205H to THP-1 macrophages.
Data are expressed as percentage of WT-VWF binding (mean ± SEM).

5.8 Investigating a role for LRP1 in mediating enhanced VWF-R1205H binding.

Macrophage LRP1 has recently been identified as a candidate VWF clearance receptor (Castro-Nunez et al. 2012; Rastegarlari et al. 2012). To investigate a role for LRP1 in mediating enhanced binding of VWF-R1205H, THP-1 macrophage binding was assessed in the presence of LRP1 inhibitor RAP. In the presence of RAP, binding of WT-VWF was significantly attenuated (WT-VWF versus WT-VWF + RAP, mean % THP-1 binding 100.0 ± 7.5% versus 20.3 ± 4.6%, $p \leq 0.001$). Similarly, binding of VWF-R1205H to THP-1 macrophages was also significantly reduced (VWF-R1205H versus VWF-R1205H + RAP, mean % THP-1 binding 130.6 ± 8.6% versus 41.1 ± 7.6%, $p \leq 0.001$). Consequently, macrophage LRP1 is important in modulating the enhanced binding of VWF-R1205H to THP-1 macrophages *in vitro*.

Interestingly, LRP1 inhibitor RAP attenuated THP-1 binding to a similar extent as that observed with the αMβ2 inhibitor NIF. To further investigate a role for LRP1 and $\alpha_M\beta_2$ integrin in mediating enhanced VWF binding, inhibition studies involving both RAP and NIF were carried out. Remarkably, no further reduction in binding was observed for either WT-VWF, or VWF-R1205H, in the presence of both RAP and NIF (mean % THP-1 binding 13.3 ± 3.5% and 29.6 ± 6.3% respectively, $p > 0.05$). Cumulatively, these data suggest that cooperative interaction between LRP1 and $\alpha_M\beta_2$ integrins may be involved in mediating VWF binding to THP-1 macrophages *in vitro*.

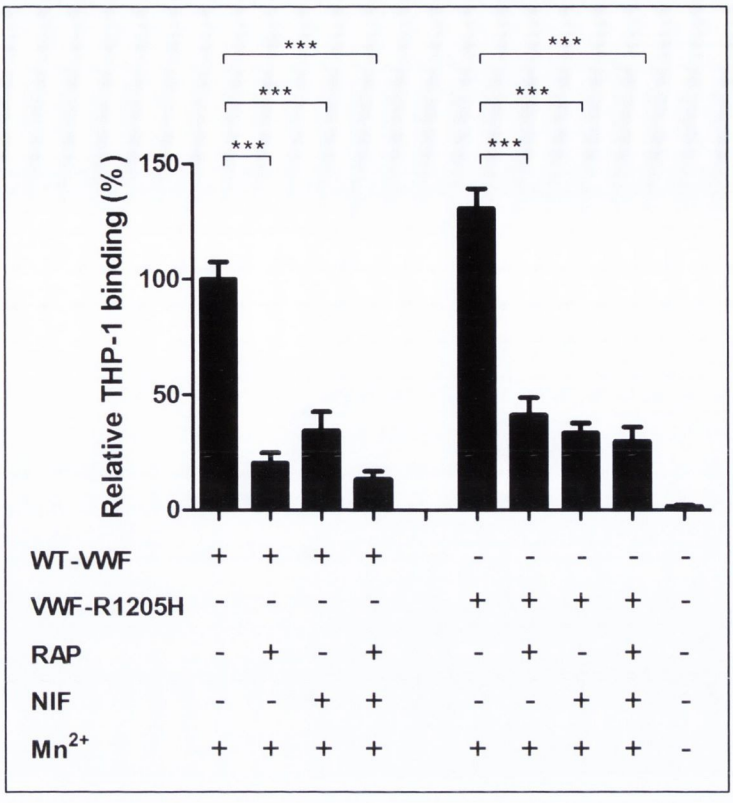


Figure 5.8 Both $\alpha_M\beta_2$ integrins and LRP1 play an essential role in mediating enhanced binding of VWF-R1205H to THP-1 macrophages.
Data are expressed as percentage of WT-VWF binding (mean ± SEM).

5.10 Discussion

Hepatic and splenic macrophages are well established mediators of VWF clearance and to date, several macrophage expressed VWF clearance receptors have been identified. However the contribution of macrophages to pathogenic VWF clearance is not well defined. Importantly, the novel data presented in this thesis demonstrates a critical role for macrophages in the pathogenesis of VWD Vicenza. Loss of R1205 leads to markedly enhanced VWF clearance which is macrophage-mediated. In keeping with this observation, *in vitro* binding studies further emphasized a role for macrophages in VWD Vicenza and showed that enhanced affinity of the R1205 variants for macrophages facilitated enhanced VWF clearance. Furthermore, macrophage LRP1 and $\alpha_M\beta_2$ integrins were identified as key modulators of this interaction.

5.10.1 Mn2+ dependent binding of VWF to THP-1 macrophages

In keeping with previous reports of the shear-stress dependent nature of the VWF/macrophage interaction, WT-VWF bound THP-1 macrophages in the presence of but not the absence of ristocetin. Remarkably, WT-VWF also bound THP-1 macrophages in the presence of Mn^{2+} ions but not in the presence of other divalent cations. Though it is well established that Mn^{2+} is a potent integrin activator, the role of Mn^{2+} in modulating binding of VWF to THP-1 macrophages is not clear. In particular, whether Mn^{2+} activates

macrophage expressed β_2 integrins to mediate VWF binding or whether Mn^{2+} can directly modulate VWF conformation is not clear.

Integrin ligand binding interactions are mediated by divalent cations present in the metal ion dependent adhesion site (MIDAS) of the integrin I-domain. Binding of Ca^{2+} has an inhibitory effect on ligand binding activity while Mn^{2+} and Mg^{2+} binding promote integrin activation and high-affinity ligand binding. Interestingly, the integrin I-domain is homologous to the VWF A domains suggesting that Mn^{2+} may be able to directly modulate VWF A domain conformation. However, the MIDAS motifs of VWF A1 and A2 domains are not conserved and the putative MIDAS motif in the VWF A3 domain is non-functional. Moreover, metal binding is not predicted for the VWF A domains. Therefore, whether Mn^{2+} can induce conformational change to activate VWF in a similar manner to β_2 integrins is not known.

5.10.2 Enhanced binding of Vicenza variants to THP-1 macrophages.

Importantly, THP-1 binding assays confirmed the findings of our *in vivo* clearance studies. In keeping with increased macrophage-mediated VWF clearance of the R1205 variants, *in vitro* THP-1 binding studies demonstrated significantly enhanced binding for these variants confirming a role for macrophages in the pathogenesis of VWD Vicenza. Moreover, in keeping with normal clearance of VWF-R1204H, no enhanced affinity of VWF-R1204H for THP-1 macrophages was observed. Consequently, these data demonstrate that enhanced affinity of R1205 variants for macrophages contributes to enhanced macrophage-mediated VWF clearance in VWD Vicenza.

5.10.3 Binding of R1205 variants to THP-1 macrophages is shear-stress dependent.

Interestingly, though enhanced binding of the R1205 variants to THP-1 macrophages was evident, loss of R1205 did not induce spontaneous macrophage binding. Like WT-VWF, binding of the R1205 variants required the presence of ristocetin or Mn^{2+} ions demonstrating that modulation of VWF conformation was critical for the interaction. Thus these data suggest that loss of R1205 does not lead to global conformational change and does not overcome the previously established requirement for shear-stress. However, these data do not exclude the possibility that loss of R1205 renders the VWF molecule less resistant to shear-stress compared with WT-VWF. If indeed R1205 serves to stabilize local D'D3 conformation via salt bridge formation, then mutation of this critical residue may sufficiently destabilize the molecule to facilitate unfolding at lower shear rates but may not be sufficient to cause larger conformational alterations. This is in keeping with the observation that patients with VWD Vicenza do not display spontaneous GPIbα binding or ADAMTS13 proteolysis suggesting that the functional A domains are not affected by mutation of this residue.

5.10.4 LRP1 and $\alpha_M\beta_2$ integrins mediate enhanced binding of VWF-R1205H to THP-1 macrophages.

The above data demonstrate cooperative-interaction between macrophage LRP1 and $\alpha_M\beta_2$ integrins to facilitate VWF binding. Inhibition of

either LRP1 or $\alpha_M\beta_2$ integrin served to abrogate VWF binding suggesting that both LRP1 and $\alpha_M\beta_2$ integrins are necessary for this interaction. These results are consistent with the recent finding that LRP1 and β2 integrins form a functional complex to regulate VWF clearance (Rastegarlari et al. 2012). Though VWF has previously been shown to independently interact with LRP1 and β2 integrins (Koivunen et al. 2001; Rastegarlari et al. 2012), it is not clear if VWF binds both LRP1 and β2 integrins of the LRP1/β2 integrin complex at the cell surface. Though LRP1 and β2 integrin complex formation appears to be essential for VWF binding, only one of these proteins may directly mediate the interaction with VWF.

In addition, these novel data highlight a role for LRP1 and $\alpha_M\beta_2$ integrins in the pathogenesis of VWD Vicenza. The LRP1/ $\alpha_M\beta_2$ integrin complex mediated enhanced binding of VWF-R1205H to THP-1 macrophages and in a similar fashion to WT-VWF, inhibition of either LRP1 or $\alpha_M\beta_2$ integrin was sufficient to abrogate binding. This finding further confirms the importance of macrophage-mediated clearance in VWD Vicenza. Given the markedly enhanced binding of VWF-R1205C and VWF-R1205S to THP-1 macrophages compared with VWF-R1205H, additional work is required to investigate whether the LRP1/$\alpha_M\beta_2$ integrin complex also plays a role in mediating enhanced macrophage binding of VWF-R1205C and R1205S.

6.0 Investigating the clinical significance of enhanced VWF clearance in two distinct patient cohorts.

According to the International Society on Thrombosis and Haemostasis (ISTH) classification criteria, von Willebrand Disease (VWD) is classified into three main categories (Sadler et al. 2006). Type 1 VWD is the most common manifestation of the disease, comprising up to 75% of all cases and is characterized by a partial quantitative deficiency of functionally normal VWF. Type 2 VWD accounts for 25% of cases, and results from the expression of a functionally abnormal VWF molecule. Finally in rare patients with type 3 VWD, there is a complete absence of VWF. Though the genetic mutations and biological mechanisms that cause type 2 and type 3 VWD are well defined, the molecular mechanisms leading to the most common form of the disease (type 1 VWD) remain poorly understood.

Recently however, several important Type 1 VWD cohort studies revealed that the majority of cases of Type 1 VWD are caused by *VWF* gene mutations (Cumming et al. 2006; Goodeve et al. 2007; James et al. 2007). Over 100 candidate missense mutations have been identified throughout the coding region of the *VWF* sequence that lead to a variety of pathogenic mechanisms including reduced VWF synthesis and/or secretion and enhanced VWF clearance. In the Type 1 cohort studies, these candidate mutations were observed in ~65% of 1 index cases. Interestingly however, candidate mutations were significantly more common in patients with plasma VWF antigen (VWF:Ag) levels less than 0.30U/ml, comprising up to 75% of cases. In

contrast, VWF candidate mutations were found in only 50% of cases with VWF:Ag levels >0.30 U/mL (James et al. 2007). Consequently, the USA National Heart, Lung and Blood Institute (NHLBI) have suggested modification of the ISTH VWD classification criteria. In particular, the NHLBI proposed that patients with reduced plasma VWF:Ag levels and bleeding phenotypes should be considered in two distinct subsets. Patients with marked reductions in plasma VWF levels (<0.3U/mL) should be considered as having Type 1 VWD, while patients with intermediate plasma VWF:Ag levels (0.3 – 0.5U/mL) should be considered in a distinct category and considered as having 'Low VWF levels'.

Importantly, while sequence variation is a key determinant of VWF plasma levels in the majority of patients with Type 1 VWD, the genetic determinants leading to "low VWF levels" are more complex. For example, the relative contribution of ABO blood group to the regulation of VWF plasma levels appears to be more significant in patients with "low VWF levels". In addition to the *VWF* and *ABO* genes, other genetic loci are also likely to contribute to the "low VWF" phenotype. Indeed, recent genome-wide association analysis studies (GWAS) identified a number of novel genetic loci that modulate VWF plasma levels (Smith et al. 2010). While the contribution of each of these individual loci is small (<5%) in healthy individuals, polymorphic variation at these loci may play a significant role in regulating VWF plasma levels in patients with "low VWF levels". However, the mechanisms by which these additional loci cause a reduction in circulating VWF:Ag levels are not known.

Following the extensive *in vitro* and *in vivo* characterization of VWF-R1205H clearance described herein, it was of interest to examine the *in vivo* biological basis underlying the enhanced VWF clearance in a VWD Vicenza cohort. Consequently, the case records of all patients (n=430) on the Irish National VWD Registry with quantitative VWD were systematically reviewed and a cohort of 17 patients were identified as having VWD Vicenza, satisfying the NHLBI criteria for Type 1 VWD. In addition, to investigate the importance of enhanced clearance in patients with low VWF levels, a cohort of 89 individuals was identified with recorded historical plasma VWF levels consistently within 0.3 –0.5U/mL range. Plasma samples were collected for patients in each cohort and baseline VWF:Ag, VWF:pp, VWF:RCo and FVIII:C were determined. DDAVP trials results were also analysed in 7 patients with VWD Vicenza (Type 1 VWD) and 10 patients with "low VWF" levels.

6.1 Examining the clinical significance of enhanced VWF clearance in a VWD Vicenza cohort.

VWD Vicenza is characterised by the shortest VWF survival reported to date. DDAVP trials conducted in patients with VWD Vicenza have reported VWF-R1205H plasma half-lives that are reduced up to 9-fold compared with those of Haemate P® (Gezsi et al. 2010). This marked reduction in VWF half-life has important clinical implications, particularly regarding selection of appropriate therapeutic agents for management of clinical events. Although plasma VWF:Ag levels increase significantly in Vicenza patients following DDAVP administration, this increase is not sustained due to rapid VWF-R1205H clearance and VWF:Ag levels return to baseline within just four hours (Casonato et al. 2002; Gezsi et al. 2010). Consequently, replacement therapy using VWF and FVIII containing concentrates has been suggested as a more appropriate treatment option for these patients (Haberichter et al. 2006).

To further investigate the biological basis underlying the enhanced VWF clearance in VWD Vicenza, VWF:Ag and FVIII:C plasma levels were measured in Vicenza patients at baseline and following DDAVP administration. DDAVP response curves were fitted to a monoexponential model of clearance and the half-lives of VWF and FVIII were calculated.

6.1.1 Baseline VWF:Ag and FVIII:C levels in a VWD Vicenza patient cohort

In the total VWD Vicenza cohort (n= 17), lowest recorded baseline VWF:Ag plasma levels as well as corresponding FVIII:C levels were reported for each patient. The median baseline VWF:Ag was 0.10U/mL (range: 0.05 – 0.29U/mL) and median baseline FVIII:C was 0.17U/mL (range: 0.05 – 0.46U/mL). These baseline VWF:Ag and FVIII:C values are consistent with those in previously published reports (Mannucci et al. 1988; Casonato et al. 2002; Castaman et al. 2011). The median VWF:Ag (0.10U/mL) satisfies the NHLBI criteria for Type 1 VWD. Remarkably, despite the marked reduction in VWF:Ag levels, the VWD Vicenza bleeding phenotype is relatively mild. This mild phenotype has been attributed to normal platelet-VWF content, as platelet-VWF can sufficiently support haemostasis in the presence of reduced plasma VWF.

	Baseline VWF:Ag (U/mL)	Baseline FVIII:C (U/mL)	Baseline FVIII:C/VWF:Ag
Patient 1-Vicenza	0.29	0.46	1.58
Patient 2-Vicenza	0.06	0.09	1.50
Patient 3-Vicenza	0.12	0.17	1.40
Patient 4-Vicenza	<0.1	0.12	1.20
Patient 5-Vicenza	0.12	0.12	1.00
Patient 6-Vicenza	0.08	0.19	2.38
Patient 7-Vicenza	0.13	0.22	1.70
Patient 8-Vicenza	<0.05	0.13	2.60
Patient 9-Vicenza	<0.1	0.27	2.70
Patient 10-Vicenza	0.05	0.17	3.40
Patient 11-Vicenza	<0.1	0.29	2.90
Patient 12-Vicenza	<0.1	0.05	0.50
Patient 13-Vicenza	<0.1	0.17	1.70
Patient 14-Vicenza	<0.1	0.15	1.50
Patient 15-Vicenza	0.07	0.08	1.14
Patient 16-Vicenza	<0.10	0.07	0.7
Patient 17-Vicenza	0.16	0.28	1.75

Table 6.0 Baseline VWF:Ag, FVIII:C and FVIII:C/VWF:Ag in the VWD Vicenza cohort.

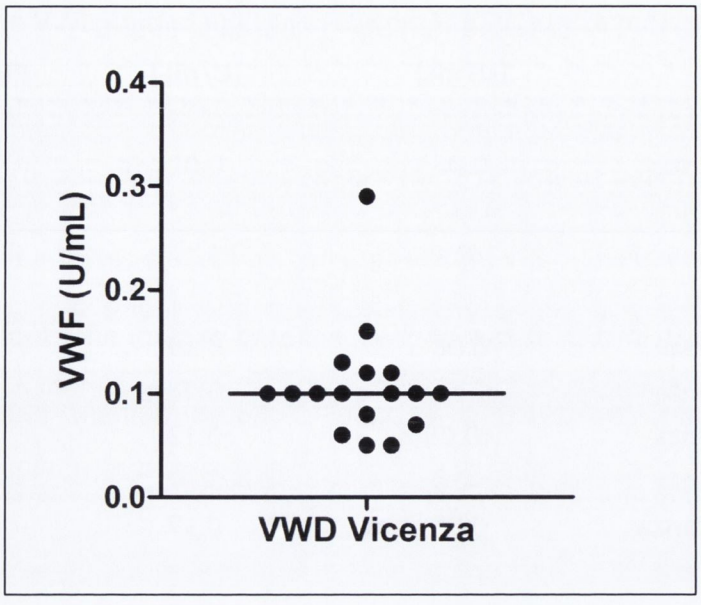


Figure 6.0 Baseline VWF:Ag levels in the VWD Vicenza cohort (n = 17).
Horizontal black line represents the median value.

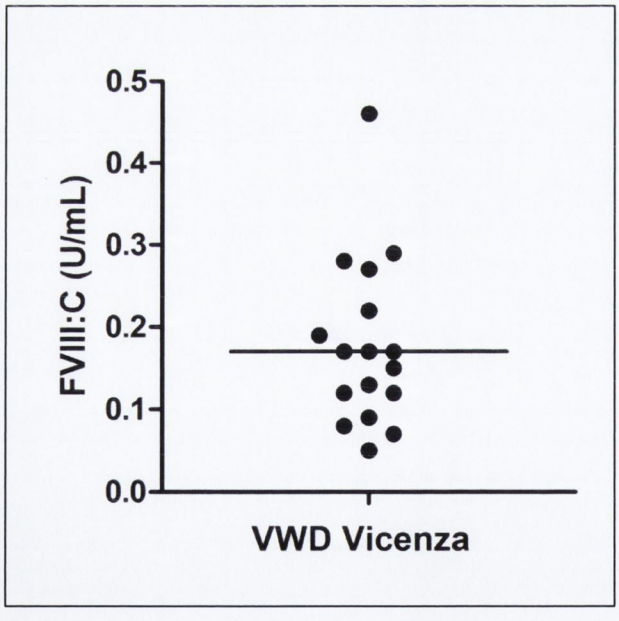


Figure 6.1 Baseline FVIII:C plasma levels in the VWD Vicenza cohort (n = 17).
Horizontal black line represents the median value.

While baseline plasma VWF:Ag levels were significantly decreased in all patients within this cohort, more variation in plasma FVIII:C levels was observed (~9-fold; table 6.1). Similar findings have been reported in a number of other VWD Vicenza cohorts (Mannucci et al. 1988; Schneppenheim et al. 2000; Casonato et al. 2006). Since the majority of plasma FVIII is believed to be cleared in complex with VWF, concordant reductions in plasma VWF:Ag and FVIII:C levels are expected in VWD Vicenza, and thus the FVIII:C/VWF:Ag ratio should equal one. These data demonstrate some discordant change in plasma VWF:Ag and FVIII:C levels in specific Vicenza patients. Consequently, elevated baseline FVIII:C/VWF:Ag levels (>1.9) were observed in some patients. Interestingly, Patient 6-Vicenza, Patient-8 Vicenza, Patient 9-Vicenza, Patient 10-Vicenza and Patient 11-Vicenza had FVIII:C/VWF:Ag ratios of 2.4, 2.6, 2.7, 3.4 and 2.9 respectively which are considered indicative of reduced VWF synthesis and/or secretion (Eikenboom et al. 2013). These clinical findings are important since *in vitro* expression studies have shown that reduced VWF-R1205H synthesis may also contribute to the pathophysiology of VWD Vicenza (Pruss et al. 2011).

6.1.2 DDAVP response kinetics in patients with VWD Vicenza

The response of VWD Vicenza patients to DDAVP was investigated in detail. DDAVP is a synthetic analog of the hormone vasopressin that is often used to treat Type 1 VWD. DDAVP acts as a specific agonist for V2 receptors expressed on endothelial cell membranes to release VWF from WPB storage. (Kaufmann et al., 2000) DDAVP treatment usually results in a 3-5 fold increase in plasma VWF:Ag and FVIII:C within 1 hour, and concentrations generally remain elevated above baseline for 6-8 hours (Rodeghiero and Castaman 2005). However, in VWD Vicenza, due to the marked reduction in plasma half-life plasma VWF:Ag return to baseline within just 4 hours (Casonato et al. 2002; Gezsi et al. 2010).

DDAVP responses were analysed in 7 patients with VWD Vicenza over a four hour time course. The half-lives of VWFpp and VWF were determined by calculation of the first-order rate constant for the elimination phase from the slope of the VWF:Ag or FVIII:C concentration against time. Peak VWF:Ag and FVIII:C levels were obtained at 1 hour post-DDAVP. Consequently, the 1 hour time point was taken as the beginning of the elimination phase after subtraction of baseline (T_0) values. Absolute peak post-DDAVP VWF:Ag and FVIII:C levels were also reported.

	VWF:Ag Baseline (U/mL)	FVIII:C Baseline (U/mL)	Baseline FVIII:C/VWF:Ag (ratio)	VWF:Ag 1 hour post (Increase above baseline) (U/mL)	FVIII:C 1 hour post (Increase above baseline) (U/mL)	VWF:Ag Fold increase over baseline	FVIII:C Fold increase over baseline	VWF:Ag $t_{1/2}$ (hours)	FVIII:C $t_{1/2}$ (hours)
Patient 3-Vicenza	0.12	0.17	1.42	0.61	1.47	6.1	9.6	0.72	0.82
Patient 4-Vicenza	<0.1	0.19	1.90	0.35	0.58	4.5	4.1	0.67	0.74
Patient 7-Vicenza	0.13	0.22	1.69	0.69	0.88	5.3	4.0	0.95	1.2
Patient 11-Vicenza	0.11	0.20	1.81	0.39	0.85	4.6	5.3	0.57	0.62
Patient 12-Vicenza	<0.10	0.05	0.50	0.36	0.53	4.6	11.6	0.49	0.89
Patient 13-Vicenza	<0.10	0.20	2.00	0.35	0.56	4.5	3.8	0.83	0.93
Patient 16-Vicenza	0.06	0.18	3.00	0.66	0.79	12.0	5.4	0.62	0.50

Table 6.1 DDAVP response kinetics in patients with VWD Vicenza.

DDAVP Time point	Absolute VWF:Ag (U/mL)	Absolute FVIII:C (U/mL)	FVIII:C/VWF:Ag (ratio)
T_0	0.12	0.17	1.42
T_1	0.73	1.64	2.25
T_2	0.35	0.74	2.11
T_4	0.16	0.38	2.38

Table 6.2 Absolute post-DDAVP values for Patient 3-Vicenza.

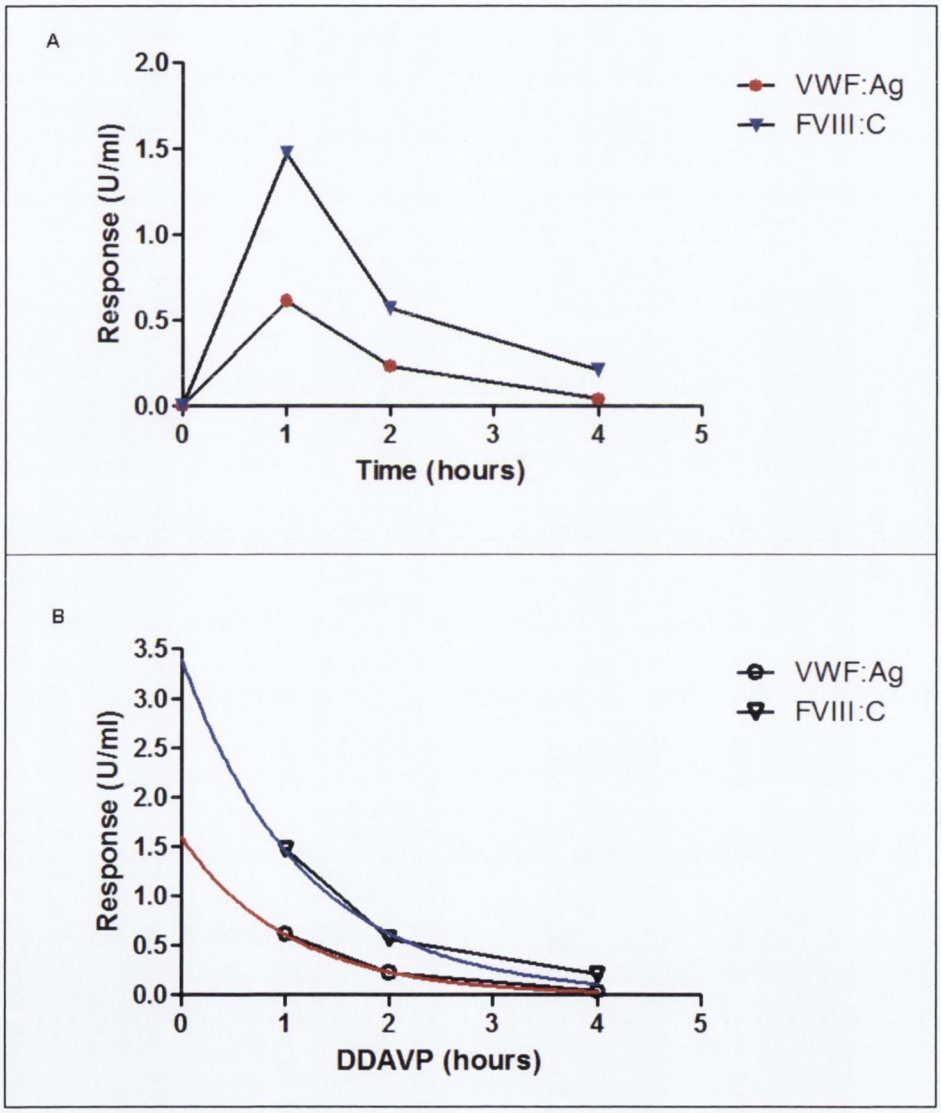


Figure 6.2 DDAVP response kinetics for Patient 3-Vicenza.

A) Time-course of DDAVP response B) Curve-fitting of VWF and FVIII response.

DDAVP Time point	Absolute VWF:Ag (U/mL)	Absolute FVIII:C (U/mL)	FVIII:C/VWF:Ag (ratio)
T_0	<0.10	0.19	1.90
T_1	0.45	0.77	1.71
T_2	0.20	0.42	2.10
T_3	0.15	-	-
T_4	0.16	0.22	1.38

Table 6.3 Absolute post-DDAVP values for Patient 4-Vicenza.

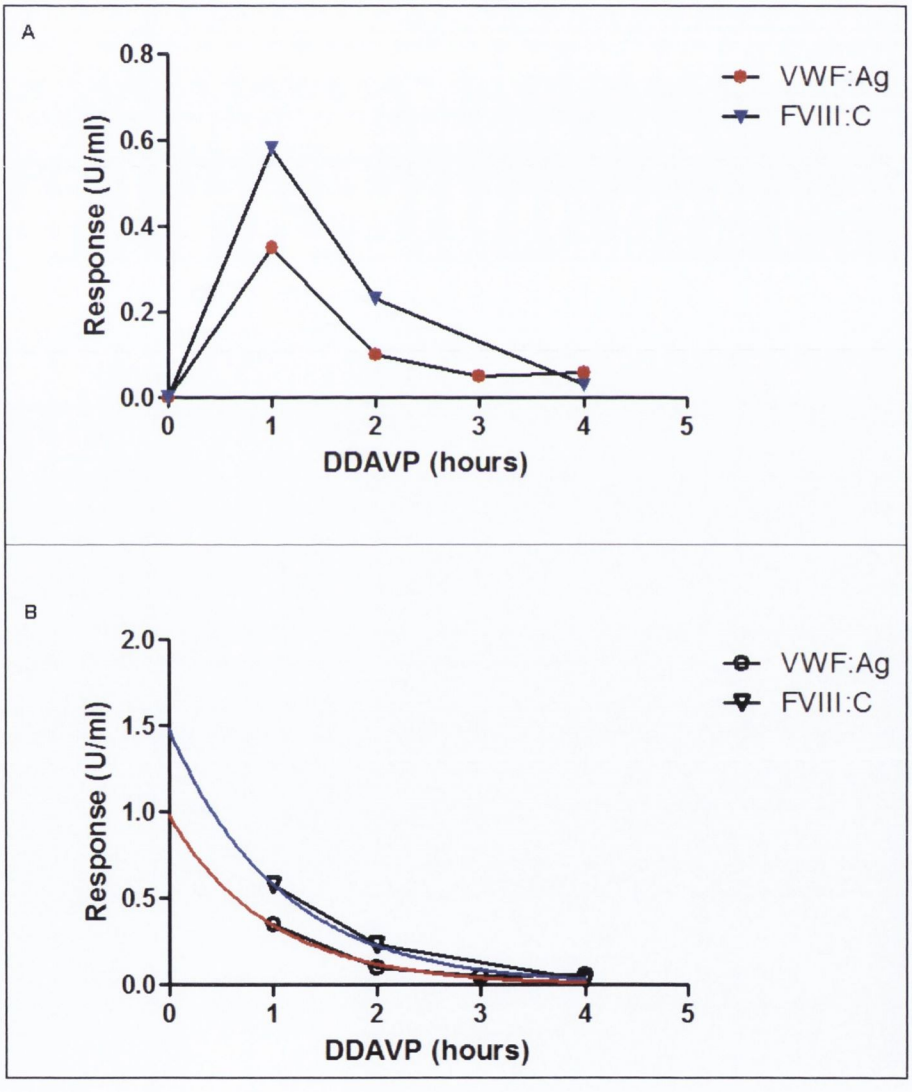


Figure 6.3 DDAVP response kinetics for Patient 4-Vicenza.
A) Time-course of DDAVP response. B) Curve-fitting of VWF and FVIII response.

DDAVP Time point	Absolute VWF:Ag (U/mL)	Absolute FVIII:C (U/mL)	FVIII:C/VWF:Ag (ratio)
T_0	0.13	0.22	1.69
T_1	0.82	1.1	1.34
T_2	0.46	0.73	1.59
T_3	0.29	0.5	1.72
T_4	0.21	0.36	1.71

Table 6.4 Absolute post-DDAVP values for Patient 7-Vicenza.

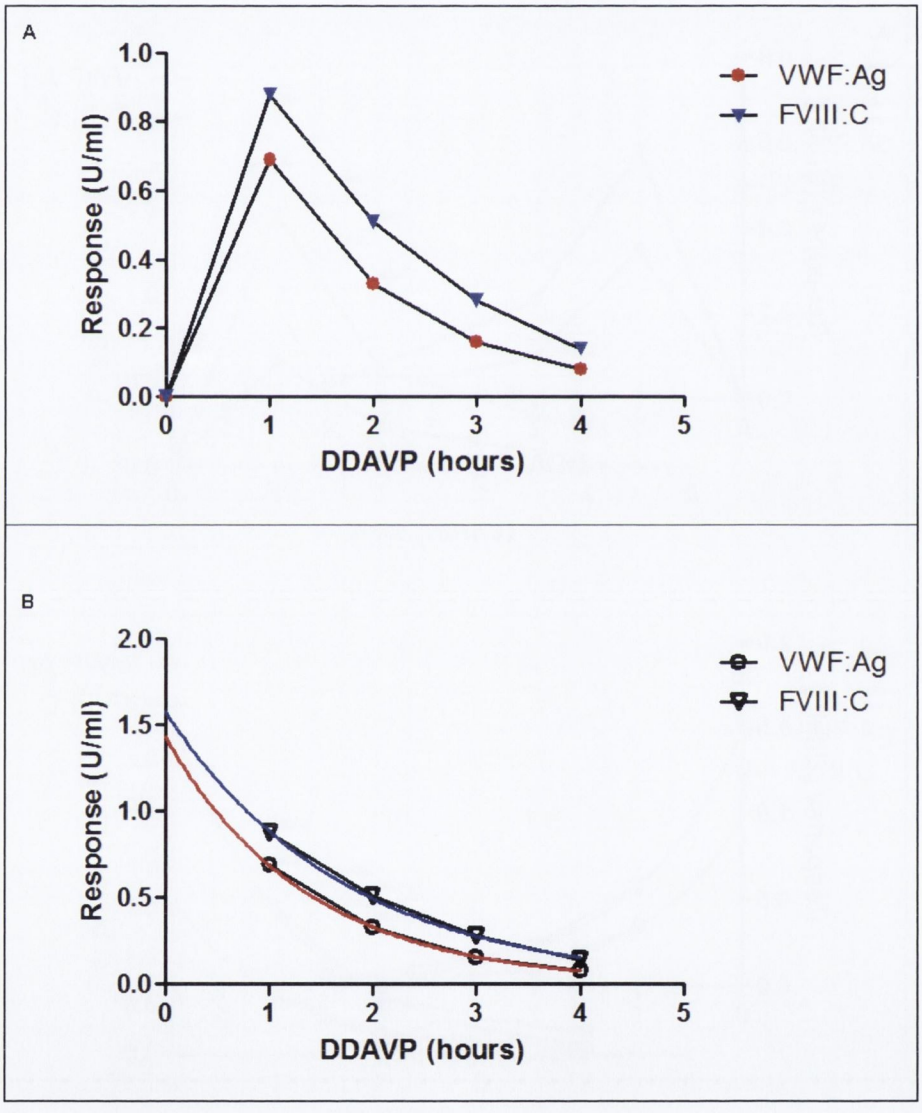


Figure 6.4 DDAVP response kinetics for Patient 7-Vicenza.
A) Time-course of DDAVP response. B) Curve-fitting of VWF and FVIII response.

DDAVP Time point	Absolute VWF:Ag (U/mL)	Absolute FVIII:C (U/mL)	FVIII:C/VWF:Ag (ratio)
T_0	0.11	0.2	1.82
T_1	0.5	1.05	2.1
T_4	0.12	0.23	1.92
T_6	<0.1	0.19	1.9

Table 6.5 Absolute post-DDAVP values for Patient 11-Vicenza.

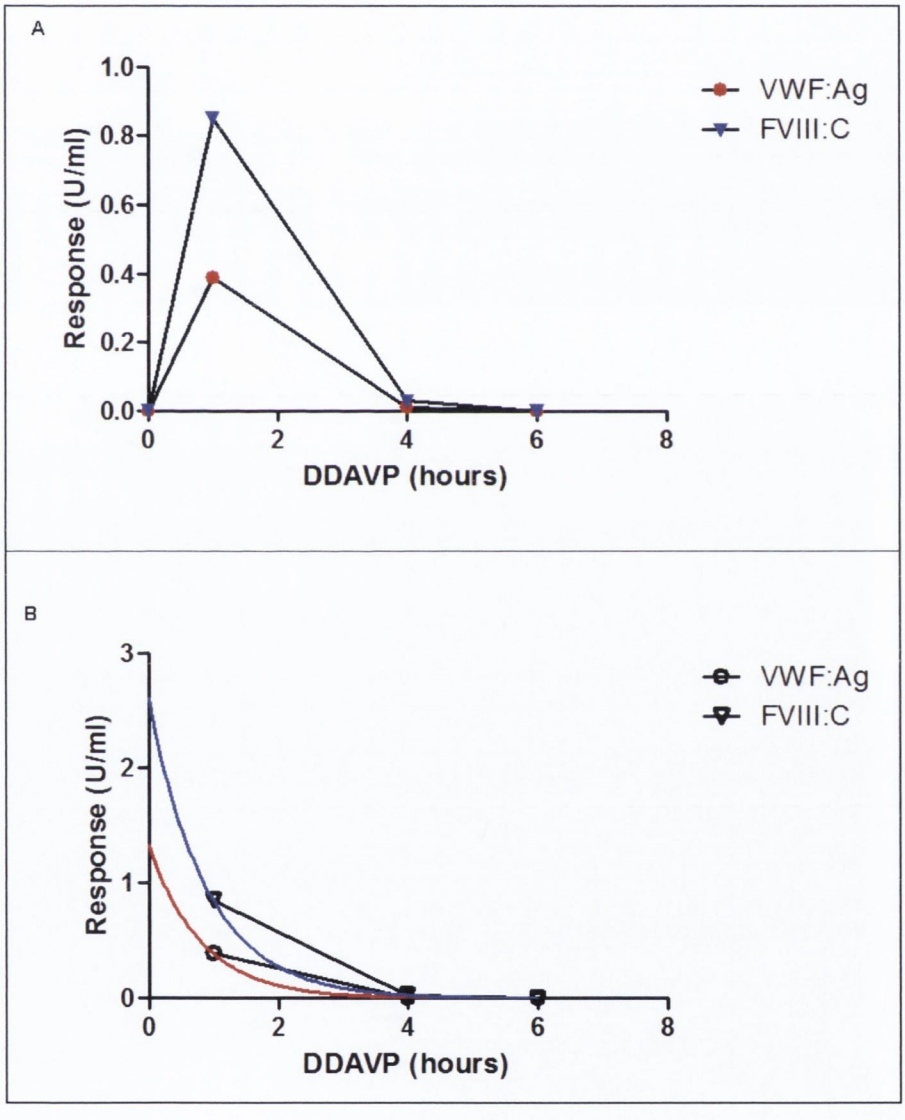


Figure 6.5 DDAVP response kinetics for Patient 11-Vicenza.
A) Time-course of DDAVP response. B) Curve-fitting of VWF and FVIII response.

DDAVP Time point	Absolute VWF:Ag (U/mL)	Absolute FVIII:C (U/mL)	FVIII:C/VWF:Ag (ratio)
T_0	<0.1	0.05	0.50
T_1	0.46	0.58	1.26
T_2	0.19	0.3	1.58
$T_{3.5}$	<0.1	0.12	1.2

Table 6.6 Absolute post-DDAVP values for Patient 12-Vicenza.

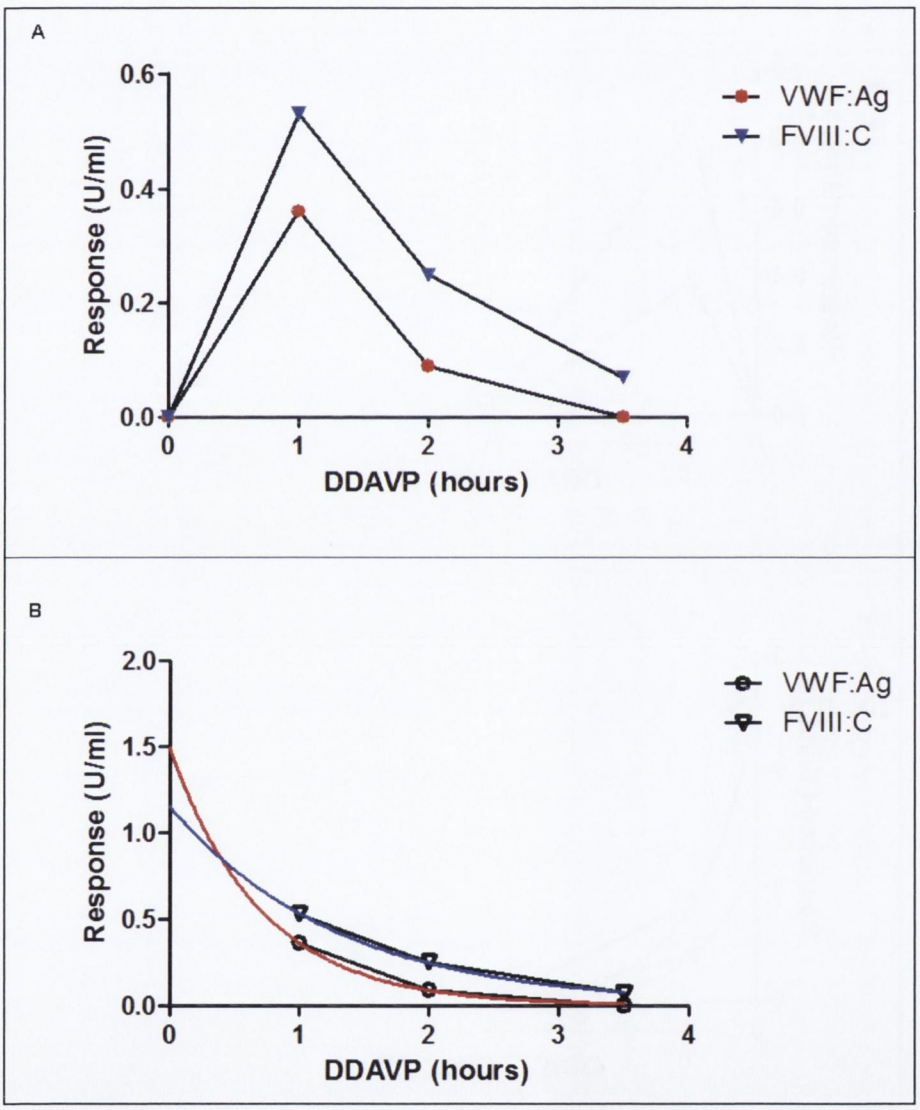


Figure 6.6 DDAVP response kinetics for Patient 12-Vicenza.
A) Time-course of DDAVP response. B) Curve-fitting of VWF and FVIII response.

DAVP Time point	Absolute VWF:Ag (U/mL)	Absolute FVIII:C (U/mL)	FVIII:C/VWF:Ag (ratio)
T_0	<0.1	0.2	2.00
T_1	0.45	0.76	1.69
T_2	0.22	0.44	2.00
T_3	0.19	0.35	1.84
T_4	0.14	0.26	1.86

Table 6.7 Absolute post-DDAVP values for Patient 13-Vicenza.

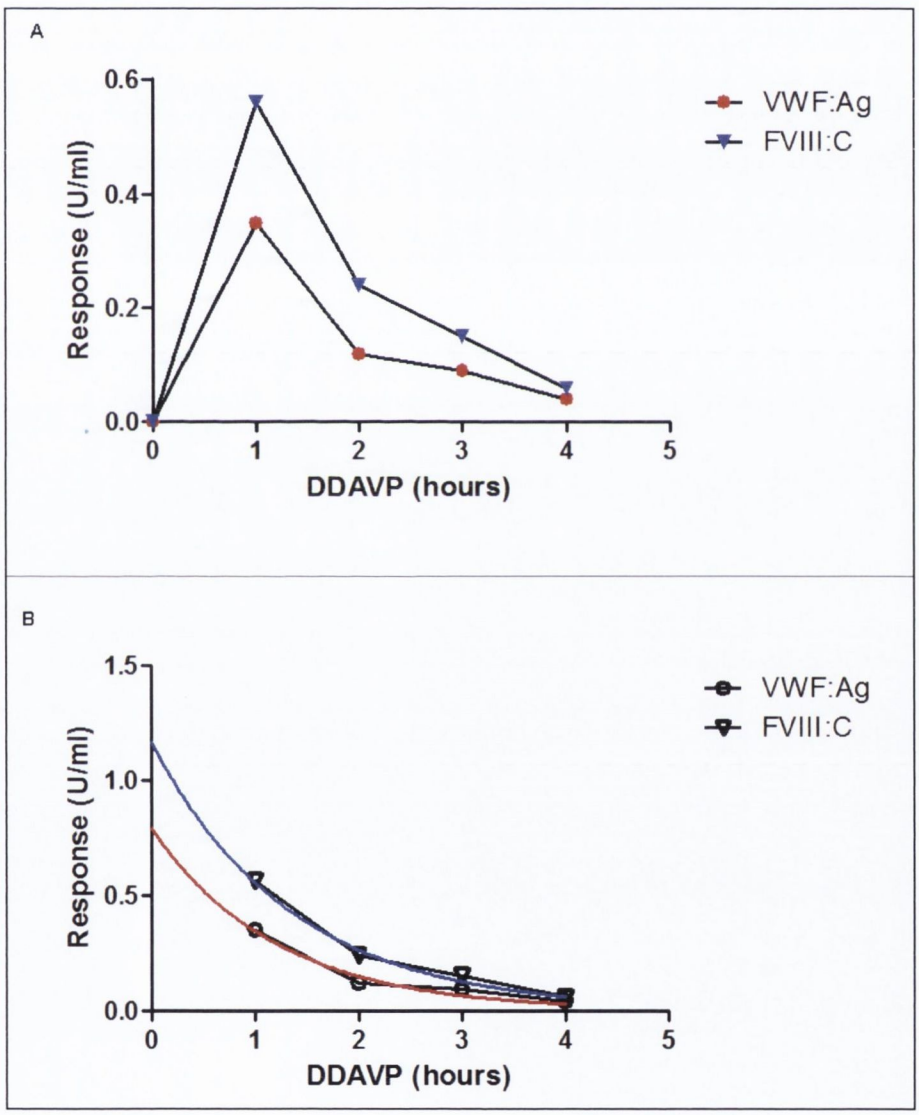


Figure 6.7 DDAVP response kinetics for Patient 13-Vicenza.
A) Time-course of DDAVP response. B) Curve-fitting of VWF and FVIII response.

DDAVP Time point	Absolute VWF:Ag (U/mL)	Absolute FVIII:C (U/mL)	FVIII:C/VWF:Ag (ratio)
T_0	0.06	0.18	3.00
T_1	0.72	0.97	1.35
T_2	0.26	0.38	1.46
$T_{3.5}$	0.13	0.19	1.46

Table 6.8 Absolute post-DDAVP values for Patient 16-Vicenza.

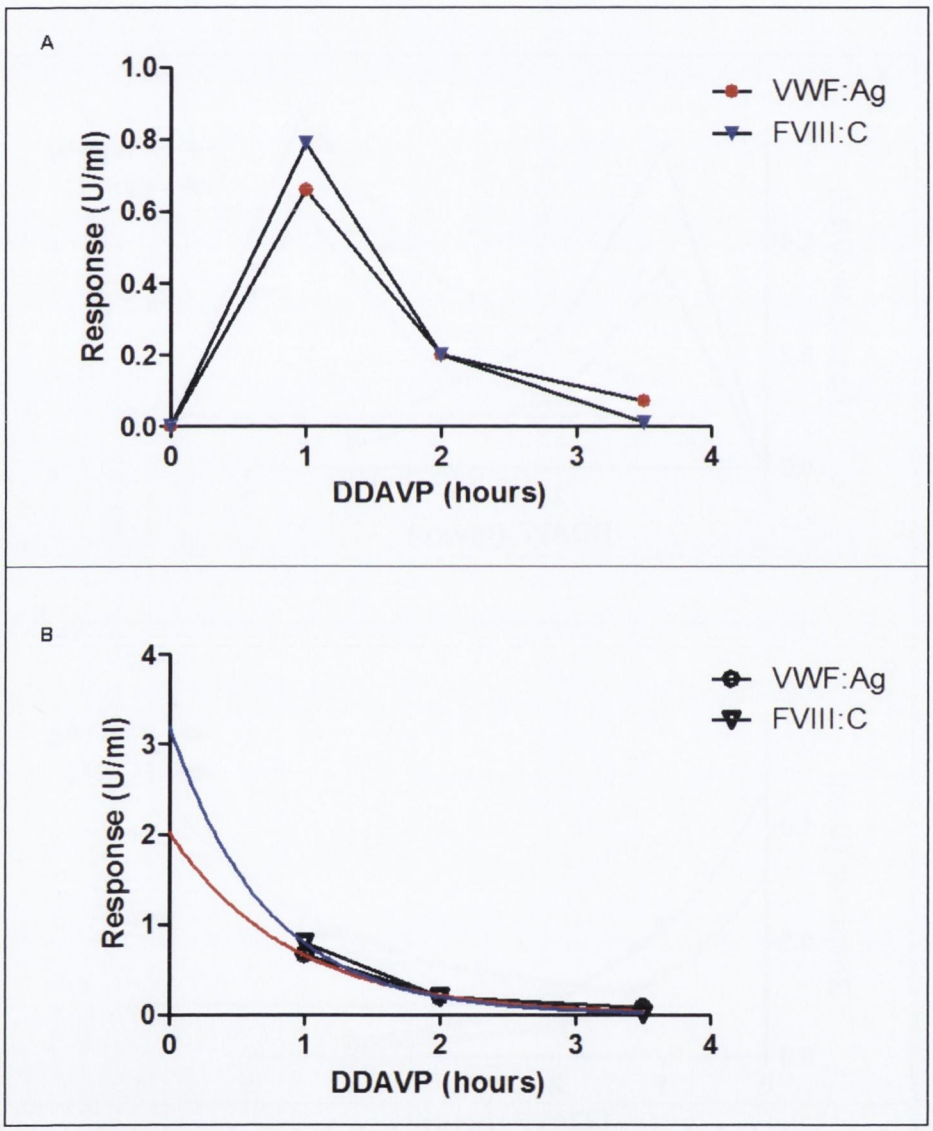


Figure 6.8 DDAVP response kinetics for Patient 16-Vicenza.
A) Time-course of DDAVP response. B) Curve-fitting of VWF and FVIII response.

In keeping with the previous finding of enhanced VWF clearance in VWD Vicenza, each of the 7 patients studied had significantly reduced VWF:Ag half-lives following DDAVP treatment (table 6.2). Though peak-post DDAVP VWF:Ag levels were elevated 4.5-12.0 fold over baseline, increases were not sustained and VWF:Ag levels returned to baseline within 4 hours in all cases. In addition, the calculated VWF:Ag half-lives were consistent with previously published VWF half-lives of approximately 1 hour in VWD Vicenza (Casonato et al. 2006; Millar et al. 2008; Gezsi et al. 2010). Markedly reduced post DDAVP FVIII:C half-lives were also observed which is in keeping with the notion that VWF and FVIII are cleared as part of a complex. While the relative response of FVIII to DDAVP (3.8 – 11.6 fold increase over baseline) was similar to that of VWF, the absolute peak post-DDAVP values of FVIII:C were significantly higher.

The efficacy of DDAVP for bleeding prophylaxis in patients with VWD Vicenza has been questioned (Haberichter et al. 2006). However, prospective studies of anti-haemorrhagic treatments in patients with VWD Vicenza reported successful management of dental extractions, deliveries and minor surgical procedures with DDAVP despite short post-infusion VWF half-lives (Castaman et al. 2006; Castaman et al. 2011). Peak post-DDAVP increases in VWF:Ag levels range from 4.5- to 7.2-fold in this cohort (table 6.2). However, despite this robust relative response, absolute plasma VWF:Ag levels failed to reach clinically useful levels (0.5U/mL) in 3 of the 7 patients suggesting that DDAVP is not a viable therapeutic option. Patient 4-Vicenza, Patient 13-Vicenza and Patient 16-Vicenza had 1 hour post-DDAVP VWF:Ag increases of 4.5-, 4.5- and 4.6-fold respectively. Nevertheless, VWF:Ag levels only reached

0.45U/mL, 0.45U/mL and 0.46U/mL in these patients. This is consistent with previous DDAVP trials that reported seemingly enhanced DDAVP responses in individuals with VWD Vicenza. However, although relative VWF:Ag increases of up to 13.5-fold were observed, in many cases, the absolute VWF:Ag increase was minimal and not clinically significant (Federici et al. 2004; Castaman et al. 2008; Haberichter et al. 2008).

Conversely, DDAVP administration appear to be a viable option for management of minor procedures in 4 of the 7 patients studied. Following DDAVP administration clinically useful VWF:Ag levels were obtained in Patient 3-Vicenza, Patient-7 Vicenza, Patient 11-Vicenza and Patient 16-Vicenza who demonstrated peak-post DDAVP VWF:Ag levels of 0.73U/mL, 0.82U/mL, 0.50U/mL and 0.72U/mL respectively. However, due to the short-lived nature of the VWF:Ag level increase, clotting factor concentrates would be required to manage major surgery or treat severe trauma in these patients.

In addition to enhanced VWF clearance, it has been proposed that quantitative defects in VWF synthesis also contribute to the pathophysiology of VWD Vicenza. *In vitro* expression studies in several cell-lines demonstrated decreased total protein production for VWF-R1205H compared with WT-VWF (Pruss et al. 2011). Indeed, defective VWF-R1205H synthesis would seemingly explain the minimal absolute increases in VWF:Ag levels in some patients with VWD Vicenza following DDAVP treatment. However, normal platelet-VWF content has consistently been observed in Vicenza patients, suggesting that at least in megakaryocytes VWF synthesis is normal (Fressinaud et al. 1994; Schneppenheim et al. 2000; Casonato et al. 2002). Crucially however, normal platelet-VWF levels do not rule out VWF secretory or storage defects within

endothelial cells. Moreover, surface plasmon resonance analysis has previously demonstrated impaired association of VWF-R1205H with the VWF propeptide at acidic pH (Lenting et al. 2004). Given that this propeptide association is critical for VWF packaging and storage, the inability of VWF-R1205H to condense into tubular structures within storage organelles may lead to impaired regulated release. Based on the absolute DDAVP responses in the above patient cohort, we postulate that the suboptimal response to DDAVP in VWD Vicenza may be due to low levels of VVWF-R1205H targeted to the WPBs for regulated secretion.

6.2 Investigating the pathogenic mechanisms responsible for "Low VWF levels" in a patient cohort.

Eighty nine patients with "low VWF levels" (0.30 – 0.50U/mL) were evaluated in this study. Seventy three (84%) patients were female and fourteen (16%) were male, with a median age of 40 years (range: 18 – 68 years). In the total cohort (n = 89), lowest recorded baseline VWF:Ag as well as corresponding VWF:RCo and FVIII:C values were reported for each patient (figure 6.10). The median baseline value for VWF:Ag was 0.46U/mL (range: 0.32 – 0.60U/mL); the median baseline value for VWF:RCo was 0.40U/mL (range: 0.30 – 0.53 U/mL) and the median baseline value for FVIII:C was 0.64 U/mL (range: 0.41 – 1.38U/mL). Of the eighty nine patients identified, twenty five had baseline plasma VWF:Ag levels above 0.5U/mL, however VWF:RCo values were reduced (0.3 – 0.5U/ml) in these patients.

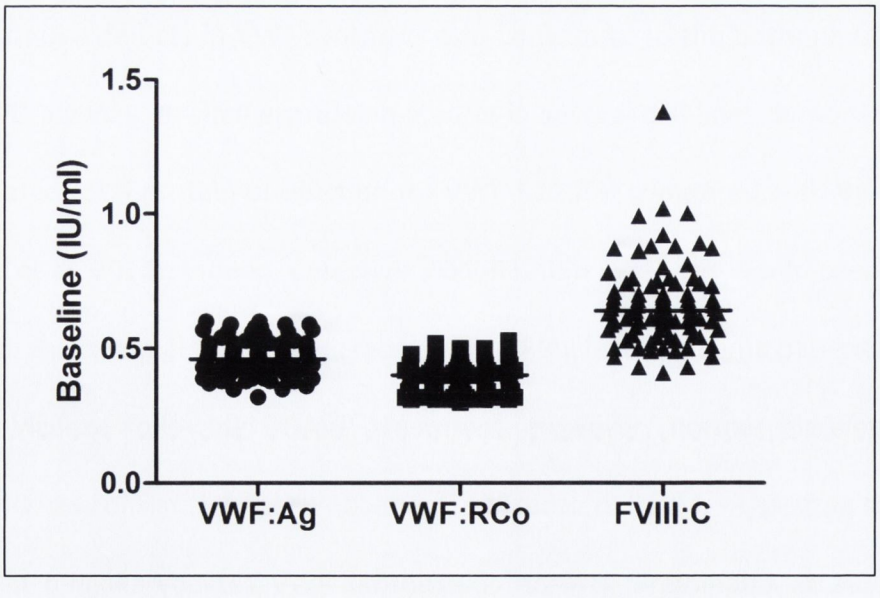


Figure 6.9 Baseline plasma VWF:Ag, VWF:RCo and FVIII:C levels in patients with "low VWF" plasma levels.
Horizontal line represents median (n=89).

In order to investigate the pathogenic mechanisms responsible for mediating the reduction in VWF plasma levels in this particular patient cohort, plasma samples were collected. Baseline VWF:Ag, VWF:pp, FVIII:C were determined for each patient and are reported in table 6.10. To investigate the contribution of reduced VWF synthesis and/or secretion and enhanced VWF clearance to the pathophysiology of "low VWF levels" FVIII:C/VWF:Ag and VWF:pp/VWF:Ag ratios were also determined. In addition, ABO blood group was established.

Patient number	ABO Blood Group	VWF:Ag	FVIII:C	VWF:pp	VWF:pp/VWF:Ag	FVIII:C/VWF:Ag
1	O	0.39	0.6	0.67	1.72	1.54
2	O	0.52	0.67	0.7	1.35	1.29
3	O	0.68	0.61	0.80	1.18	0.90
4	O	0.62	0.74	0.86	1.39	1.19
5	O	0.43	0.5	0.83	1.93	1.16
6	A	0.72	1.17	0.69	0.96	1.63
7	O	0.5	0.77	1.38	2.76	1.54
8	O	0.52	0.78	0.99	1.90	1.50
9	O	0.43	0.61	0.46	1.07	1.42
10	O	0.42	0.53	0.76	1.81	1.26
11	O	0.66	ND	0.68	1.03	ND
12	O	0.52	0.69	0.65	1.25	1.33
13	O	0.55	0.8	0.98	1.78	1.45
14	O	0.52	0.71	0.68	1.31	1.37
15	O	0.5	0.86	0.74	1.48	1.72
16	O	0.59	0.71	0.66	1.12	1.20
17	O	0.49	0.67	0.59	1.20	1.37
18	O	0.61	0.61	0.82	1.34	1.00
19	O	0.43	0.59	0.72	1.67	1.37
20	O	0.42	0.58	0.80	1.90	1.38
21	O	0.58	ND	1.01	1.74	ND
22	O	0.6	0.57	0.84	1.40	0.95
23	O	0.7	0.9	0.87	1.24	1.29
24	O	0.44	0.64	0.65	1.48	1.45
25	O	0.66	0.85	0.75	1.14	1.29
26	O	0.6	ND	0.65	1.08	ND
27	O	0.5	0.68	0.55	1.10	1.36
28	O	0.38	0.7	0.48	1.26	1.84
29	O	0.52	0.73	0.42	0.81	1.40
30	O	0.6	0.8	1.39	2.32	1.33
31	A	0.49	0.59	0.67	1.37	1.20
32	O	0.51	0.74	0.81	1.59	1.45
33	O	0.57	0.75	0.79	1.39	1.32
34	O	0.62	0.69	0.87	1.40	1.11
35	O	0.57	0.57	1.37	2.40	1.00
36	O	0.39	0.5	0.66	1.69	1.28
37	O	0.58	0.75	1.35	2.36	1.29
38	A	0.38	0.55	0.93	2.20	1.45
39	O	0.64	0.73	0.67	1.05	1.14
40	O	0.43	0.5	0.8	1.86	1.16

Patient number	ABO Blood Group	VWF:Ag	FVIII:C	VWF:pp	VWF:pp/VWF:Ag	FVIII:C/VWF:A
41	O	0.5	0.63	0.73	1.46	1.26
42	O	0.53	0.89	0.74	1.40	1.68
43	O	0.6	0.87	0.59	0.98	1.45
44	O	0.6	0.61	0.95	1.58	1.02
45	O	0.49	0.85	0.55	1.12	1.73
46	O	0.53	0.83	0.71	1.34	1.57
47	O	0.43	0.6	0.89	2.07	1.40
48	A	0.47	0.84	0.59	1.26	1.79
49	O	0.72	0.89	1.56	2.17	1.24
50	O	0.46	0.7	0.92	2.00	1.52
51	O	0.56	0.62	0.68	1.21	1.11
52	O	0.57	0.94	0.84	1.47	1.65
53	A	0.45	0.76	0.39	0.87	1.69
54	O	0.64	0.52	0.98	1.53	0.81
55	O	0.52	0.54	1.09	2.10	1.04
56	O	0.69	0.91	0.95	1.38	1.32
57	O	1.05	0.93	0.8	0.76	0.89
58	O	0.8	0.83	0.94	1.18	1.04
59	O	0.43	0.68	0.55	1.28	1.58
60	O	0.46	0.63	0.44	0.96	1.37
61	O	0.54	0.88	1.21	2.24	1.63
62	A	0.67	0.68	1.32	1.97	1.01
63	O	0.56	0.89	0.75	1.34	1.59
64	O	0.47	0.68	0.89	1.89	1.45
65	O	0.46	0.64	0.71	1.54	1.39
66	O	0.48	0.73	0.8	1.67	1.52
67	O	0.55	0.7	0.58	1.05	1.27
68	O	0.59	0.76	0.94	1.59	1.29
69	O	0.4	0.78	0.36	0.90	1.95
70	O	0.61	0.73	1.42	2.33	1.20
71	A	0.72	0.74	0.85	1.18	1.03
72	O	0.67	0.65	0.99	1.48	0.97
73	O	0.5	ND	1.01	2.02	ND
74	O	0.45	0.67	0.49	1.09	1.49
75	O	0.56	0.92	0.43	0.77	1.64
76	O	0.63	0.88	0.65	1.03	1.40
77	A	0.6	0.73	0.94	1.57	1.22
78	O	0.54	ND	1.12	2.07	ND
79	O	0.45	0.29	0.46	1.02	0.64
80	B	0.45	0.68	0.44	0.98	1.51

Patient number	ABO Blood Group	VWF:Ag	FVIII:C	VWF:pp	VWF:pp/VWF:Ag	FVIII:C/VWF:Ag
81	A	0.58	0.68	0.67	1.16	1.17
82	O	0.54	0.86	0.64	1.19	1.59
83	O	0.46	0.61	0.82	1.78	1.33
84	O	0.61	0.68	0.81	1.33	1.11
85	A	0.5	0.64	0.73	1.46	1.28
86	O	0.8	0.65	0.84	1.05	0.81
87	O	0.57	0.43	1.1	1.93	0.75
88	A	0.64	0.77	1.06	1.66	1.20
89	O	0.49	1.02	0.72	1.47	2.08

Table 6.9 Phenotypic characteristics of low VWF cohort.

6.2.1 Contribution of ABO blood group to the regulation of VWF plasma levels in the "Low VWF" cohort.

The most well-defined genetic regulator of VWF plasma levels is the ABO locus located on chromosome 9q34. Up to 30% of the genetic variability in VWF plasma levels is directly attributed to this locus (Orstavik et al. 1985). Consequently, VWF plasma levels are ABO dependant such that group O individuals have significantly lower levels of circulating VWF than non-O individuals (Gallinaro et al. 2008). To investigate the contribution of ABO blood group to the regulation of VWF plasma levels in the "low VWF" cohort, ABO status was determined by reverse grouping.

Out of eighty nine individuals with "low VWF levels", a total of seventy six individuals had blood group O (87.4%), with ten having blood group A (11.5%). In addition, one individual had blood group B (1.1%). No individuals had blood group AB. This compared with frequencies of 57% (group O), 29% (group A), 11% (group B) and 3% (group AB) in the general Irish population (Tills et al. 1977). Thus, blood group O was significantly over-represented in the "low VWF" cohort.

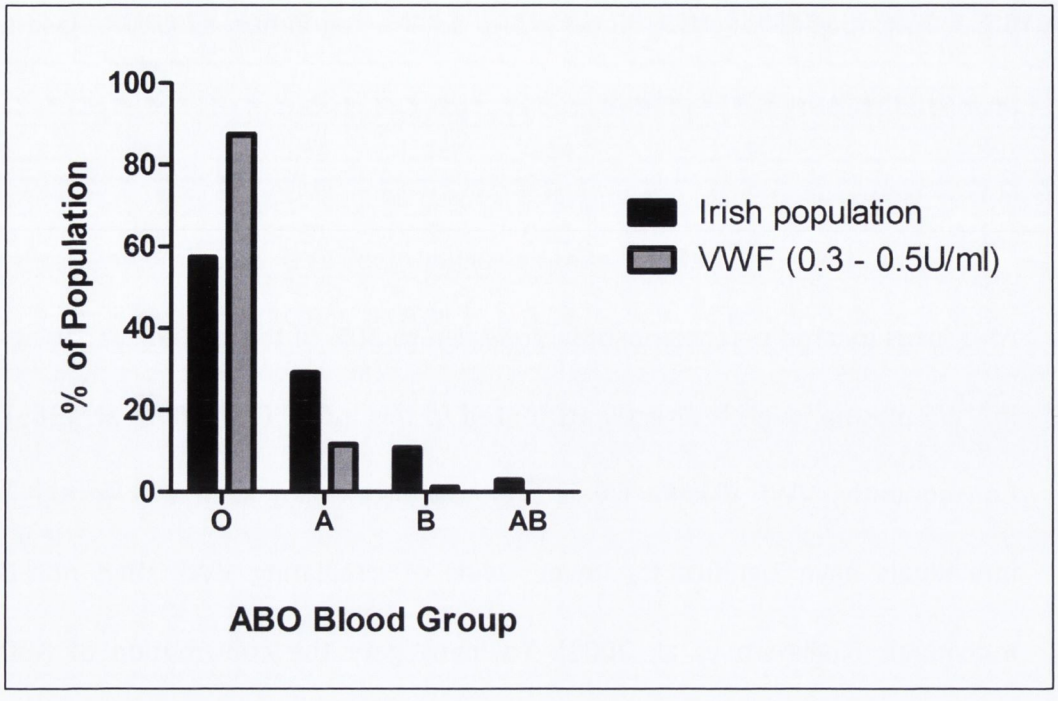


Figure 6.10 Relative proportion of ABO blood groups in "Low VWF" cohort compared with the general Irish population.

The finding that blood group O is significantly over represented in the "low VWF" cohort is in agreement with the results of two recent multicentre studies. In the Canadian Type 1 cohort study, approximately 66% of index cases with "low VWF levels" had blood group O compared with 46% of the general Canadian population. (James et al. 2007). Importantly, this study also noted that in Type 1 index cases (VWF plasma levels <0.30 U/mL), the prevalence of blood group O was not different to that of the general population. Taken together, these data suggest that relative contribution of ABO blood group to the regulation of VWF plasma levels is more significant in the "low VWF" cohort than in Type 1 VWD.

Similarly, over-representation of blood group O was also reported by the Molecular and Clinical Markers for the Diagnosis and Management of

Type 1 Von Willebrand Disease (MCMDM-1 VWD) study. Up to 76% of mutation negative Type 1 index cases were blood group O compared with only 38% of healthy controls (Goodeve et al. 2007). Furthermore, segregation analysis in this particular cohort revealed that the prevalence of blood group O was significantly greater in index cases from families in which VWD incompletely cosegregated with the *VWF* gene compared with completely cosegregating index cases (89% versus 63% respectively). Since individuals with "low VWF levels" are significantly less likely to have a *VWF* gene mutation, these data suggest that ABO blood group is a key determinant of "low VWF plasma levels" in these individuals.

6.2.2 Contribution of reduced VWF synthesis/secretion to "low VWF levels".

The molar ratio of FVIII:C to VWF:Ag (FVIII:C/VWF:Ag) in normal plasma is 1:50. However, when FVIII:C and VWF:Ag concentrations are expressed as units, the FVIII:C/VWF:Ag ratio in normal plasma equals one. Since VWF acts as a molecular chaperone for FVIII in the circulation, decreases in circulating VWF result in concordant change in FVIII levels. For example, when VWF clearance is increased, the FVIII:C/VWF:Ag ratio remains stable as FVIII is cleared in complex with VWF. However, clinical data have revealed discordant changes in plasma VWF:Ag and FVIII:C levels in particular subtypes of VWD. Consequently, an increased FVIII:C/VWF:Ag ratio (>1.9) was associated with reduced VWF synthesis and/or secretion, a common pathogenic mechanism in Type 1 VWD (Eikenboom et al. 2002; Eikenboom et al. 2013).

To establish whether reduced VWF synthesis/secretion contributed to reduced VWF plasma levels in the "low VWF" cohort, plasma FVIII:C levels were determined in eighty four of the eighty nine patients and were expressed as a ratio relative to VWF:Ag. FVIII:C/VWF:Ag ratios were also analyzed following ABO blood group stratification.

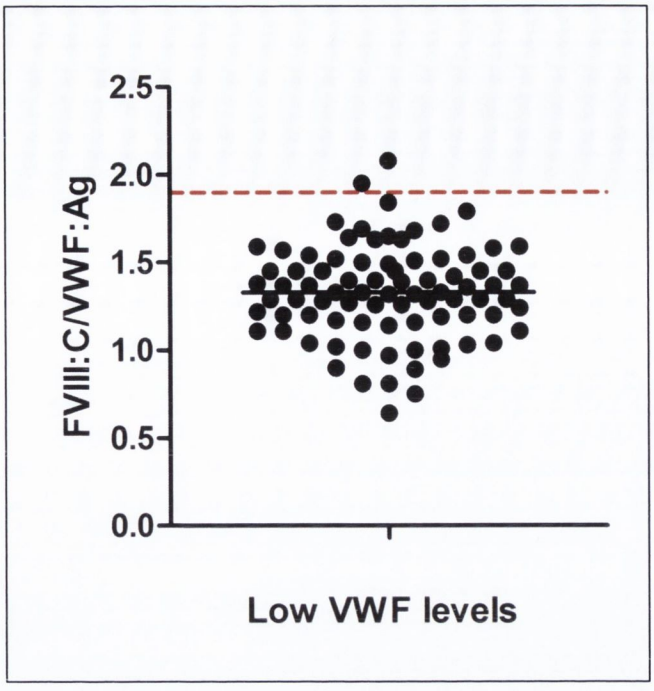


Figure 6.11 FVIII:C/VWF:Ag ratio in patients with "low VWF" plasma levels.
The horizontal black line indicates median values (n = 84). The dashed red line represents the upper limit of the normal range for FVIII:C/VWF:Ag (1.9).

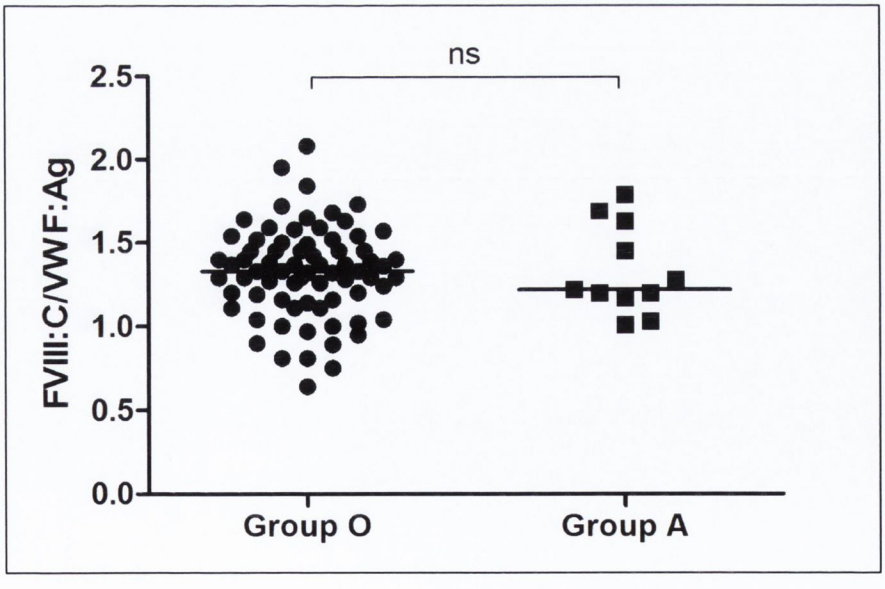


Figure 6.12 Influence of ABO blood group on FVIII:C/VWF:Ag ratios in patients with "low VWF levels".
The horizontal black line indicates median values.

The median value for FVIII:C/VWF:Ag was 1.33 (range: 0.64 – 2.08). This value is in agreement with a previously published report in which the median FVIII:C/VWF:Ag ratio of mutation negative Type 1 index cases was 1.4 (range: 1.1-1.7) (Eikenboom et al. 2013). Of the eighty four patients for which FVIII:C/VWF:Ag ratios were determined, only two individuals (~2%) had ratios that exceeded the upper limit of the previously established normal range (0.6 – 1.9) (Eikenboom et al. 2013). Patients 69 and 89 had very marginally elevated steady-state FVIII:C/VWF:Ag ratios of 1.95 and 2.08 respectively. Overall, these data suggest that reduced VWF synthesis and/or secretion, at least as assessed using steady state FVIII:C/VWF:Ag ratios, is not an important pathogenic mechanism leading to reduced plasma VWF levels in the "Low VWF" cohort.

In addition, median baseline FVIII:C/VWF:Ag ratio in group O individuals did not differ significantly to that in group A individuals (1.33 compared with 1.22, $p > 0.05$). Thus ABO blood group did not influence FVIII:C/VWF:Ag ratios, supporting the notion that expression of ABO blood group antigens on VWF does not impact synthesis or secretion.

6.2.3 Contribution of enhanced VWF clearance to "low VWF levels" in a patient cohort.

In the Weibel-Palade bodies VWF:pp remains non-covalently associated with VWF and the two proteins are found in a 1:1 stoichiometric ratio. Following secretion into plasma, VWF:pp circulates at a concentration of ~1 μg/ml with a half-life of 2 to 3 hours, whereas mature VWF circulates at ~10 μg/ml with a plasma half-life of 8-12 hours. Due to the discordant clearance rate, the ratio between VWF:pp and VWF:Ag can be used as a marker for altered VWF clearance.

By definition, 1ml of normal plasma contains 1U of VWF:pp and 1U of VWF:Ag with a VWF:pp/VWF:Ag ratio of 1. Consequently, an increased ratio (>2.2) has been described as being indicative of enhanced VWF clearance (Haberichter et al. 2006; Eikenboom et al. 2013). To determine if enhanced VWF clearance was responsible for the reduction in VWF plasma levels in the 'low VWF' cohort, VWF:pp and VWF:Ag plasma levels were measured by ELISA. VWF:pp and VWF:Ag concentrations in plasma were then expressed as a ratio. Since VWF:pp/VWF:Ag varies between blood groups, with higher ratios observed for blood group O compared with non-O individuals (Haberichter et al. 2006), VWF:pp/VWF:Ag ratios were also analyzed following stratification for ABO blood group.

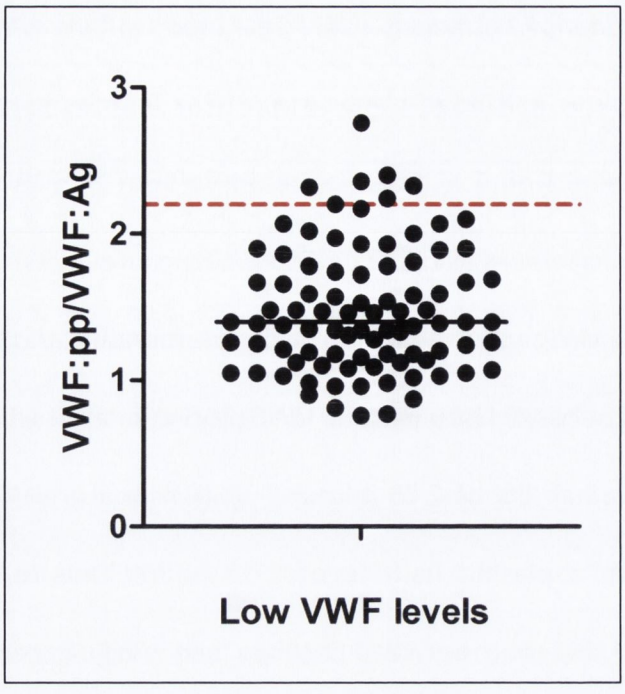


Figure 6.13 VWF:pp/VWF:Ag ratios in patients with "low VWF levels".
The horizontal black line indicates median values (n=89). The dashed red line represents the upper limit of the normal range for VWF:pp/VWF:Ag (2.2).

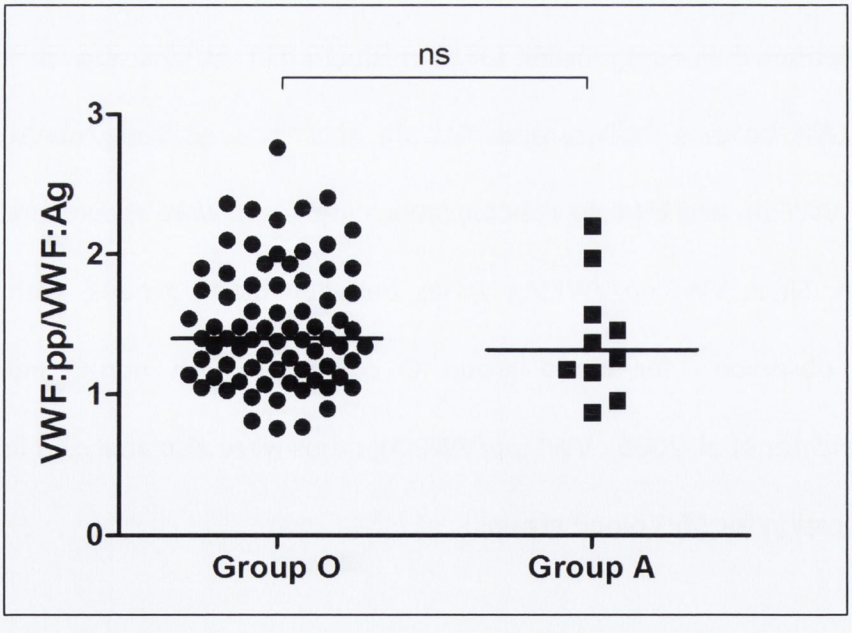


Figure 6.14 Influence of ABO blood group on VWF:pp/VWF:Ag ratios in patients with "low VWF" plasma levels.
The black horizontal line indicates median values.

The median value for VWF:pp/VWF:Ag was 1.40 (range: 0.76 – 2.36). This value is in agreement with a median VWF:pp/VWF:Ag value of 1.60 (range: 1.3 - 1.9) that was previously reported in a cohort of mutation negative Type 1 patients (Eikenboom et al. 2013). Of the eighty nine individuals for whom VWF:pp/VWF:Ag was determined, six individuals displayed VWF:pp/VWF:Ag that were slightly elevated above the upper limit of the normal range. Patients 7, 30, 35, 37, 61 and 70 had base-line VWF:pp/VWF:Ag ratios of 2.76, 2.32, 2.40, 2.36, 2.24, and 2.33 respectively. However, enhanced VWF clearance, at least as assessed by VWF:pp/VWF:Ag ratios does not appear to constitute a major pathogenic mechanism leading to reduced VWF:Ag levels in this particular patient cohort. This is in agreement with the previous observation that elevated VWF:pp/VWF:Ag ratios are more likely to be found in individuals with VWF plasma levels <0.30U/mL (Haberichter et al. 2008).

Interestingly ABO blood group did not influence VWF:pp/VWF:Ag as the median VWF:pp/VWF:Ag value for group O individuals was not significantly different to that of group A individuals (1.40 compared with 1.32, $p > 0.05$). This is surprising given that VWF:pp/VWF:Ag values are known to differ between the groups (Haberichter et al. 2006). However, this may be due to the fact that the group A data set comprised a relatively small number of individuals (n=10).

6.2.4 Responsiveness to DDAVP in "low VWF cohort".

To further investigate the pathogenic mechanisms in the "low VWF cohort" DDAVP trails results were examined in ten patients. In particular, VWF:Ag, VWF:pp and FVIII:C response kinetics were investigated in detail. Given that 1ml of normal plasma contains 1 unit of VWF:Ag and 1 unit of FVIII:C, data pertaining to FVIII:C are reported in units (table 6.11). However, since VWF:Ag and VWF:pp are stored and secreted in a 1:1 stoichiometric ratio, VWF:pp data are reported in molar terms (table 6.12). In all ten patients, approximate equimolar release of VWF:Ag and VWF:pp was observed. Moreover, significant fall-off in VWF:pp plasma levels was observed over the 4 hour time-course which is consistent with the 2-3 hour half-life of the propeptide.

Clinically useful post-DDAVP VWF:Ag levels were observed in all patients in the "low VWF" cohort. However, on the basis of their DDAVP response pattern, the ten patients were subsequently divided into 3 distinct subgroups. Subgroup-1 displayed a good response (~3-fold elevation over baseline) to DDAVP, but reduced post-DDAVP VWF:Ag survival (VWF:Ag $t_{1/2}$ < 8 hours). Subgroup-2 displayed a poor response to DDAVP treatment (<3-fold elevation over baseline) and finally, subgroup-3 demonstrated an apparently normal response.

Patient Number	ABO Blood Group	VWF:Ag Baseline (U/mL)	FVIII:C Baseline (IU/mL)	FVIII:C/ VWF:Ag Baseline	VWF:Ag 1 hour post (Increase above baseline) (U/mL)	FVIII:C 1 hour post (Increase above baseline) (U/mL)	VWF:Ag 1 hour post (Fold increase over baseline)	FVIII:C 1 hour post (Fold increase over baseline)	VWF:Ag $t_{1/2}$ (Hours)	FVIII:C $t_{1/2}$ (Hours)
19	O	0.43	0.77	1.37	1.25	1.19	3.9	3.0	4.8	7.2
28	O	0.47	0.6	1.28	0.42	0.77	2.0	2.3	6.7	4.0
43	O	0.54	1.06	1.96	0.43	0.54	1.8	1.5	11.5	3.7
50	O	0.47	0.72	1.53	0.67	0.89	2.4	2.2	3.8	2.1
60	O	0.4	0.68	1.7	0.77	0.72	2.9	2.1	4.5	2.6
64	O	0.47	0.68	1.45	1.00	1.03	3.1	2.5	3.8	2.3
79	O	0.54	0.28	0.52	1.16	0.49	3.2	2.8	6.6	3.4
81	A	0.45	0.66	1.47	1.00	0.92	3.2	2.4	10.6	34.69*
82	O	0.57	0.75	1.32	1.45	1.24	3.5	2.7	15.6	6.1
88	A	0.64	0.77	1.2	1.13	1.01	2.8	2.3	12.1	6.0

Table 6.10 DDAVP response kinetics for VWF:Ag and FVIII:C in patients with low VWF levels.

Patient Number	ABO Blood Group	VWF:Ag Baseline (nm)	VWF:pp Baseline (nm)	VWF:pp/VWF:Ag Baseline	VWF:Ag 1 hour post (Increase above baseline) (nm)	VWF:pp 1 hour post (Increase above baseline) (nm)	VWF:Ag 1 hour post (Fold increase over baseline)	VWF:pp 1 hour post (Fold increase over baseline)	VWF:Ag $t_{1/2}$ (Hours)	VWF:pp $t_{1/2}$ (Hours)
19	O	17.2	7.4	0.43	50.0	47.1	3.9	7.4	4.8	1.8
28	O	18.0	5.3	0.29	17.6	15.8	2.0	4.0	6.7	2.6
43	O	21.6	6.4	0.30	17.2	15.7	1.8	3.5	11.5	2.3
50	O	18.8	7.9	0.42	26.8	20.0	2.4	3.5	3.8	2.7
60	O	16.0	4.9	0.30	30.8	28.1	2.9	6.8	4.5	2.0
64	O	18.8	12.1	0.64	40.0	55.1	3.1	5.6	3.8	1.2
79	O	21.6	6.6	0.31	46.4	39.3	3.2	6.7	6.6	2.3
81	A	18.0	7.4	0.41	40.0	30.0	3.2	5.0	10.6	2.9
82	O	22.8	7.5	0.33	58.0	54.0	3.5	8.2	15.6	1.7
88	A	25.6	10.9	0.43	45.2	61.2	2.8	6.6	12.1	4.3

Table 6.11 DDAVP response kinetics for VWF:Ag and VWF:pp in patients with low VWF levels.

6.2.4.1 DDAVP response kinetics in the low VWF cohort: subgroup-1.

The first subgroup (n = 4) was composed of patients with low VWF levels who responded well to DDAVP treatment but had reduced post-DDAVP VWF:Ag half-lives (table 6.13). Good peak plasma VWF:Ag responses (> 3-FOLD over baseline) were observed following DDAVP for Patient 19, Patient 60, Patient 64 and Patient 79. However, these increases in plasma VWF:Ag levels were not sustained, as VWF:Ag half-lives were significantly reduced. Importantly, each of the four patients in this particular subgroup with reduced VWF half-lives had blood group O.

	VWF:Ag response (fold increase over baseline)	VWF:Ag $t_{1/2}$ (hours)
Patient 19	3.9	4.8
Patient 60	2.9	4.5
Patient 64	3.1	3.8
Patient 79	3.2	6.6

Table 6.12 DDAVP response and post DDAVP VWF:Ag half-lives – subgroup 1.

DDAVP Time point	Absolute VWF:Ag (U/mL)	Absolute FVIII:C (U/mL)	Absolute VWF:Ag (nm)	Absolute VWF:pp (nm)
T_0	0.43	0.59	17.2	7.4
T_1	1.68	1.78	67.2	54.5
T_2	1.47	1.62	58.8	46.9
T_3	1.38	1.64	55.2	38.2
T_4	1.23	1.44	49.2	13

Table 6.13 Absolute post-DDAVP VWF:Ag, VWF:pp and FVIII:C values for Patient 19.

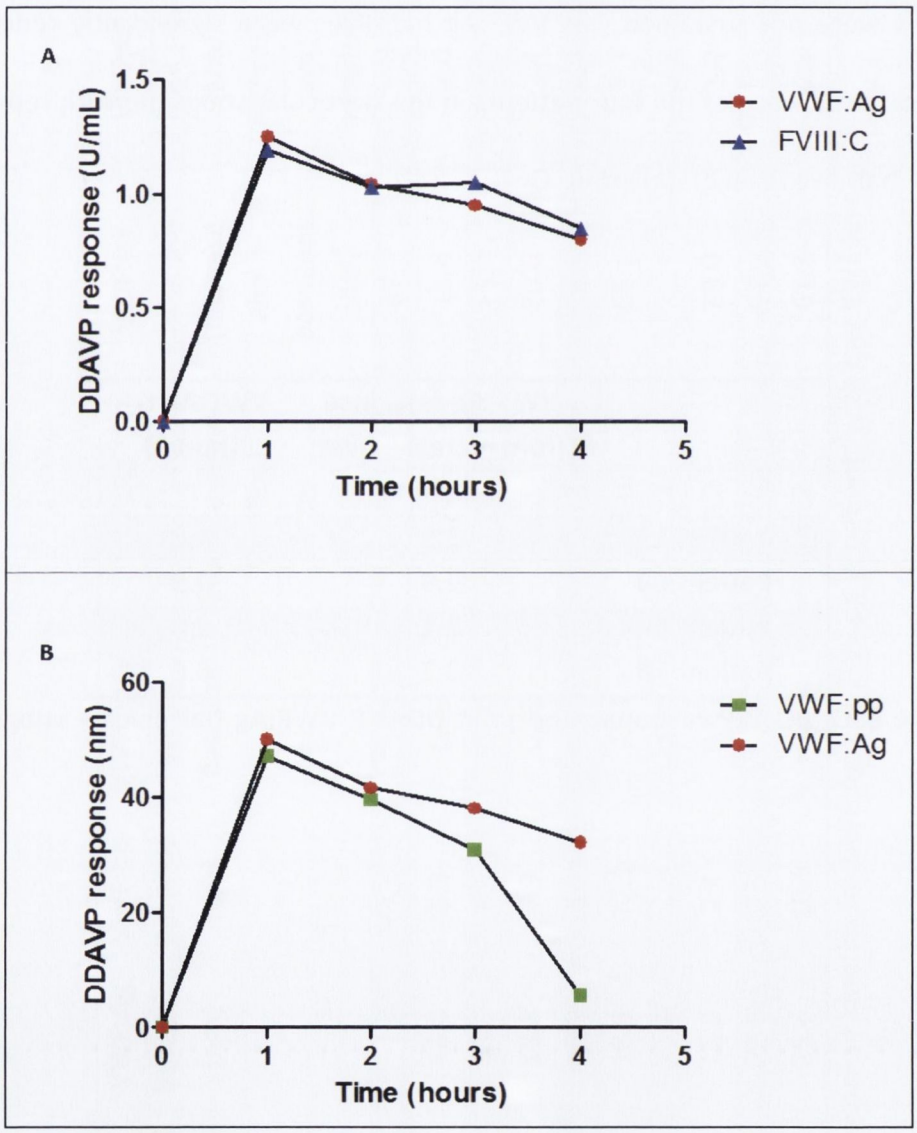


Figure 6.15 DDAVP response for VWF:Ag, VWF:pp and FVIII:C in patient 19 with "low VWF levels".

A) Time-course of post-DDAVP VWF:Ag and FVIII:C response B) Time-course of post-DDAVP VWF:Ag and VWF:pp response.

DDAVP Time point	Absolute VWF:Ag (U/mL)	Absolute FVIII:C (U/mL)	Absolute VWF:Ag (nm)	Absolute VWF:pp (nm)
T_0	0.4	0.68	16	4.85
T_1	1.17	1.4	46.8	32.99
T_2	1.12	1.28	44.8	27.93
T_3	1.01	1.14	40.4	18.56
T_4	0.87	0.98	34.8	14.85

Table 6.14 Absolute post-DDAVP VWF:Ag, VWF:pp and FVIII:C values for Patient 60.

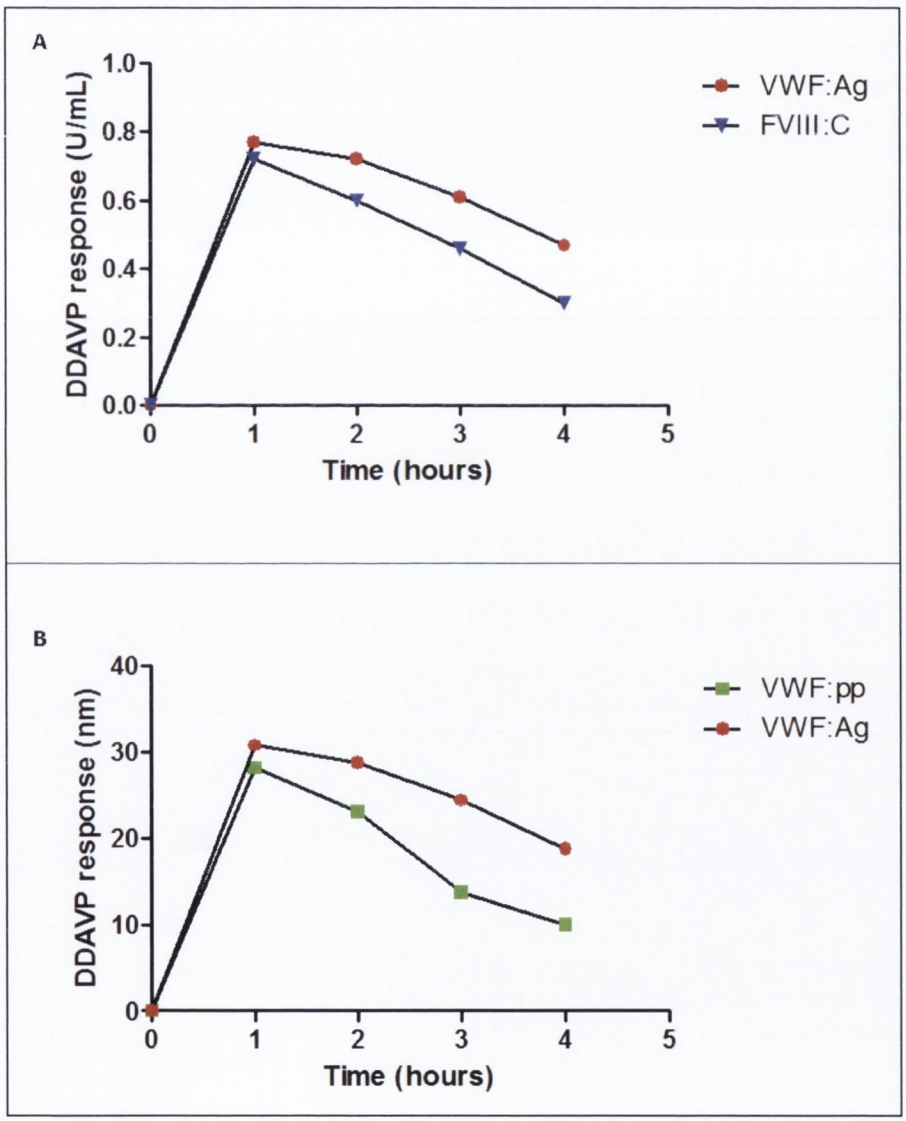


Figure 6.16 DDAVP response for VWF:Ag, VWF:pp and FVIII:C in patient 60 with "low VWF levels".

A) Time-course of post-DDAVP VWF:Ag and FVIII:C response B) Time-course of post-DDAVP VWF:Ag and VWF:pp response.

DDAVP Time point	Absolute VWF:Ag (U/mL)	Absolute FVIII:C (U/mL)	Absolute VWF:Ag (nm)	Absolute VWF:pp (nm)
T_0	0.47	0.68	18.8	12.06
T_1	1.47	1.71	58.8	67.11
T_2	1.38	1.34	55.2	41.03
T_3	1.14	1.23	45.6	27.32
T_4	1.06	1.11	42.4	23.71

Table 6.15 Absolute post-DDAVP VWF:Ag, VWF:pp and FVIII:C values for Patient 64.

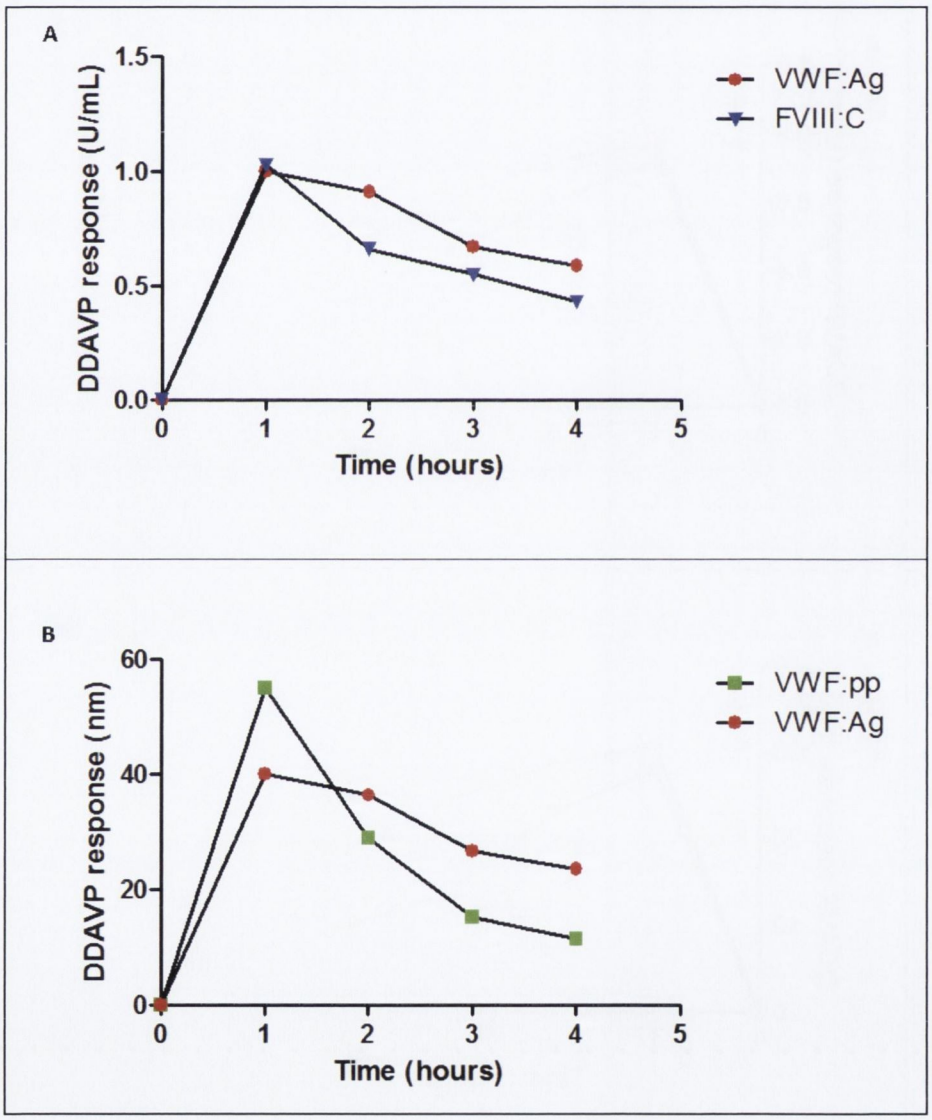


Figure 6.17 DDAVP response for VWF:Ag, VWF:pp and FVIII:C in patient 64 with "low VWF levels".

A) Time-course of post-DDAVP VWF:Ag and FVIII:C response B) Time-course of post-DDAVP VWF:Ag and VWF:pp response.

DDAVP Time point	Absolute VWF:Ag (U/mL)	Absolute FVIII:C (U/mL)	Absolute VWF:Ag (nm)	Absolute VWF:pp (nm)
T_0	0.54	0.28	21.6	6.6
T_1	1.7	0.77	68	45.88
T_2	1.66	0.72	66.4	43.71
T_3	1.42	0.62	56.8	25.15
T_4	1.43	0.54	57.2	23.09

Table 6.16 Absolute post-DDAVP VWF:Ag, VWF:pp and FVIII:C values for Patient 79.

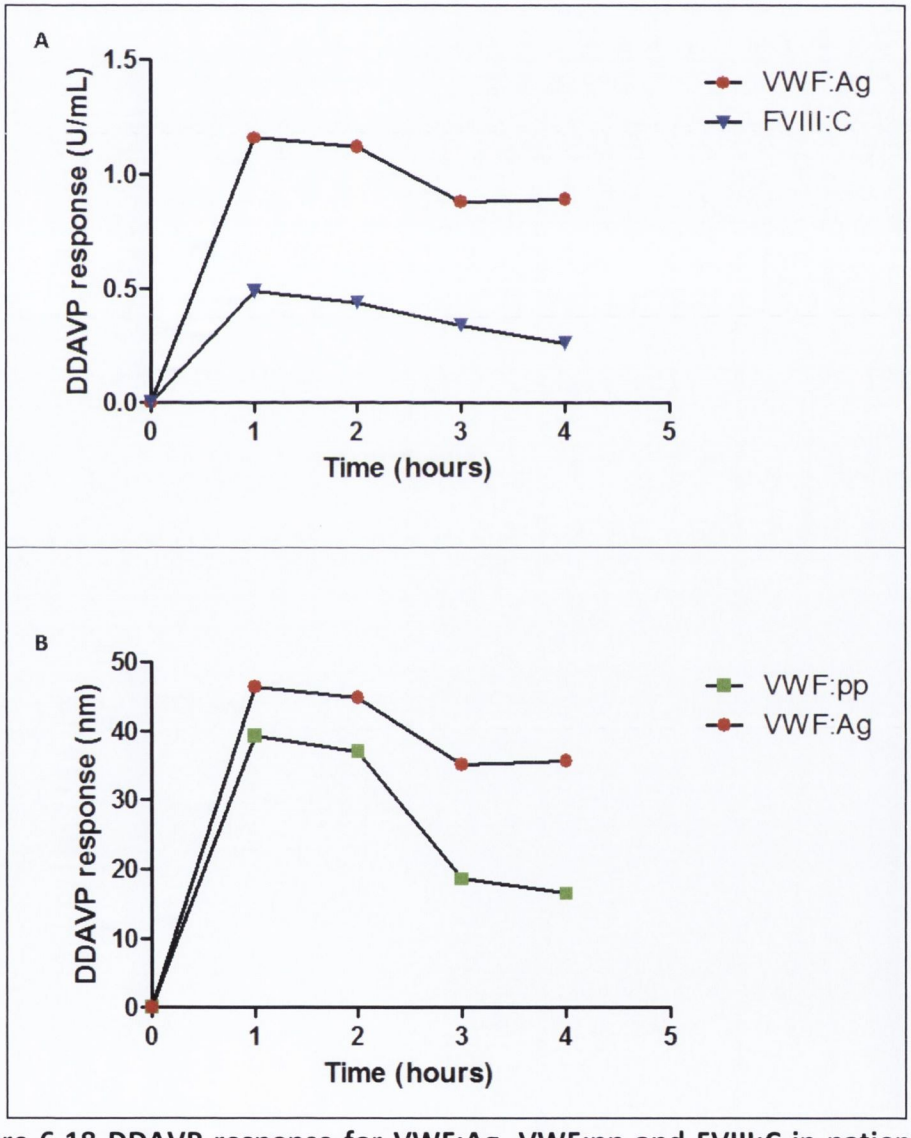


Figure 6.18 DDAVP response for VWF:Ag, VWF:pp and FVIII:C in patient 79 with "low VWF levels".

A) Time-course of post-DDAVP VWF:Ag and FVIII:C response B) Time-course of post-DDAVP VWF:Ag and VWF:pp response.

The increase in plasma VWF:Ag levels observed following DDAVP in this patient subgroup are indicative of normal VWF synthesis, storage and secretion. Consequently, reduced VWF synthesis and/or secretion do not appear contribute to the pathophysiology of low VWF levels in these patients. However, despite the fact that normal steady state VWF:pp/VWF:Ag ratios were reported for these patients, the data suggest that increased VWF:Ag clearance may contribute to their reduced plasma VWF:Ag levels. This is also consistent with the observation that all of the patients in this particular subgroup had blood group O, as group O individuals have significantly reduced VWF plasma levels due to increased clearance of group O VWF.

Though large increases in VWF:pp/VWF:Ag ratio have been reported in cases where VWF:Ag survival in plasma is dramatically reduced, these findings suggest that VWF:pp/VWF:Ag ratio may not constitute a sufficiently sensitive marker for detection of more minor reductions in VWF half-life. This is in keeping with the observation that although blood group O individuals have significantly shorter VWF half-lives compared with group AB individuals, the plasma VWF:pp/VWF:Ag ratios for both groups lie within the limits of the normal range (Haberichter et al. 2006). Therefore, while markedly enhanced VWF clearance does not constitute a major pathogenic mechanism in the low VWF cohort, the above data suggest that less marked but significantly increased VWF clearance may still be an important aetiology underlying the reduced plasma VWF:Ag levels observed in some patients with 'low VWF' levels.

6.2.4.2 DDAVP response kinetics in the low VWF cohort: subgroup-2.

The second subgroup (n = 3) consisted of patients who demonstrated a relatively poor increase in plasma VWF:Ag levels following DDAVP administration. For Patients 28, 43 and 50 relative increases of 2.0-, 1.8- and 2.4-fold in plasma VWF:Ag levels were recorded following DDAVP treatment compared with increases of up to 3.9-fold in other patients in the low VWF cohort. Poor relative increases in post-DDAVP VWF:pp levels were also observed.

	VWF:Ag response (fold increase over baseline)	VWF:Ag $t_{1/2}$ (hours)
Patient 28	2.0	6.7
Patient 43	1.8	11.5
Patient 50	2.4	3.8

Table 6.17 DDAVP response and post-DDAVP VWF:Ag half-lives – subgroup 2.

DDAVP Time point	Absolute VWF:Ag (U/mL)	Absolute FVIII:C (U/mL)	Absolute VWF:Ag (nm)	Absolute VWF:pp (nm)
T_0	0.47	0.6	18	5.25
T_1	0.89	1.37	35.6	21
T_2	0.86	1.31	34.4	17.11
T_3	0.81	1.18	32.4	13.51
T_4	0.78	1.04	31.2	13.19

Table 6.18 Absolute post-DDAVP values for Patient 28.

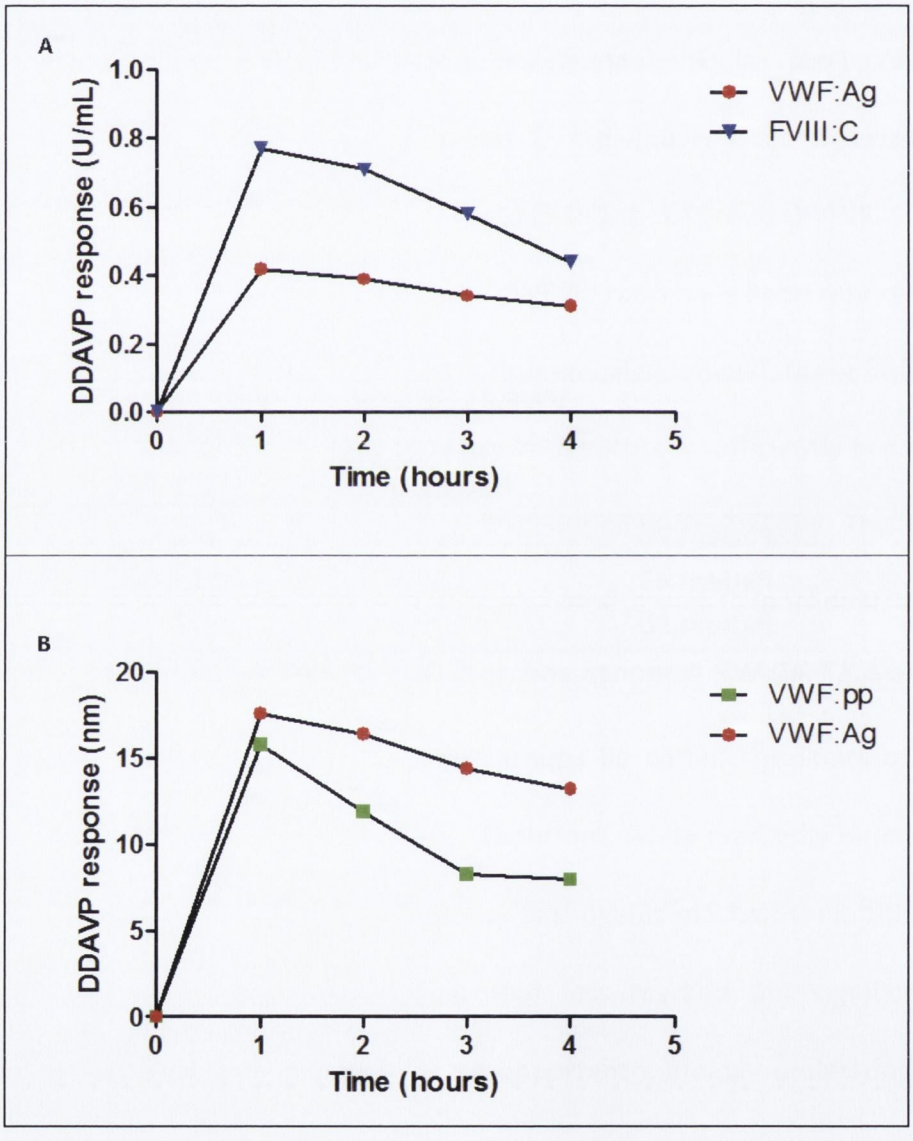


Figure 6.19 DDAVP response for VWF:Ag, VWF:pp and FVIII:C in patient 28 with "low VWF levels".

A) Time-course of post-DDAVP VWF:Ag and FVIII:C response B) Time-course of post-DDAVP VWF:Ag and VWF:pp response.

DDAVP Time point	Absolute VWF:Ag (U/mL)	Absolute FVIII:C (U/mL)	Absolute VWF:Ag (nm)	Absolute VWF:pp (nm)
T_0	0.54	1.06	21.6	6.39
T_1	0.97	1.6	38.8	22.06
T_2	0.93	1.5	37.2	16.28
T_3	0.91	1.41	36.4	14.9
T_4	0.9	1.38	36	13.09

Table 6.19 Absolute post-DDAVP values for Patient 43.

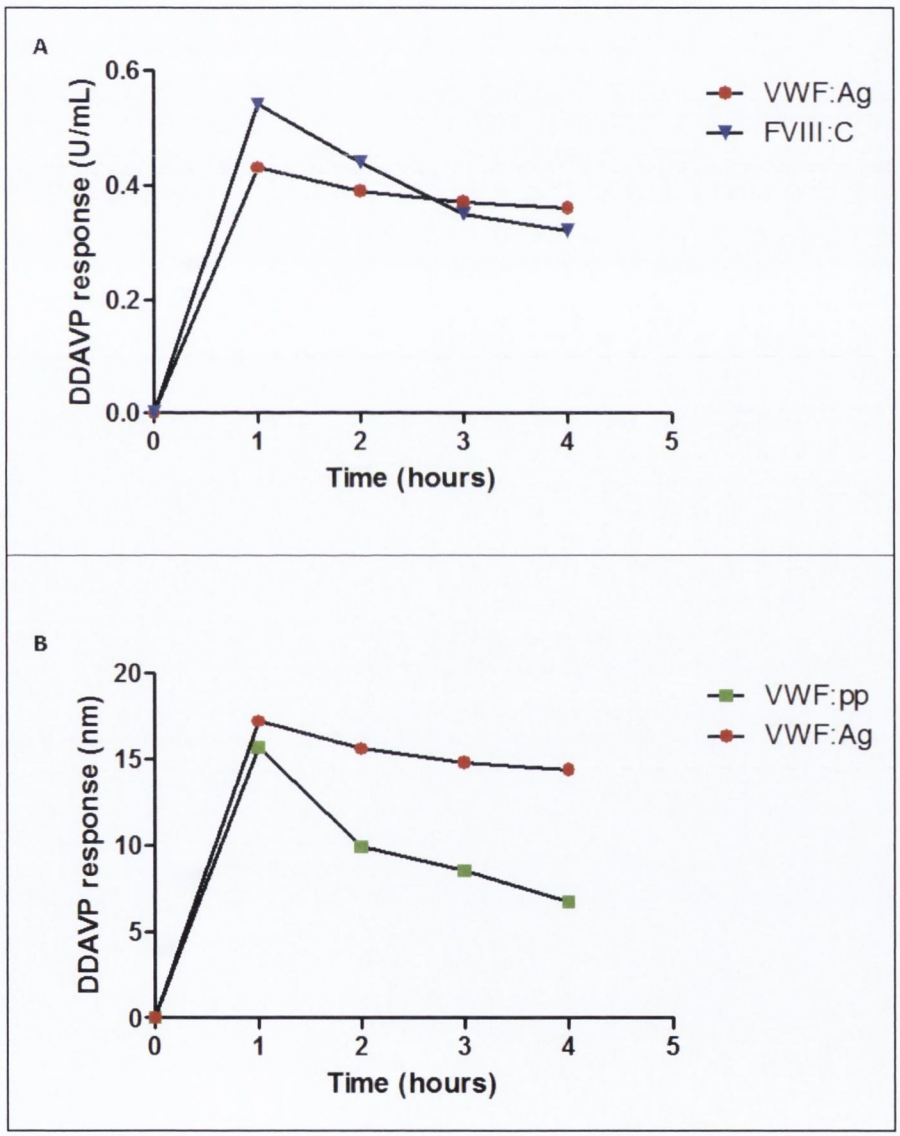


Figure 6.20 DDAVP response for VWF:Ag, VWF:pp and FVIII:C in patient 43 with "low VWF levels".

A) Time-course of post-DDAVP VWF:Ag and FVIII:C response B) Time-course of post-DDAVP VWF:Ag and VWF:pp response.

DDAVP Time point	Absolute VWF:Ag (U/mL)	Absolute FVIII:C (U/mL)	Absolute VWF:Ag (nm)	Absolute VWF:pp (nm)
T_0	0.47	0.72	18.8	7.94
T_1	1.14	1.61	45.6	27.84
T_2	1.03	1.42	41.2	21.13
T_3	0.95	1.26	38	29.69
T_4	0.85	1.15	35	17.6

Table 6.20 Absolute post-DDAVP values for Patient 50.

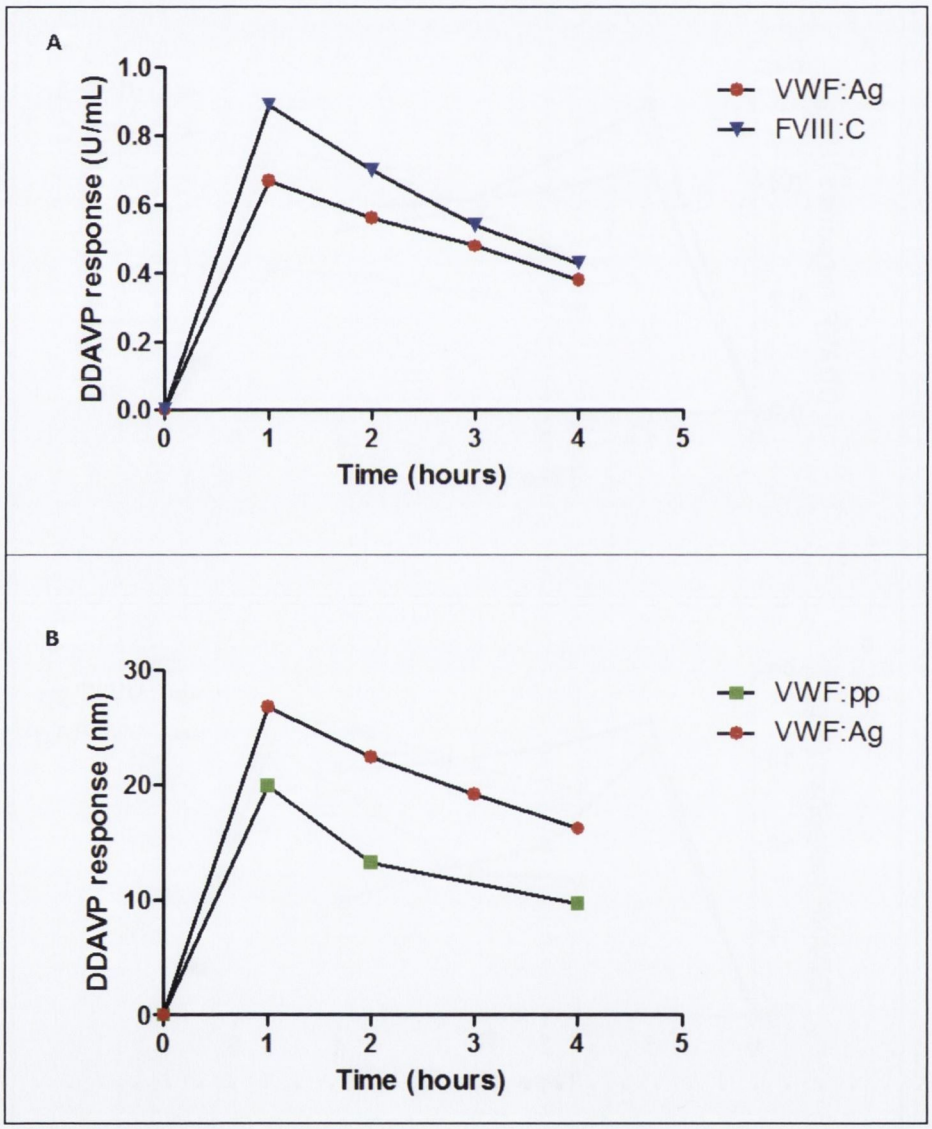


Figure 6.21 DDAVP response for VWF:Ag, VWF:pp and FVIII:C in patient 50 with "low VWF levels".

A) Time-course of post-DDAVP VWF:Ag and FVIII:C response B) Time-course of post-DDAVP VWF:Ag and VWF:pp response.

The releasable pool of VWF is stored within the WPBs of the endothelium. Secretagogue DDAVP activates cAMP-mediated signalling to release VWF from WPB storage which typically results in a 3-5 fold increase in circulating VWF:Ag. Consequently, DDAVP responses of a lesser magnitude are suggestive of synthetic or secretory VWF defects. In this context, these data suggest that reduced VWF synthesis and/or secretion may also contribute to the reduction in circulating VWF:Ag levels in this subgroup of patients with 'low VWF' levels.-2.

In addition, to a poor DDAVP response, reduced post-DDAVP VWF:Ag survival was also observed in Patient 28 and Patient 50 who displayed VWF:Ag half-lives of 6.7 hours and 3.8 hours respectively. These data suggest that increased VWF clearance also contributes to the pathophysiology of reduced VWF plasma levels in these patients. In contrast, normal post-DDAVP survival was observed for Patient 43 suggesting that reduced synthesis and/or secretion is a more dominant pathogenic mechanism in this patient. Interestingly, Patient 43 appeared to have an inconsistently low FVIII:C half-life when compared to the VWF:Ag half-life. Though the absolute increase in FVIII:C exceeded that of VWF:Ag following DDAVP administration, the increase was not maintained over time.

Despite these results observed following DDAVP administration suggesting that reduced VWF synthesis and/or secretion is important in the aetiology of low VWF levels in some patients, it is interesting that steady-state FVIII:C/VWF:Ag ratios in these patients were within the normal range. Consequently, these data suggest that like VWF:pp/VWF:Ag, steady-state FVIII:C/VWF:Ag may not be sufficiently sensitive to detect more subtle

changes in VWF biology. This is consistent with the observation that the majority of mutation negative Type 1 index cases in the MCMDM-1 cohort had FVIII:C/VWF:Ag and VWF:pp/VWF:Ag ratios within the normal range (Eikenboom et al. 2013).

6.2.4.3 DDAVP response kinetics in the low VWF cohort: subgroup-3.

The third subgroup (n = 3) included patients with a robust response to DDAVP and normal post-DDAVP VWF half-lives. Patient 81, Patient 82 and Patient 88 demonstrated VWF:Ag half-lives of 10.6 hours, 15.6 hours and 12.1 hours respectively, values that are in agreement with the reported VWF:Ag half-life of 8-12 hours (Borchiellini et al. 1996).

	VWF:Ag response (fold increase over baseline)	VWF:Ag $t_{1/2}$ (hours)
Patient 81	3.2	10.6
Patient 82	3.5	15.6
Patient 88	2.8	12.1

Table 6.21 DDAVP response and post-DDAVP VWF:Ag half-lives – subgroup 3.

DDAVP Time point	Absolute VWF:Ag (U/mL)	Absolute FVIII:C (U/mL)	Absolute VWF:Ag (nm)	Absolute VWF:pp (nm)
T_0	0.45	0.66	18	7.42
T_1	1.45	1.58	58	37.32
T_2	1.33	1.68	53.2	33.3
T_3	1.3	1.55	52	25.77
T_4	1.27	1.56	50.8	21.98

Table 6.22 Absolute post-DDAVP values for Patient 81.

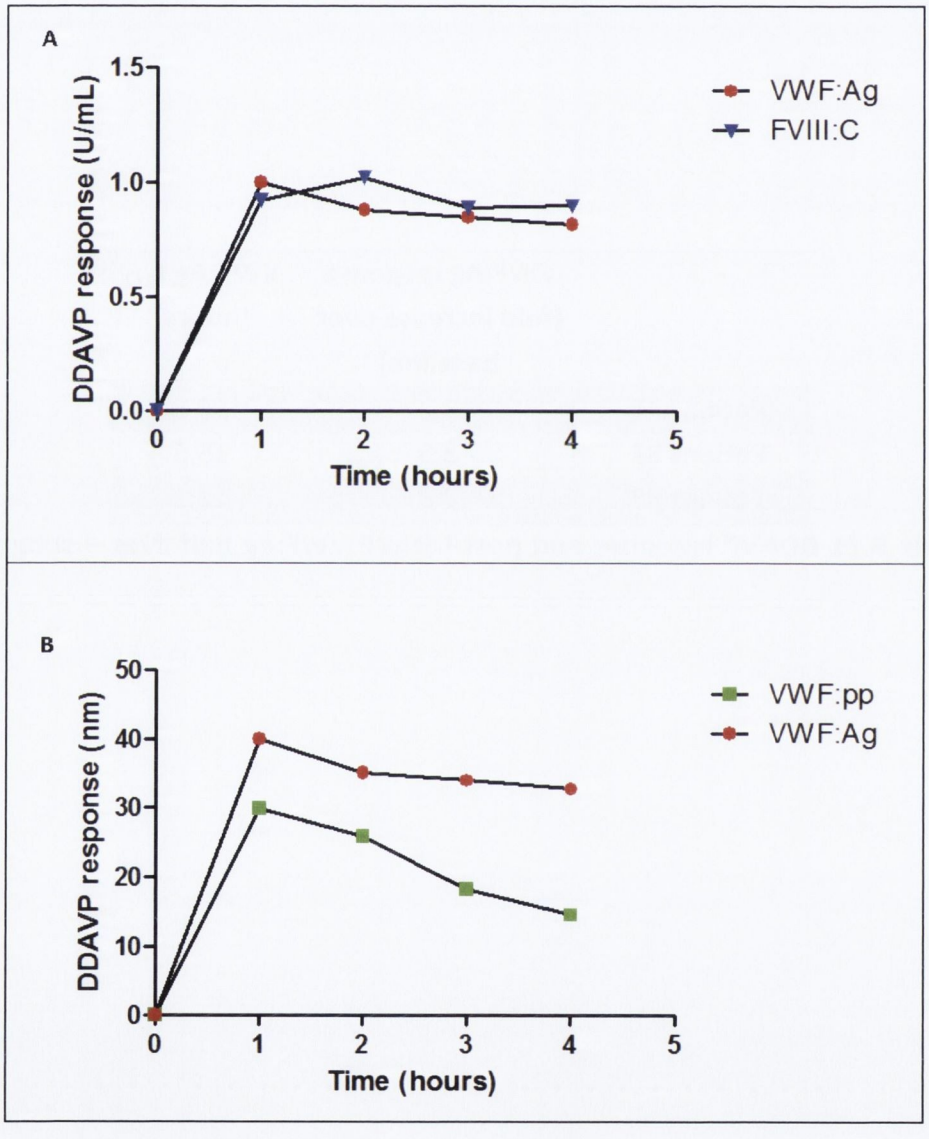


Figure 6.22 DDAVP response for VWF:Ag, VWF:pp and FVIII:C in patient 81 with "low VWF levels".

A) Time-course of post-DDAVP VWF:Ag and FVIII:C response B) Time-course of post-DDAVP VWF:Ag and VWF:pp response.

DDAVP Time point	Absolute VWF:Ag (U/mL)	Absolute FVIII:C (U/mL)	Absolute VWF:Ag (nm)	Absolute VWF:pp (nm)
T_0	0.57	0.75	22.8	7.53
T_1	2.02	1.99	80.8	61.55
T_2	1.91	1.87	76.4	40.21
$T_{2.5}$	1.94	1.79	77.6	38.04

Table 6.23 Absolute post-DDAVP values for Patient 82.

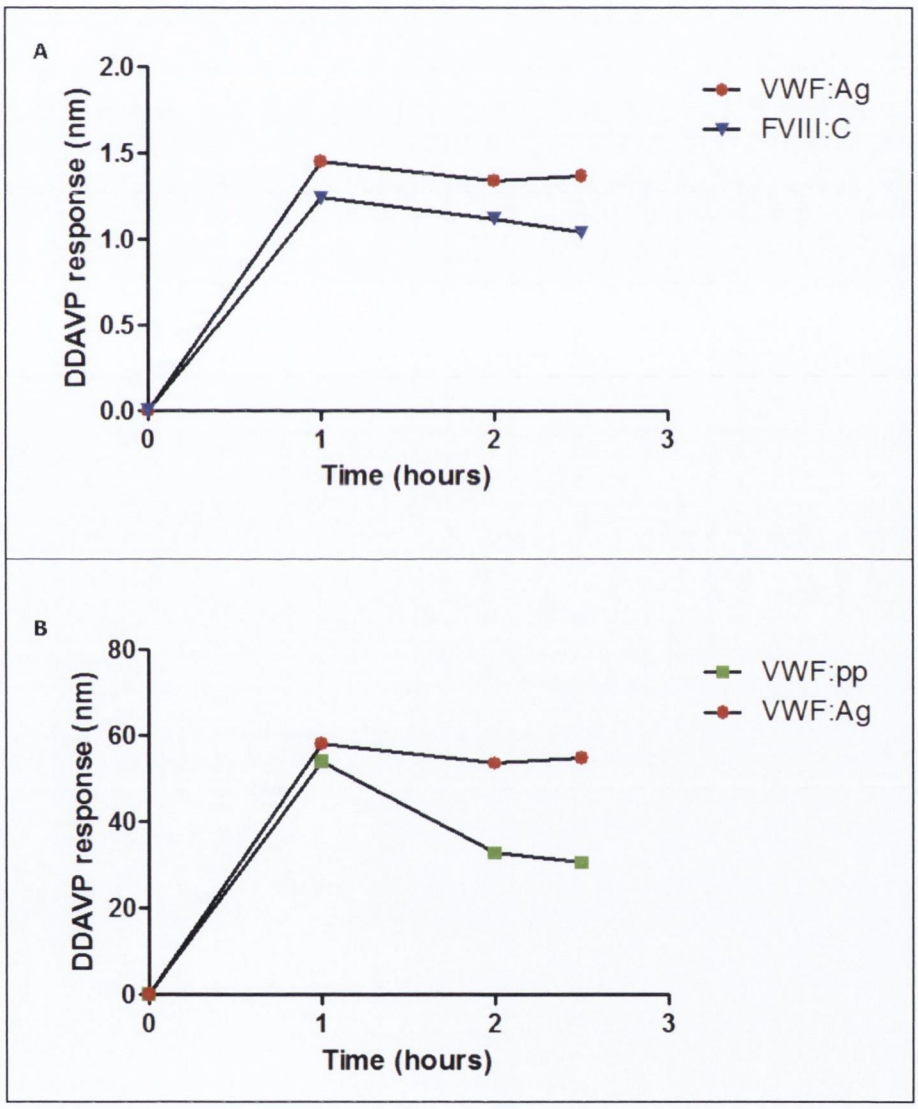


Figure 6.23 DDAVP response for VWF:Ag, VWF:pp and FVIII:C in patient 82 with "low VWF levels".

A) Time-course of post-DDAVP VWF:Ag and FVIII:C response B) Time-course of post-DDAVP VWF:Ag and VWF:pp response.

DDAVP Time point	Absolute VWF:Ag (U/mL)	Absolute FVIII:C (U/mL)	Absolute VWF:Ag (nm)	Absolute VWF:pp (nm)
T_0	0.64	0.77	25.60	10.90
T_1	1.77	1.78	70.80	72.10
T_2	1.75	1.62	70.00	67.60
T_3	1.7	1.64	68.00	54.50
T_4	1.58	1.44	63.20	49.50

Table 6.24 Absolute post-DDAVP values for Patient 88.

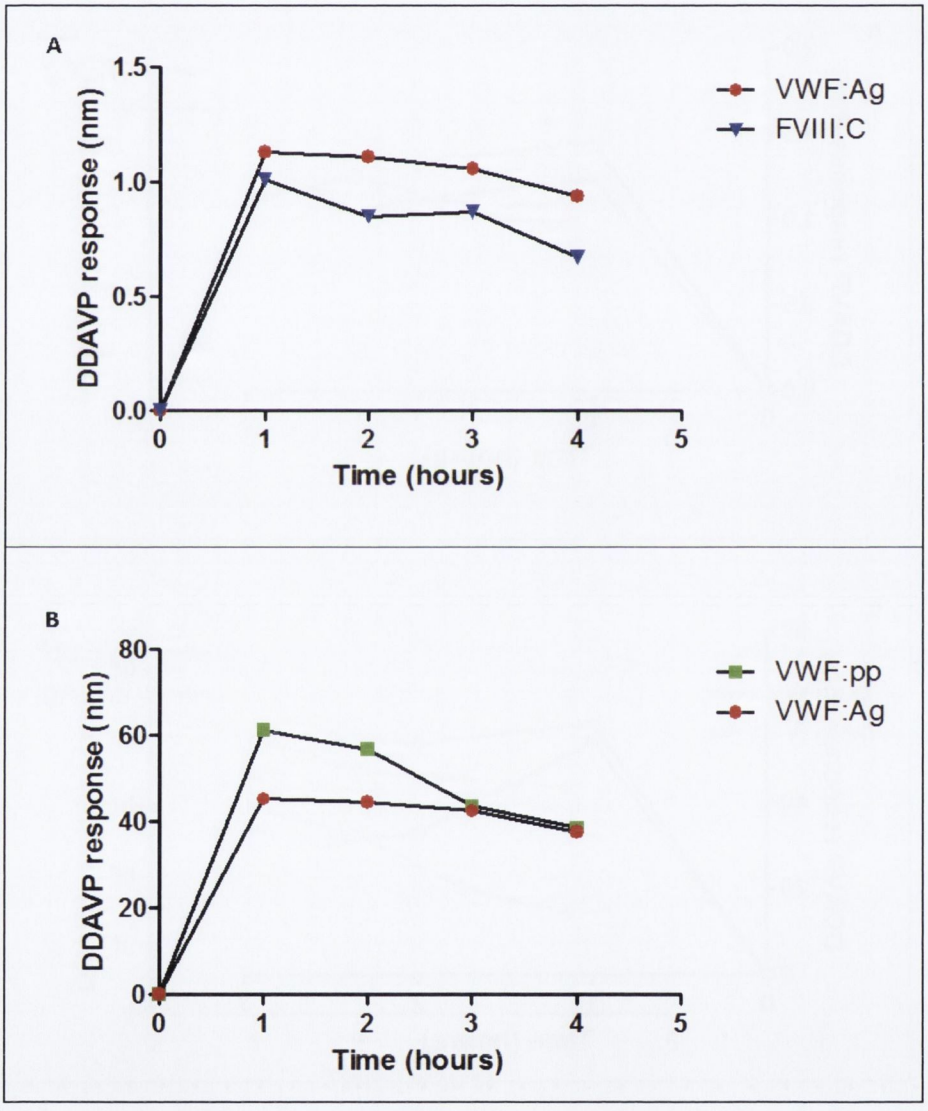


Figure 6.24 DDAVP response for VWF:Ag, VWF:pp and FVIII:C in patient 88 with "low VWF levels".

A) Time-course of post-DDAVP VWF:Ag and FVIII:C response B) Time-course of post-DDAVP VWF:Ag and VWF:pp response.

Both VWF and FVIII parameters of Patients 81, 82 and 88 responded well to DDAVP treatment. Baseline VWF:Ag values were increased by ~3-fold, while baseline FVIII:C values were increased by ~2.5-fold. This robust response suggests adequate VWF synthesis and subsequent storage within WPBs for regulated release. Coupled with the normal VWF:Ag half-life values observed in these patients, these data appear to rule out defective VWF synthesis and enhanced VWF clearance as modulators of the low VWF phenotype in these patients. However, since examination of DDAVP response kinetic provides insight only into regulated VWF release, defects in basal or constitutive VWF secretion may contribute to the low steady-state VWF levels observed.

6.3 Discussion

A diagnosis of VWD is difficult to establish in individuals in whom plasma VWF:Ag levels are not markedly reduced, but are near to the lower limit of the normal range. Therefore, the NHLBI have suggested modification of the current ISTH classification criteria to distinguish these individuals from those with true Type 1 VWD.

Importantly, improved understanding of the biological mechanisms that underlie the pathogenesis of Type 1 VWD and "low VWF" levels is essential to develop a better understanding of the relationship between VWF levels and clinical bleeding phenotypes and also to define optimal treatment plans for these groups of patients. Based on the results of this study, the same pathogenic mechanisms that lead to marked reductions in VWF:Ag plasma levels are also responsible for intermediate reductions in plasma VWF:Ag level. Reduced VWF:Ag synthesis and/or secretion and enhanced VWF:Ag clearance were consistently observed in both the Type 1 and "low VWF" cohorts, though genetic modulators of these effects are likely to differ between these two cohorts. In addition, these novel data also indicate that although steady-state FVIII:C/VWF:Ag and VWF:pp/VWF:Ag ratios are clinically useful tools in the Type 1 VWD cohort, they may not be sufficiently sensitive to detect the more subtle synthetic/secretory defects or the less pronounced reductions in VWF:Ag half-life that lead to "low VWF" plasma levels.

6.3.1 Over-representation of female gender in the "low VWF" cohort.

Females outnumbered males by 84% to 16% in the "low VWF" cohort. This is in keeping with several previous studies in which females consistently outnumbered males by at least 2:1 in many VWD populations (Lillicrap 2013). Although the autosomal inheritance of VWD predicts an equal frequency of gene transmission to both genders, low VWF levels are more likely to be diagnosed in females who present with bleeding complications such as menorrhagia, mucocutaneous bleeding, or post-partum bleeding.

6.3.2 Over-representation of blood group O in the "low VWF" cohort.

The ABO blood group locus has long since been established as a critical determinant of plasma VWF:Ag levels. Group O individuals are known to have lower circulating VWF:Ag levels and a significantly shorter VWF:Ag half-life compared with non-O individuals. Since the ABO effect on VWF plasma levels is believed to be the result of differential VWF clearance rather than effects on VWF synthesis, secretion or proteolysis, over-representation of blood group O in the low VWF cohort, suggests that increased VWF clearance is an important pathogenic mechanism in these patients. Though the clearance receptor and mechanisms responsible for mediating this effect are not known, it is likely that polymorphic variation in receptor expression and/or activity contributes to the reduction in VWF:Ag plasma levels in the "low VWF" cohort.

6.3.3 Several pathogenic mechanisms contribute to the "low VWF" phenotype.

Detailed examination of DDAVP response data in the "low VWF cohort" highlights the contribution of several pathogenic mechanisms to this phenotype. While reduced VWF synthesis/secretion or increased VWF clearance (or indeed a combination of both) contributed to the reduction in VWF:Ag levels in the majority of patients, there exists a patient subgroup in which no obvious pathogenic mechanism could be identified. In patient subgroup-3, robust post-DDAVP responses as well normal post-DDAVP VWF:Ag half-lives were observed and appear to exclude a role for reduced VWF synthesis and/or secretion and increased VWF clearance as pathogenic mechanisms in these individuals. Therefore, it seems likely that defects in basal and/or constitutive secretion may contribute to the low VWF:Ag plasma levels in these patients. The involvement of several pathogenic mechanisms underscores the heterogeneity and complexity of the "low VWF" trait. This complexity is further emphasized by the findings of recent GWAS meta-analysis that identified several novel candidate loci other than the *VWF* locus that influence plasma VWF:Ag levels (Smith et al. 2010). The plausibility of these loci playing a role in regulating VWF:Ag levels in the "low VWF" cohort is supported by the observation that two of these loci encode proteins involved in vesicular trafficking and exocytosis (syntaxin-binding protein 5 and syntaxin 2) and three encode lectin (CLEC4M) or scavenger clearance receptors (Scara5 and stab-2). Furthermore direct evidence confirming the involvement of some of these proteins in regulating VWF:Ag levels has recently emerged as

polymorphic variation at the *CLEC4M* locus was shown to significantly contribute to quantitative variation in plasma VWF:Ag levels in humans (Rydz et al. 2013).

Since the relative contribution of genetic loci outside of the VWF gene appears to be more significant in the "low VWF" cohort, polymorphic variation at these novel loci may significantly influence VWF plasma levels in this particular cohort. Indeed, the notion that polymorphic variation in clearance receptor expression or activity of intracellular trafficking proteins appear to be consistent with the underlying pathogenic mechanisms of reduced VWF synthesis/secretion and increased VWF clearance observed in the "low VWF" cohort. Moreover, polymorphic variation at several of these loci may be responsible for the combination of pathogenic mechanisms that is evident in some of these patients.

6.3.4 The biological basis underlying enhanced VWF clearance in VWD Vicenza.

In keeping with previous reports, increased VWF:Ag clearance was found to contribute to the pathophysiology of VWD Vicenza. Though robust relative increases in VWF:Ag levels were observed following DDAVP administration, increased VWF:Ag levels were not sustained over the DDAVP time-course. Interestingly however, despite having the same causal mutation, there is some variation in plasma VWF:FVIII levels amongst patients with VWD Vicenza. Detailed assessment of DDAVP response kinetics suggests

involvement of an additional pathogenic mechanisms in VWD Vicenza, as absolute peak post-DDAVP VWF:Ag levels failed to reach clinically useful levels in some patients. These reduced DDAVP responses suggest that targeting of VWF to WPBs for storage and subsequent regulated release may also be sub-optimal in patients with VWD Vicenza, which is consistent with the findings from previous *in vitro* expression studies (Lenting et al. 2004; Pruss et al. 2011). Cumulatively therefore, these data suggest that while DDAVP may be a viable option for management of minor procedures in specific Vicenza patients, VWF/FVIII containing concentrates are a more appropriate option in many cases.

7.0 Overall conclusions and future studies

7.1 Conclusions

VWF deficiency results in the most common inherited bleeding disorder, von Willebrand disease (VWD) affecting up to 1% of the general population. The most common form of this disease is Type 1 VWD, which results in partial quantitative deficiency of plasma VWF. Up to 75% of all Type 1 cases are caused by single amino acid substitutions in the *VWF* coding sequence that cause defective VWF synthesis, secretion, or clearance. Interestingly however, in up to 30% of Type I cases, no direct link to the *VWF* gene has been confirmed, indicating that other factors such as VWF glycosylation are important

Although substantial progress has been achieved in understanding VWF biology in recent years, the molecular mechanisms responsible for the regulation of VWF plasma levels remain poorly understood. Despite the critical importance of VWF glycan structures, the specific mechanisms through which the glycan effects are mediated remain unknown. In particular, the details of the blood group-dependent differential VWF clearance have yet to be elucidated and the clearance receptor responsible remains unknown. Similarly, the receptors and cell-types responsible for mediating increased clearance of mutant VWF molecules are not known.

This thesis focuses on the role of VWF clearance pathways in the regulation of VWF plasma levels. Moreover, the work presented herein

highlights the critical roles played by both glycosylation and sequence variation. In particular, this work focuses on characterization of the specific glycan determinants responsible for mediating the recently documented physiologically relevant interaction between VWF and galectin-3.

In addition, this thesis also investigates the molecular mechanisms through which single amino acid substitutions cause enhanced VWF clearance in Type 1 VWD. More specifically, the significance of R1205 substitution was studied in an *in vivo* mouse model, in which the critical importance of macrophage-mediated clearance was established. This was further confirmed using a series of *in vitro* THP-1 macrophage binding assays. Finally, the occurrence of enhanced VWF clearance was investigated in two distinct patient cohorts. Analysis of DDAVP response kinetics confirmed enhanced VWF clearance as the predominant pathogenic mechanism in VWD Vicenza and also demonstrated its occurrence as a common pathogenic mechanism in the "low VWF" cohort.

7.1.2 The N-linked and O-linked VWF glycans modulate its interaction with galectin-3.

Modification of VWF glycosylation using specific exoglycosidases demonstrated that both the N-linked and O-linked VWF glycans regulate its interaction with galectin-3. Terminal N-linked sialic acid and ABO(H) blood group antigen expression were of particular importance in this regard. Removal of terminal sialic acid via α2-3,6,8,9 neuraminidase digestion significantly enhanced galectin-3 binding. Conversely, specific removal of O-

linked sialic acid had no effect. Furthermore, galectin-3 displayed significantly enhanced affinity for group AB VWF. However, no significant difference in binding was observed between Group O and Bombay VWF. Consequently, although terminal A (GalNAc) and B (Gal) sugar moieties promote galectin binding, expression of terminal α1-2 fucose residues is not important.

Importantly, VWF multimer composition was also a critical determinant of this interaction as galectin-3 displayed markedly enhanced affinity for higher molecular weight multimers compared with lower molecular weight forms. In addition, a role for the VWF A domains was also established. Modification of A domain conformation with ristocetin markedly enhanced galectin-3 binding. Similarly, introduction of type 2B mutation R1450E also enhanced binding. This most likely resulted from improved access to the complex N-linked glycans of the VWF A2 domain.

7.1.3 R1205 is a critical regulator of VWF clearance.

The novel data presented in this thesis also provide insight into the mechanisms by which sequence variation modulates VWF plasma levels. In particular, the molecular mechanisms underlying enhanced VWF clearance in VWD Vicenza were studied. The results outlined herein demonstrate a critical role for R1205 in modulating VWF clearance as substitution of R1205 with histidine, cysteine or serine resulted in markedly reduced survival of full-length recombinant human VWF in the $VWF^{-/-}$ mouse. Moreover, introduction of these mutations into the D3 domain of the truncated D'A3-VWF fragment

also significantly enhanced clearance. Collectively these data demonstrate that the reduced survival associated with substitution of arginine 1205 is modulated through effects within the D'A3 region. Subsequent clearance studies utilizing A1 domain-deleted VWF (VWF-ΔA1) further demonstrated that these effects are locally mediated within the D'D3 domains. VWF-ΔA1 was cleared at the same rate as WT-VWF suggesting that the VWF A1 domain is not important in the context of VWF clearance. Conversely, VWF-ΔA1 R1205H was cleared significantly faster than VWF-ΔA1. Therefore, the R1205H mutation critically regulates VWF clearance in the absence of the VWF A1 domain demonstrating that enhanced VWF clearance associated with the R1205H mutation is not due to disruption of D'D3 shielding effects on the neighbouring A1 domain.

7.1.4 Substitution of R1205 leads to enhanced macrophage mediated VWF clearance.

Clearance studies in $ASGPR^{-/-}$ mice and WT C57/Bl6 mice demonstrated that VWF-R1205H was cleared at the same rate in both strains. Consequently, these data exclude a role for ASGPR in mediating enhanced clearance of VWF-R1205H. Additional clearance studies in $VWF^{-/-}$ mice demonstrated prolonged survival of VWF-R1205H following macrophage-depletion using clodronate liposomes. Importantly, macrophage-depletion also significantly prolonged the survival of VWF-R1205C and VWF-R1205S. Interestingly, macrophage-depletion inhibited VWF-R1205H clearance to a greater degree than that observed for WT-VWF or the other R1205 variants,

suggesting that macrophage-mediated clearance is the predominant pathway through which VWF-R1205H is cleared.

7.1.5 LRP1 and $\alpha_M\beta_2$ integrins mediate enhanced binding of R1205 variants to THP-1 macrophages.

The novel data describe herein also identify a role for LRP1 and $\alpha_M\beta_2$ integrins in the pathogenesis of VWD Vicenza. Both LRP1 and $\alpha_M\beta_2$ integrins mediated enhanced binding of VWF-R1205H to THP-1 macrophages, and inhibition of either LRP1, or $\alpha_M\beta_2$ integrin was sufficient to attenuate binding. Moreover, no further reduction in macrophage binding was observed when both LRP1 and $\alpha_M\beta_2$ were inhibited. Consequently, LRP1 and $\alpha_M\beta_2$ integrins act as part of a functional surface expressed complex to mediate enhanced VWF-R1205H binding. In addition, these data further confirm the *in vivo* finding that macrophages mediate enhanced VWF-R1205H clearance.

7.1.6 Pathogenic mechanisms contributing to the "low VWF" phenotype.

Detailed examination of DDAVP response data in the "low VWF cohort" highlights the contribution of several pathogenic mechanisms to this phenotype. Reduced VWF synthesis/secretion or increased VWF clearance (or indeed a combination of both) were found to contribute to the reduction in VWF:Ag levels in the majority of patients. The occurrence of increased VWF clearance as a pathogenic mechanism was further emphasized by the over-

representation of blood group O in this patient cohort. Interestingly however, no clear pathogenic mechanism was identifiable in a specific subset of patients, emphasizing the heterogeneity and complexity of the "low VWF" trait.

7.1.7 The biological basis underlying enhanced VWF clearance in VWD Vicenza.

Following examination of DDAVP trial results, enhanced VWF clearance was confirmed as the principle pathogenic mechanism underlying marked reductions in plasma VWF:Ag levels in VWD Vicenza. Remarkably however, considerable variation in the magnitude of the post DDAVP VWF:Ag response was observed in Vicenza patients despite the presence of a common VWF mutation. The suboptimal DDAVP responses observed in some Vicenza patients suggest that reduced VWF secretion may also contribute to the marked reduction in VWF plasma levels in VWD Vicenza.

7.2 Future studies

7.2.1 Characterizing the biochemical basis through which VWF interacts with galectin-3

1. Though galectin-3 is expressed as a soluble lectin it is also abundantly expressed at the macrophage cell surface. Consequently, galectin-3 is a candidate VWF clearance receptor. To investigate a role for galectin-3 in mediating VWF clearance, VWF binding and uptake by THP-1 macrophages and primary monocyte-derived macrophages will be analyzed under galectin-3 blocking conditions. In particular, lactose and monoclonal anti galectin-3 antibodies will be employed.

2. Preliminary data suggest that both ADAMTS13 and FVIII bind galectin-3. Thus the glycan determinants that modulate these interactions will be determined using modified immunosorbent assays following specific glycosidase digestion.

3. To investigate the significance of these interactions, functional assays will be carried out in the presence of galectin-3. These will include static ADAMTS13 cleavage assays and FRETS VWF-73 assays for assessment of ADAMTS13 function as well as one-stage clotting assays for assessment of FVIII activity.

7.2.2 Investigating the molecular mechanisms underlying enhanced VWF clearance in VWD Vicenza.

1. Clearance studies utilizing the D'D3 VWF fragment incorporating the R1205H mutation will provide useful information regarding the mechanism by which the mutation exerts its effects. Comparison of half-life data for D'D3 and D'D3 R1205H will confirm whether the R1205H mutation exerts local conformational effects.

2. Further site-directed mutagenesis and clearance studies will provide additional insight into the critical importance of the arginine residue at position 1205 in the VWF D3 domain. For example, to confirm the specific importance of preserving the positive charge at position 1205, substitution for a lysine residue will be carried out. Conversely, reversing the charge at position 1205 may also be informative. Therefore, substitution for a glutamic acid residue will also be undertaken.

3. Leading on from *in vitro* inhibition studies, hydrodynamic expression of LRP1 inhibitor RAP and $\alpha_M\beta_2$ integrin inhibitor NIF in $VWF^{-/-}$ mice will be undertaken to confirm a role for both these receptors in mediating enhanced VWF-R1205H clearance. *In vivo* inhibition studies will also be useful to delineate the mechanisms and receptors responsible for differential clearance of the VWF-R1205C and VWF-R1205S variants,

particularly given that the mechanisms involved appear to differ to those mediating VWF-R1205H clearance. Additional *in vitro* RAP and NIF inhibition studies utilising these variants may also be beneficial in this regard.

4. To further elucidate a role for $\alpha_M\beta_2$ integrins, removal of the LLG integrin binding motifs located in the VWF D3 and A2 domains will be carried out by site directed mutagenesis. Single and double LLG mutants will be generated in both WT-VWF and VWF-R1205H expression plasmids. Following expression and purification, clearance will be studied in $VWF^{-/-}$ mice.

7.2.3 Investigating the pathogenic mechanisms responsible for "Low VWF levels" in a patient cohort

1. To gain further insight into the relative roles of decreased VWF synthesis and enhanced VWF clearance in modulating reduced plasma VWF levels, platelet-VWF content will be determined for each patient and the ratio of platelet-VWF to plasma-VWF will be investigated.

2. In patients in whom impaired VWF synthesis appears to be the dominant pathogenic mechanism leading to low VWF levels, the

underlying biology will be further investigated by analyzing VWF synthesis and secretion from blood outgrowth endothelial cells (BOECs). In addition, in patients in whom the predominant pathogenic mechanism appears to be enhanced VWF clearance, the molecular mechanisms responsible will be further investigated by analyzing VWF binding and uptake by primary monocytes isolated from the patient.

3. Systematic analysis of the VWF promoter region, exons comprising the coding region, and flanking intronic regions will be carried out to determine whether common single nucleotide polymorphisms (SNPs) in the *VWF* gene influence VWF plasma levels Furthermore, genotyping of additional select loci known to contribute to the regulation of VWF plasma levels will also be undertaken. These will include *CLEC4M, STAB2, SCARA5, STXBP5* and *STX2.*

4. Finally, to assess the contribution of glycan variation to the pathophysiology of low VWF levels, glycoprofiling via lectin microarray will be carried out using purified VWF isolated from patients within the low VWF cohort.

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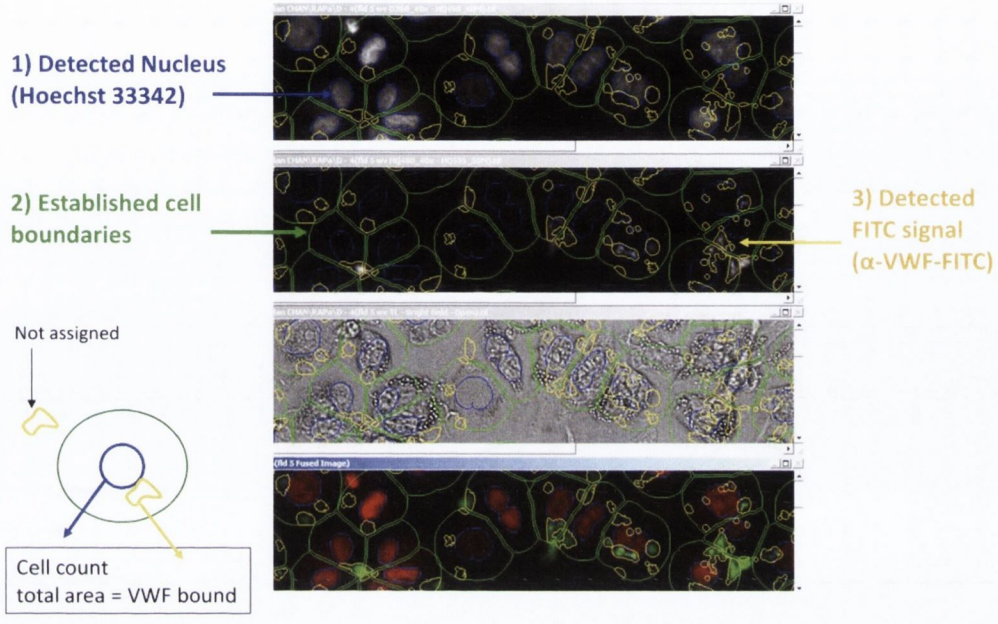
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Appendix I: Image acquisition and analysis



Appendix II: Abstracts leading to poster presentations

1. DEFINING THE MOLECULAR BASIS UNDERLYING THE PHYSIOLOGICAL INTERACTION BETWEEN VON WILLEBRAND FACTOR AND GALECTINS IN NORMAL PLASMA

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BACKGROUND

Von Willebrand Factor (VWF) is abundantly glycosylated with 12 N-linked and 10 O-linked glycans, which are heavily sialylated. Importantly, these structures influence VWF functional properties, including plasma clearance and susceptibility to ADAMTS13 proteolysis. Although the molecular mechanisms through which glycans modulate VWF biology remain poorly understood, recent studies have demonstrated that plasma VWF circulates in complex with specific members of the galectin family. Moreover, these galectin-interactions modulate VWF-mediated thrombus formation *in vivo*.

AIMS

In this study, we sought to define the molecular basis underlying the interactions between VWF and galectins -1 and -3 respectively.

METHODS

VWF was purified from human plasma (pdVWF) by cryoprecipitation and 2BCL gel filtration. VWF glycosylation was then modified using specific exoglycosidases. Blood group specific VWF was purified from pooled group AB, O or Bombay plasmas. Mutated full length VWF and a series of truncated VWF domain fragments were transiently expressed in HEK293T cells. Recombinant galectin-1 and -3 were expressed in *E. coli* and purified via nickel affinity chromatography. Binding interactions were characterized via modified immunosorbent assay.

RESULTS

Both galectin-1 and galectin-3 bound to pdVWF in a dose-dependent manner. Pre-incubation with PNGase F markedly decreased binding to both galectin-1 and galectin-3 (13±1% and 57±2%, $p<0.001$). Moreover, removal of both N- and O-linked glycans (PNGase F and O-glycosidase treatment) further attenuated galectin-3 binding (21±1%, $p<0.0001$). ABO blood group antigen expression significantly influenced interaction with both galectins. In particular, group AB VWF bound to both galectin-1 and galectin-3 significantly better compared to group O VWF. In keeping with its lack of AB antigen expression, platelet-derived VWF bound both galectins with significantly

reduced affinity compared to pdVWF. Terminal sialic acid and sub-terminal galactose expression on VWF also modulated galectin interaction. For example, enzymatic desialylation of pdVWF with α2-3,6,8,9 neuraminidase markedly enhanced binding to galectin-1 and galectin-3 (231±6% and 136±6%, $p<0.05$). Importantly, both galectins -1 and -3 bound with higher affinity to HMWM VWF multimers compared to LMWM of the same blood group. The relative importance of specific VWF domains in regulating galectin interaction was investigated using a series of recombinant truncated domain fragments. Differential galectin binding was evident across the individual domains. Interestingly, a key role for the VWF A domains was observed. In keeping with these findings, incubation with ristocetin significantly enhanced pdVWF binding to both galectin-1 and galectin-3 (914±50% and 205±23%, $p<0.05$). Moreover, both galectins bound to the VWD type 2B mimic R1450E with significantly enhanced affinity (165±10% and 117±4%, $p<0.05$). Finally, targeted removal of the individual N-linked glycan located at N1515 in the VWF A2 domain via site directed mutagenesis lead to dramatically decreased galectin-3 binding (23.86±3%, $p<0.0001$).

CONCLUSIONS

These novel data define the molecular basis underlying the physiological VWF-galectin interaction. In particular, we have demonstrated that both N- and O-linked glycan determinants modulate VWF-galectin binding through terminal sialic acid and ABO blood group expression, with an additional role for specific N-linked glycans. Furthermore, we have identified a critical role for the VWF A domains in modulating these interactions.

XXIV Congress of the International Society on Thrombosis and Haemostasis, 2013.
Oral poster presentation.

2. SPECIFIC N- AND O-LINKED CARBOHYDRATE STRUCTURES MEDIATE VON WILLEBRAND FACTOR INTERACTION WITH GALECTINS -1 AND -3.

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Von Willebrand Factor (VWF) is extensively glycosylated with both N- and O-linked carbohydrates. Moreover, these complex glycan structures influence VWF functional properties, including susceptibility to ADAMTS13 proteolysis, and plasma clearance. The molecular mechanisms through which VWF glycosylation (including ABO blood group antigens) act to influence VWF physiology remains unexplained. However, recent data suggest that VWF circulates in normal plasma bound to various carbohydrate-binding proteins, including specific members of the galectin family. In addition, galectin-3 binding has been reported to influence VWF cleavage by ADAMTS13. In this context, we sought to elucidate the role of specific VWF glycan determinants in modulating galectin interaction.

VWF was purified from human plasma (pdVWF) by cryoprecipitation and gel filtration. VWF glycosylation was then modified using exoglycosidases and quantified by specific lectin ELISAs. Blood group specific VWF was also purified from pooled group AB, O, or Bombay plasmas. Galectins-1 and -3 were transiently expressed in competent *E-coli* cells with an N-terminal histidine tag, and purified by nickel chromatography. Finally, binding interactions were characterized via modified immunosorbant assay.

In keeping with the previous report of Lenting *et al,* human pdVWF bound to both galectin-1 and galectin-3 in a dose-dependent manner. Enzymatic desialylation of pdVWF with α2-3,6,8,9 neuraminidase (Neu-VWF) markedly enhanced binding to galectin-1 (231±6%, $p<0.0001$). Similarly, removal of terminal sialic acid also increased binding to galectin-3, albeit to a lesser extent (136±6%, $p<0.05$). To further define the role of VWF glycans in regulating galectin binding, pdVWF was exposed to sequential neuraminidase and galactosidase digestions to remove terminal sialic acid and sub-terminal galactose residues (NeuGal-VWF). In contrast to the enhanced binding of Neu-VWF, binding of NeuGal-VWF to both galectin -1 and -3 was significantly reduced (51±5% and 52±6% compared to pdVWF; $p<0.005$). Cumulatively these findings suggest that loss of capping sialic acid and exposure of sub-terminal galactose critically regulates VWF-galectin binding. Treatment with PNGase F to completely remove N-linked carbohydrate structures (PNG-VWF) markedly decreased binding to galectin -1 and -3 (13±1% and 57±2%, $p<0.001$). Moreover, combined PNGase F and O-glycosidase digestions further attenuated galectin-3 binding (21±1%, $p<0.001$), suggesting that both the N- and O-linked glycans are involved in mediating the VWF-galectin interaction.

ABO(H) blood group antigens are expressed on both the N-linked and O-linked glycans of human VWF. Moreover, ABO(H) determinants influence VWF susceptibility to ADAMTS13 proteolysis and plasma VWF half-life, through unknown mechanisms. Purified VWF from normal group AB individuals bound to both galectin-1 and galectin-3 significantly better than group O VWF (146±8% and 483±19%; $p<0.01$). Conversely, no significant difference in binding was observed between Group O and Bombay VWF. Consequently, although terminal A (GalNAc) and B (Gal) sugar moieties promote galectin binding, expression of terminal α1-2 fucose residues is not important. The glycosylation profile of platelet-VWF differs from that of pdVWF. In particular, platelet-VWF expresses reduced levels of both capping sialic acid and sub-terminal galactose residues (~50%), and lacks AB blood group antigens. To characterize the effects of this differential sugar expression on galectin binding, platelet-derived VWF was isolated and purified (platelet freeze-thawing followed by immuno-affinity chromatography with monoclonal CLB-Rag20). In keeping with the reduction in Gal and AB blood group antigen expression, platelet VWF bound less well to galectin-1 and galectin-3 (72±6% and 67±7% versus pdVWF; $p<0.05$).

53rd American Society of Haematology Annual Meeting, 2011.

3. THE N-LINKED GLYCANS OF VON WILLEBRAND FACTOR MEDIATE NOVEL INTERACTIONS WITH THE CARBOHYDRATE RECOGNITION DOMAINS OF GALECTINS-1 AND -3.

Jamie O'Sullivan, Orla Rawley, Gudmundur Bergsson, Emily McRae, Rachel McGrath, Alain Chan, and James O'Donnell.

Haemostasis Research Group, Trinity College Dublin.

INTRODUCTION

Von Willebrand factor (VWF) is a large mulitmeric sialoglycoprotein involved in normal haemostasis. We have recently demonstrated that the N- and O-linked sugars of VWF play critical roles in mediating its function [1,2]. However, the molecular mechanism(s) through which carbohydrate determinants modulate VWF activity remains unknown. Galectins are a family of soluble lectins that bind plasma glycoproteins, and have important roles in cell-matrix adhesions, cell proliferation, inflammation, and cancer.

MATERIALS AND METHODS

To determine whether galectins can bind VWF, galectins 1 and 3 were transiently expressed in competent *E-coli* cells using the pET302 vector with an N-terminal histidine tag. Each galectin was purified using a nickel-coated chromatography column. Plasma-derived VWF (pd-VWF) was purified from Haemate P using a Sepharose CL-2B gel filtration column as before [1].

RESULTS

In plate-binding assays, pdVWF demonstrated significant binding to both galectin 1 and 3 (Kd=4.7 and 3.8 nM). Treatment of pdVWF with PNGase F to remove N-linked glycans markedly attenuated binding to both galectin 1 and 3 (45% and 10% of wild type; $P<0.05$). Moreover, enzymatic desialylation (α2-3,6,8,9 neuraminidase) of pdVWF was associated with significant enhanced binding to both galectins. In contrast, specific removal of VWF O-linked sugars had no effect.

CONCLUSIONS

We have identified galectins 1 and 3 as two novel plasma binding partners for human VWF. In addition, we demonstrate that the N-linked sugars of VWF (particularly α2-6 linked sialic acid) play critical roles in regulating these interactions.

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[1] O'Donnell JS et al., Blood. 2005; 106(6), 1988-1991.
[2] Mc Grath RA et al. Blood. 2010; 115(13); 2666-2673.

Haematology Association of Ireland, Annual Meeting 2010.
Oral poster presentation.

Winner: Best Oral Poster.

Appendix III: Abstracts leading to oral presentations

1. ELUCIDATING THE ROLE OF CARBOHYDRATE DETERMINANTS IN REGULATING HAEMOSTASIS – INSIGHTSAND OPPORTUNITIES

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Abstract: Significant developments in modern analytical technologies have enabled an explosive growth in studies relating to the field of glycobiology. These studies have lead to a deeper understanding of the critical roles played by both N-and O-linked carbohydrate structures in directing the functional properties of human glycoproteins. It is now well established that glycans regulate protein synthesis and secretion, together with plasma half-life and specific functional properties. Given these data, it is perhaps unsurprising that both gross and subtle glycosylation defects have been implicated in the pathogenesis of a number of different human diseases. In this presentation, we will review the accumulating evidence for the significance of glycosylation in mediating the structure and functions of the plasma glycoproteins involved in haemostasis and thrombosis. In particular, the role of naturally occurring coagulation protein glycoforms and inherited defects in carbohydrate attachment in modulating coagulation will be considered. Finally, the therapeutic opportunities presented by new insights into the roles of attached carbohydrates in shaping coagulation protein function will be examined, together with the promise for carbohydrate modification in the delivery of novel therapeutic biologics with enhanced functional properties for the treatment of hemostatic disorders.

2. IDENTIFICATION OF GALECTIN-1 AND GALECTIN-3 AS NOVEL BINDING PARTNERS FOR FVIII.

Jamie O' Sullivan, Orla Rawley, P. Vince Jenkins, Alain Chion, Teresa Brophy and James S. O' Donnell.

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During biosynthesis, Factor VIII (FVIII) undergoes complex post-translational modification including significant glycosylation. Consequently each FVIII molecule can contains 25 N- and 6 O-linked glycans. These carbohydrate structures are of physiological significance. For example, FVIII glycan expression modulates intracellular trafficking and also regulates FVIII clearance by dendritic cells. Nevertheless, the molecular mechanisms through which glycan structures influence FVIII biology remains poorly defined. Interestingly, carbohydrate-binding galectins (Gal) -1 and -3 have recently been reported to bind human VWF. Moreover, these galectin interactions significantly influence VWF function. In this study, based upon similar glycans expression profiles, we hypothesised that galectins might also constitute novel binding partners for human FVIII.

In brief, His-tagged Gal-1 and Gal-3 were expressed in *E-coli* and purified using nickel chromatography. Recombinant FVIII (rFVIII) was purified from different commercial concentrates. Subsequently, FVIII glycosylation was modified using specific exoglycosidases and quantified by lectin-binding ELISA. Galectin-FVIII interaction was characterised using modified immunosorbant assays and surface plasmon resonance (SPR).

In plate–binding assays using purified proteins and SPR studies, both Gal-1 and Gal-3 bound to full length rFVIII in a time- and dose-dependent manner. Interestingly the apparent affinities of the galectin-FVIII interactions (Kd of 0.11 ± 0.02nM for Gal-1 and 0.21 ± 0.1nM for Gal-3 respectively) were unusually high for these lectins. Digestion with PNGase F to remove N-linked glycans ablated FVIII binding to Gal-1 (8.6 ± 1%; $p<0.0001$). In contrast, PNG-FVIII retained significant ability to bind Gal-3 (30.3 ± 3%; $p<0.0001$). However, combined FVIII digestion with both PNGase F and O glycosidase further attenuated Gal-3 binding (16.5 ± 2%; $p<0.05$). Cumulatively these findings suggest that whilst Gal-1 binding is mediated predominantly through the N-linked glycans of FVIII, both N- and O-linked glycans modulate its interaction with Gal-3.

The majority of FVIII glycans are contained within the B domain. Unsurprisingly, Gal-1 and Gal-3 binding were both markedly attenuated for B domain deleted rFVIII compared to full length rFVIII (42 ± 1% and 26 ± 0.8%; $p<0.0001$). Previous studies have described different glycosylation profiles for specific full length commercial rFVIII products. To investigate the relevance of this differential glycosylation, we compared the galectin-binding properties of Advate® (CHO cell line) and Helixate® (BHK cell line). Interestingly, Gal-1 and Gal-3 both displayed significantly enhanced affinity for Helixate (107 ± 2% and 124 ± 1%; $p<0.05$). These findings are consistent with the fact that the N-linked glycans of BHK-derived FVIII express galactose α1-3 galactose epitopes which constitute preferential galectin-binding ligands.

To determine whether FVIII interacts with galectins *in vivo*, immunoprecipitation studies were performed using plasma from VWF-/- mice. We observed that that both Gal-1 and Gal-3 were co-precipitated with FVIII even in the absence of VWF. Consequently, both the VWF-FVIII complex and free FVIII in plasma are likely to circulate in a complex with galectins. Importantly, recent studies have reported a prothrombotic phenotype in Gal-1/Gal-3 double deficient mice compared to wild type controls following ferric chloride injury. To investigate whether galectin-binding influences FVIII function, FVIII activity was assessed using a one-stage clotting assay in the presence of increasing galectin concentrations. Interestingly, preincubation of FVIII with Gal-1 (0.5-17μM) resulted in a significant dose-dependent prolongation of the APTT (58 ± 0.2 sec compared to 26 ± 3 secs, $p<0.001$) In contrast, no such effect was observed for galectin-3 up to 20μM, suggesting these galectins may have differential effects on FVIII biology.

In conclusion, we identify Gal-1 and Gal-3 as novel direct ligands for human FVIII. Both the N- and O-linked carbohydrates of FVIII contribute to galectin binding. Importantly, different commercial FVIII concentrates do not interact with galectins in the same manner. Finally, we also demonstrate that plasma FVIII can circulate in complex with both Gal-1 and Gal-3, and that Gal-1 binding can inhibit the procoagulant function of FVIII.

3. DEFINING THE PATHOLOGICAL MECHANISMS UNDERLYING ENHANCED CLEARANCE IN VWD TYPE VICENZA.

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INTRODUCTION

Enhanced clearance has emerged as a critical pathophysiologic mechanism in type 1 von Willebrand disease (VWD). The most severe example of which is VWD Vicenza caused by an arginine to histidine substitution at position 1205 in the von Willebrand factor (VWF) D3 domain. In this study, we sought to investigate the underlying molecular mechanism using a VWF $^{-/-}$ mouse model.

MATERIALS & METHODS

Wild-type VWF (WT-VWF) and a series of mutants incorporating the R1205H substitution or patient mutations R1205C and R1205S were transiently expressed in HEK293T cells. Recovery, half-life and mean residence time (MRT) were determined for each variant in the VWF $^{-/-}$ mouse.

RESULTS

WT-VWF was cleared in a monophasic fashion with a mean MRT of 11.4±0.8mins. VWF-R1205H was cleared significantly faster with a MRT of 7.9±0.5 mins ($p<0.05$). Residual plasma VWF levels indicated that both VWF-R1205C and VWF-R1205S were also rapidly cleared compared with WT-VWF (8.4±3.2%, 6.0±1.5% and 37.9±1.9% respectively). In contrast, mutation of the highly conserved neighbouring arginine residue (R1204) to histidine had no significant effect on VWF survival (MRT = 15.2±2.5 mins). Interestingly, however, comparison of initial recovery values for VWF-R1205H, VWF-R1205C and VWF-R1205S (95.3±4.6%, 48.7±2.4% and 24.1±5.8% of total injected VWF respectively, $p<0.05$), revealed that the molecular mechanism through which VWF-R1205C and VWF-R1205S are rapidly cleared differs markedly to the mechanism through which VWF-R1205H is cleared.

CONCLUSIONS

These novel data identify a specific and significant role for R1205 in modulating VWF clearance. These data also characterize for the first time, clearance of patient mutations R1205C and R1205S in the VWF $^{-/-}$ mouse.

Haematology Association Ireland, Annual Meeting 2013.

4. DEFINING THE MOLECULAR BASIS UNDERLYING THE PHYSIOLOGICAL INTERACTION BETWEEN VWF AND GALECTIN-3 IN NORMAL PLASMA.

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INTRODUCTION

Recent reports have shown that von Willebrand Factor (VWF) circulates in normal plasma bound to galectin-3. Moreover, galectin-binding critically regulates VWF multimer proteolysis by ADAMTS13. In this study, we have defined for the first time the molecular mechanisms through which this critical interaction between VWF and galectin-3 is mediated.

METHODS

Plasma VWF (pdVWF) was purified from commercial concentrate Haemate P by gel filtration. VWF glycosylation was then modified using specific exoglycosidases. Recombinant VWF and a series of VWF mutants were expressed in HEK293T cells. Recombinant galectin-3 was expressed in competent *E-coli* cells and purified by nickel chromatography. Finally, binding interactions were characterized via modified immunosorbant assay.

RESULTS

Galectin-3 binds to pdVWF in a glycan-dependent manner. Complete removal of both N- and O-linked sugar structures via enzymatic digestion significantly decreased binding to galectin-3 (21±1%, $p<0.001$). Further enzymatic modification of N- and O-linked sugars demonstrated that loss of N-linked capping sialic acid significantly enhanced galectin-3 binding (136±6%, $p<0.05$). Additionally, preferential binding to blood group AB vs group O VWF (483±19%, vs 116±7%, $p<0.0001$) was observed. Interestingly, targeted removal of individual N-linked sugars 1515 and 1574 within the VWF A2 domain led to dramatically reduced binding (24±3%, and 44±3%). Conversely, structural modification of VWF via incubation with ristocetin or introduction of VWD type 2B mutant R1450E led to markedly enhanced binding to galectin-3.

CONCLUSIONS

These novel data define the molecular mechanism underlying the physiological interaction between galectin-3 and human pdVWF. In particular, we have demonstrated that both N- and O-linked glycan determinants critically modulate VWF-galectin binding and describe a role for specific N-linked sugars.

Haematology Association Ireland, Annual Meeting 2012.
Oral presentation, President's Symposium.
Winner: President's Prize, Best Overall Abstract.

5. A CRITICAL ROLE FOR N- AND O-LINKED CARBOHYDRATE DETERMINANTS IN MODULATING VON WILLEBRAND FACTOR CLEARANCE IN VIVO.

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VWF is a multimeric plasma sialoglycoprotein essential for normal haemostasis. Although the biosynthesis, structure and functional properties of VWF have been well characterized, the molecular mechanism(s) underlying its clearance remain poorly understood. Nevertheless, it is well established that enhanced VWF clearance is important in the pathophysiology of VWD. Moreover, emerging data suggest that variation in VWF glycosylation (notably ABO blood group) may constitute an important regulator of in vivo clearance rates. To define the role of VWF glycans in modulating clearance, VWF was purified from human plasma (pdVWF) by cryoprecipitation and gel filtration. Subsequently, VWF glycosylation was modified using specific exoglycosidases and quantified by lectin-binding ELISA. Finally, the effect of altered glycan expression on VWF plasma half-life was characterized in the $VWF^{-/-}$ mouse model.

Wild type pdVWF was cleared in biphasic manner, characterized by a rapid initial phase followed by a slower secondary phase ($t_{1/2}$ = 46.9 min). Complete enzymatic desialylation of VWF (α2-3,6,8,9 neuraminidase; Neu-VWF) markedly enhanced VWF clearance ($t_{1/2}$ = 3.7 min; $p<0.01$). Digestion of pdVWF with α2-3 neuraminidase to remove predominantly O-linked sialic acid (which constitutes less than 20% total VWF sialylation) was also sufficient to markedly enhance VWF clearance ($t_{1/2}$ = 13.1 min; $p<0.05$). In the presence of the asialoglycoprotein receptor (ASGPR)-antagonist ASOR, the mean residence time of Neu-VWF was identical to that of pd-VWF. Recent studies have shown that macrophages may be important in VWF clearance. Since the ASGPR is expressed on both hepatocytes and macrophages, the effect of macrophage depletion on VWF clearance was assessed. Pre-treatment with liposome-encapsulated clodronate depleted F4/80+CD11b+ murine macrophages by 75%, and significantly prolonged Neu-VWF survival. However Neu-VWF survival was not corrected to that observed in the presence of ASOR. Cumulatively, these findings demonstrate that both N- and O-linked sialylation are critical in protecting VWF against ASGPR-mediated clearance. Moreover, ASGPR-modulated clearance is at least in part macrophage-dependent.

ß-galactose residues exposed following removal of capping sialic acid are recognised by the ASGPR. To further define the role of VWF glycosylation in regulating clearance, the effect of removing both terminal sialic acid and sub-terminal galactose (sequential neuraminidase and galactosidase digestions to generate NeuGal-VWF) was studied. Surprisingly, in $VWF^{-/-}$ mice, NeuGal-VWF was cleared rapidly from the plasma in a monophasic fashion ($t_{1/2}$ = 4.8 min). Moreover, treatment with PNGase F to completely remove N-linked

carbohydrate structures also markedly decreased the plasma half-life (PNG-VWF; $t_{1/2}$ = 2.1 min). In keeping with their lack of exposed galactose residues, the enhanced clearance of NeuGal-VWF and PNG-VWF were not mediated via the ASGPR (ASOR had no significant effect). In contrast, macrophage depletion by liposomal clodronate significantly inhibited the enhanced clearance of both NeuGal-VWF and PNG-VWF respectively. These data suggest that the ASGPR is not the only macrophage receptor involved in modulating VWF clearance, which is consistent with the relatively minor prolongation in VWF survival previously reported in $Asgpr1^{-/-}$ mice.

These novel data demonstrate that variation in the N- or O-linked carbohydrate structures of VWF can significantly modulate its half-life in vivo. Moreover, these glycans effects are not mediated solely through the ASGPR. Rather, additional as yet unidentified macrophage receptors are also important. Therefore, qualitative variation in VWF glycosylation is likely to represent to a key regulator of VWF clearance, and as such to be of direct pathophysiological significance.

53rd American Society of Haematology Annual Meeting, 2011.
Oral presentation.

Appendix IV: Manuscripts submitted for publication

von Willebrand factor arginine 1205 substitution results in accelerated macrophage-dependent clearance *in vivo*.

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KEY POINTS

1. Conservative and non-conservative arginine 1205 amino acid substitutions alleviate D'D3-mediated inhibition of VWF clearance in vivo.
2. VWF arginine 1205 substitutions specifically enhance macrophage-mediated clearance.

ABSTRACT

Enhanced von Willebrand factor (VWF) clearance is important in the etiology of von Willebrand disease (VWD), and has been associated with a number of different VWF point mutations, including the R1205H Vicenza mutation. In this study, we have investigated the patho-biological mechanisms underlying the enhanced clearance associated with VWF-R1205H. Our findings demonstrate that substitutions of R1205 with histidine, cysteine or serine all result in markedly reduced survival of full-length recombinant VWF in the $VWF^{-/-}$ mouse. Furthermore, D'A3 fragments containing these same R1205 substitutions also demonstrated significantly enhanced clearance compared to wild-type. Interestingly, another conserved arginine is located at 1204 in human VWF. In contrast to the reduced in vivo survival observed with R1205H, clearance of R1204H was not enhanced. Finally, our data further demonstrate that the pathological enhanced clearance associated with VWF-R1205 substitutions is mediated predominantly through a macrophage-dependent pathway. These findings therefore support the hypothesis that macrophages play a key role in modulating the reduced survival of VWF in patients with Type IC VWD.

INTRODUCTION

Increased plasma clearance of von Willebrand factor (VWF) has been implicated as an important mechanism in the etiology of both type 1 and type 2 von Willebrand disease (VWD).[1,2] In particular, accumulating data suggest that enhanced VWF clearance may be a relatively common pathological mechanism in patients with type 1 VWD.[1,3,4] To date, more than 20 different VWF point mutations have been associated with enhanced clearance.[5-9] Of these, the most common is the VWD-Vicenza variant, which is characterized by an Arg1205His substitution in the E3 module of the VWF D3 domain.[10] Patients with VWD-Vicenza typically have significantly reduced plasma VWF levels, but retain normal platelet-VWF content.[7,10] In addition, their steady-state VWF propeptide (VWF:pp) to VWF antigen (VWF:Ag) ratio is markedly elevated. Following DDAVP administration, plasma VWF:Ag levels increase significantly in VWD-Vicenza individuals.[11] However the half-life of the secreted VWF-R1205H is markedly reduced compared to that of wild type (WT-) VWF.[10,11]

The direct role of the R1205H substitution in regulating enhanced VWF clearance has been confirmed in several studies using $VWF^{-/-}$ mice. Lenting *et al* demonstrated that the mean residence time of human VWF-R1205H was markedly reduced compared to that of human WT-VWF.[12] Subsequently, Pruss *et al* showed that the half-life of murine VWF-R1205H was also significantly reduced following intravenous injection.[13] In addition, enhanced clearance of R1205H-VWF was also observed following hydrodynamic expression in these VWF-deficient mice.[13] Cumulatively, these data

demonstrate that the R1205H substitution results in significantly enhanced VWF clearance from the circulation. Critically however, the molecular and cellular basis through which this single amino acid substitution results in enhanced clearance of multimeric VWF has not been defined. Recent studies have identified a role for hepatic and splenic macrophages in modulating VWF clearance,[14,15] and have described a number of putative macrophage receptors including the low-density lipoprotein-receptor related protein-1 (LRP1) and β2-integrins.[15] Furthermore, variation in VWF glycans including terminal sialic acid and ABO(H) blood group antigen expression have also been shown to regulate VWF clearance.[16-18] Importantly, the asialoglycoprotein receptor (ASGPR) which is predominantly expressed on hepatocytes has also been found to modulate *in vivo* clearance of VWF.[16,18] In this study, we have utilized a series of recombinant VWF variants and truncations to investigate the molecular basis underlying the enhanced clearance of the VWD-Vicenza variant. Our findings demonstrate a specific and critical role for the R1205 residue in modulating macrophage-mediated clearance of VWF.

MATERIALS AND METHODS

VWF expression and purification

The expression vector pcDNA-VWF was described previously.[19] The Kapa HiFi HotStart PCR Kit (Anachem, United Kingdom) was used to generate point mutations (R1204H, R1205C, R1205H, and R1205S) in both full-length VWF and D'A3-VWF constructs. Full-length VWF and D'A3-VWF variants were then transiently expressed in HEK293T cells using polyethylenimine (PEI) as a transfection reagent. Conditioned serum free medium was harvested after 72 hours and concentrated via anion exchange chromatography as before.[20] Full length VWF variants were further concentrated using 100-kDa cut-off spin filters (Amicon, United Kingdom). D'A3-VWF variants were further purified via nickel affinity chromatography. VWF concentration was subsequently determined by ELISA as previously described.[21]

VWF clearance studies in $VWF^{-/-}$ and $ASGPR^{-/-}$ mice.

$VWF^{-/-}$ and $Asgr1^{-/-}$ mice were obtained from the Jackson Laboratory (Sacramento, USA), both on a C57Bl/6 background. All animal experiments were approved by the Animal Research Ethics Committee, Trinity College Dublin and were performed in compliance with the Irish Medicines Board regulations, on mice between 6 to 8 weeks of age. Briefly, $VWF^{-/-}$ mice were intravenously injected with 300nM VWF or fragments thereof, diluted in 100µl sterile-filtered PBS. At specific time-points, mice were anaesthetized with 2.5% tribromethanol (0.2ml per 10g of body weight) and exsanguinated

by subclavicle incision Blood was collected into lithium heparin-coated microcontainers (BD Unitech, Ireland) and plasma VWF:Ag levels were determined by ELISA. Three to five mice were used per time point and each mouse was bled only once. Percentage recovery, mean residence time and plasma half-life were determined for each variant. Clearance values were expressed and graphed as percentage residual VWF:Ag against time. Clearance experiments in $Asgpr^{-/-}$ mice were performed using biotin-labelled wild type and R1205H VWF respectively. VWF was biotinylated using EZ-link NHS-Biotin (Pierce) according to manufacturer's instructions. Biotinylated VWF was quantified using a modified ELISA. Polyclonal anti-VWF (Dako) was used as a capture antibody and bound biotinylated VWF was subsequently detected using HRP-conjugated streptavidin. For macrophage depletion, mice were intravenously injected with clodronate liposomes (100µl/10g body weight) 24 hours prior to injection of VWF as previously described.[22] Control mice were injected with PBS liposomes.

Statistical analysis

Data were analysed using GraphPad Prism software (GraphPad Prism version 5.0 for Windows; GraphPad Software, San Diego, CA). All data are expressed as mean ± SEM. To assess statistical differences, the data were analyzed using one way ANOVA with post-hoc Dunnett's test for multiple comparisons.

RESULTS AND DISCUSSION

In preliminary experiments, we confirmed that recombinant human VWF-R1205H was cleared significantly faster than human WT-VWF in $VWF^{-/-}$ mice (Figure 1A). Two other substitutions of arginine 1205 (with cysteine and serine respectively) have also been reported in individual patients with significantly reduced plasma VWF:Ag levels, and elevated VWFpp/VWF:Ag ratio.[23] To determine whether these substitutions also influenced VWF survival *in vivo*, we further characterised the clearance of recombinant VWF-R1205C and VWF-R1205S in the $VWF^{-/-}$ mouse. Interestingly, both VWF-R1205C and VWF-R1205S were also associated with significantly enhanced clearance compared to WT-VWF (Figure 1B). Indeed, VWF-R1205C and VWF-R1205S were actually cleared more rapidly than VWF-R1205H. The arginine 1205 residue in human VWF is highly conserved across other species. Of note, another conserved arginine is located at residue 1204. In view of this proximity, we proceeded to investigate *in vivo* clearance of recombinant human VWF-R1204H. Surprisingly, in contrast to the markedly enhanced clearance phenotype observed with the R1205H substitution, clearance of VWF-R1204H was not significantly different from that of WT-VWF (Figure 1C). Cumulatively, these data highlight a specific critical role for the arginine 1205 residue within the D3 domain of VWF in regulating clearance *in vivo*.

Although the precise regions of the VWF protein responsible for modulating clearance have not been defined, the majority of reported patient mutations associated with enhanced VWF clearance are clustered within the D'D3 and A1A2A3 domains (Figure 2A).[1] In addition, previous studies using

recombinant VWF truncations demonstrated that the fragment A1A2A3 was cleared at a similar rate to full length VWF.[12] In contrast, the fragment D'D3A1A2A3 (D'A3) was cleared significantly more slowly, suggesting that the D'D3 domains may have a role in regulating VWF clearance.[12] To investigate the molecular mechanism(s) responsible for the reduced plasma survival associated with R1205 substitution, we further characterised the effects of introducing the R1205H, R1205S and R1205C substitutions into the D3 domain of the D'A3 fragment. Interestingly, in keeping with their effects in reducing the survival of full length VWF, each of these R1205 substitutions resulted in significantly enhanced clearance of the D'A3 fragment (Figure 2B). Collectively these data demonstrate that the reduced survival associated with loss of arginine 1205 is modulated through local effects within the D'A3 region. We postulate that the enhanced clearance phenotype may be due to abrogation of the inhibitory effect of the D'D3 region upon A1A2A3-mediated VWF clearance.[24] Alternatively, R1205 substitutions may serve to enhance VWF clearance by creating a novel receptor-binding site within the D'D3 region.

Recent studies have identified an important role for the ASGPR in modulating clearance of both hyposialylated and normally sialylated VWF *in vivo*.[18] To further elucidate the mechanism underlying the reduced survival associated with R1205H substitution, we investigated clearance of biotin-labelled VWF-R1205H in $Asgr1^{-/-}$ compared to wild type C57Bl/6 mice respectively. Interestingly, the rapid clearance of biotin-labelled VWF-R1205H was partially attenuated in the $Asgr1^{-/-}$ mice (Figure 2C). In addition, pre-treatment of $VWF^{-/-}$ mice with the ASGPR-antagonist asialo-orosomucoid

(ASOR) prior to VWF-R1205H infusion also served to significantly reduce the enhanced clearance of VWF-R1205H (data not shown). Collectively, these data suggest that the ASGPR contributes to modulating the enhanced clearance of VWF-R1205H.

Emerging data suggest that hepatic and splenic macrophages can also bind and endocytose VWF, and thus play a role in regulating VWF clearance.[14,15] In addition, although the ASGPR receptor is predominantly expressed on hepatocytes, recent data suggest that it may also be expressed on macrophages.[25] In keeping with previous reports, we confirmed that macrophage depletion with clodronate significantly attenuated *in vivo* clearance of WT-VWF in $VWF^{-/-}$ mice (Figure 2D). Importantly, macrophage-depletion also significantly prolonged the survival of VWF-R1205H, R1205S and R1205C respectively. Interestingly, macrophage-depletion inhibited VWF-R1205H clearance to a greater degree than that observed with WT-VWF. Moreover, the magnitude of the effect of macrophage depletion in reversing the enhanced clearance of VWF-R1205H was significantly greater than that observed following ASGPR inhibition. Cumulatively therefore, these findings suggest that macrophage-mediated clearance represents the predominant pathway through which R1205H is cleared. Moreover, these data also support the hypothesis that macrophages can play critical roles in modulating the reduced survival of VWF in patients with Type IC VWD.

In conclusion, our findings emphasize the specific critical role played by arginine 1205 in modulating VWF clearance. Thus, substitutions of R1205 with

histidine, cysteine or serine all resulted in markedly reduced survival of full length VWF or D3-containing VWF fragments. This was predominantly mediated through a macrophage-dependent clearance mechanism. The unique sensitivity of VWF-R1205H clearance to macrophage depletion rather than other proposed routes of VWF endocytosis illustrates the presence of multiple, distinct VWF clearance mechanisms that can be variously accelerated by the presence of select VWF amino acid substitutions.

AUTHORSHIP

Contribution: O. R., J. M. O'S., A. C., S. K., R. J. S. P., and T. M. B. performed experiments; O. R., J. M. O'S., A. C., S. K., M. L., N. V. R., P. F., T. M. B. and J. S. O'D. designed the research and analyzed the data. All authors were involved in writing and reviewing the paper.

Conflict-of-interest disclosure:

J.S.O'D has served on the speaker's bureau for Baxter, Bayer, Novo Nordisk, Leo Pharma and Octapharma. He has also served on the advisory boards of Baxter, Bayer, Octapharma CSL Behring, and Pfizer. J.S.O.D has also received research grant funding awards from Baxter, Bayer and Novo Nordisk.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Arginine 1205 in the D3 domain plays a critical role in regulating von Willebrand factor clearance *in vivo*

A) Recombinant wild type (WT-) and R1205H- human VWF were expressed in HEK293T cells. To study *in vivo* survival, $VWF^{-/-}$ mice were intravenously injected with purified VWF, and blood samples collected at specified subsequent time points. Mice were sampled only once. Residual circulating VWF concentration was determined at each time point using VWF:Ag ELISA. All results are plotted as percentage residual VWF:Ag levels relative to the amount injected. Three to five mice were used per time point, and data are represented as mean ± SEM. In some cases, the SEM cannot be seen due to its small size.

B) In addition to the R1205H substitution, R1205C and R1205S mutations have also been described in patients with VWD. Recombinant human VWF-R1205C and VWF-R1205S were expressed and *in vivo* clearance assessed in $VWF^{-/-}$ mice. Plasma samples were collected at 3 and 10 minutes following intravenous administration and clearance compared to that of WT-VWF. Both VWF-R1205C and VWF-R1205S were cleared significantly faster than WT-VWF (* $P < 0.05$, ** $P < 0.001$, and *** $P < 0.0001$ respectively).

C) The human VWF D3 domain contains conserved arginine residues at positions 1204 and 1205. An asterisk (*) indicates positions which have a single, fully conserved residue. Positively charged residues are highlighted in green and negatively charged residues are highlighted in red.

D) To further investigate the mechanism through which R1205 substitutions result in enhanced VWF clearance, recombinant VWF-R1204H was expressed, and *in vivo* survival measured in $VWF^{-/-}$ mice. Four mice were used for each time point. In contrast to the enhanced clearance phenoytpes associated with the R1205H, R1205S and R1205C substitutions, VWF-R1204H clearance did not differ significantly to that of WT-VWF. In some cases, SEM cannot be seen due to its small size.

Figure 2. Macrophages play a major role in modulating the enhanced clearance associated with VWF R1205 substitutions.

A) Diagram illustrates the domain structure of full-length VWF. In addition, the domains present in the truncated VWF variants expressed in this study are also shown. In the amplified section, the recently defined subdomains within the N-terminal D'D3 domains have been indicated, together with the location of R1205 which lies within the E3 subdomain of D3.

B) Previous studies have suggested that the A1A2A3 domains of VWF may contain a critical clearance receptor binding site, whilst the D'D3 domains act to inhibit VWF clearance. To investigate whether the enhanced clearance associated with R1205 substitutions resulted from disruption of this D'D3 inhibitory effect, *in vivo* clearance of VWF D'A3 fragments incorporating the R1205H, R1205S and R1205C substitutions was compared to wild type D'A3. Plasma samples were collected at 3 and 10 minutes following intravenous administration. Three mice were used for each time point. Ten minutes following infusion, residual plasma levels of the D'A3 truncations containing R1205H, R1205S and R1205C were significantly reduced compared to wild type D'A3. (** $P < 0.001$, and *** $P < 0.0001$ respectively).

C) The ASGPR has been shown to be important in regulating the normal physiological clearance of VWF. To determine whether the ASGPR contributes to the reduced survival of VWF-R1205H, we studied biotin-labelled VWF-R1205H clearance in $Asgr1^{-/-}$ mice (on a C57Bl/6 background) compared to wild type C57Bl/6 mice. After 15 minutes, residual plasma VWF-R1205H levels

in the Asgr1$^{-/-}$ were significantly higher than those in the wild type C57Bl/6 mice.

D) Recent studies have demonstrated that hepatic macrophages play an important role in mediating VWF clearance *in vivo*. To investigate whether macrophages are involved in mediating the enhanced clearance of R1205 variants, hepatic macrophages were depleted in VWF$^{-/-}$ mice by intravenous administration of clodronate liposomes. *In vivo* clearance studies for VWF-R1205H, VWF-R1205S and R1205C were then repeated 24 hours following clodronate administration. Three mice were used for each time point. VWF$^{-/-}$ mice injected with PBS-containing liposomes were used as controls. Clodronate-mediated macrophage depletion significantly prolonged survival of VWF-R1205H, VWF-R1205C and VWF-R1205S. (** $P < 0.001$ and *** $P < 0.0001$ respectively).

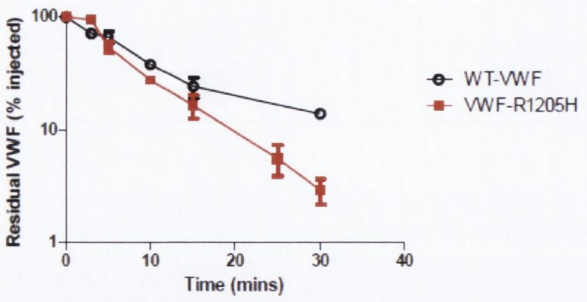
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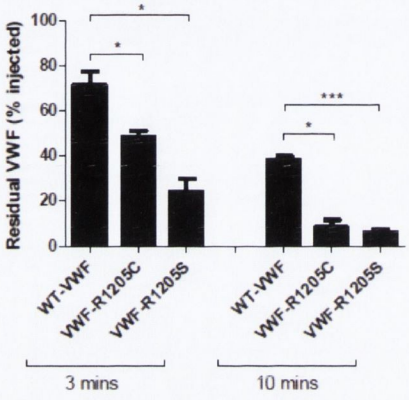
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FIGURE 1

A



B



C

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HUMAN   1198  VCEVAGRRFASGKKVTLNPSDPE  1220
CHIMP   1198  VCEVAGRRFASGKKVTLNPSDPE  1220
MOUSE   1198  VCEVAGRRLAPGKKITLSPDDPA  1220
CAT     1198  VCEVAGRRLASGKKITLNPDDPE  1220
DOG     1198  VCEVAGRRLAPGKKIILNPSDPE  1220
PIG     872   VCEAAGRRLAPGKKIILNPRDPA  894
COW     1195  VCQVEGRRLASGKKVTLNPGDPE  1217
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D

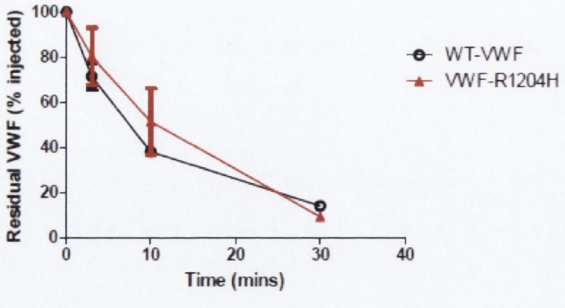
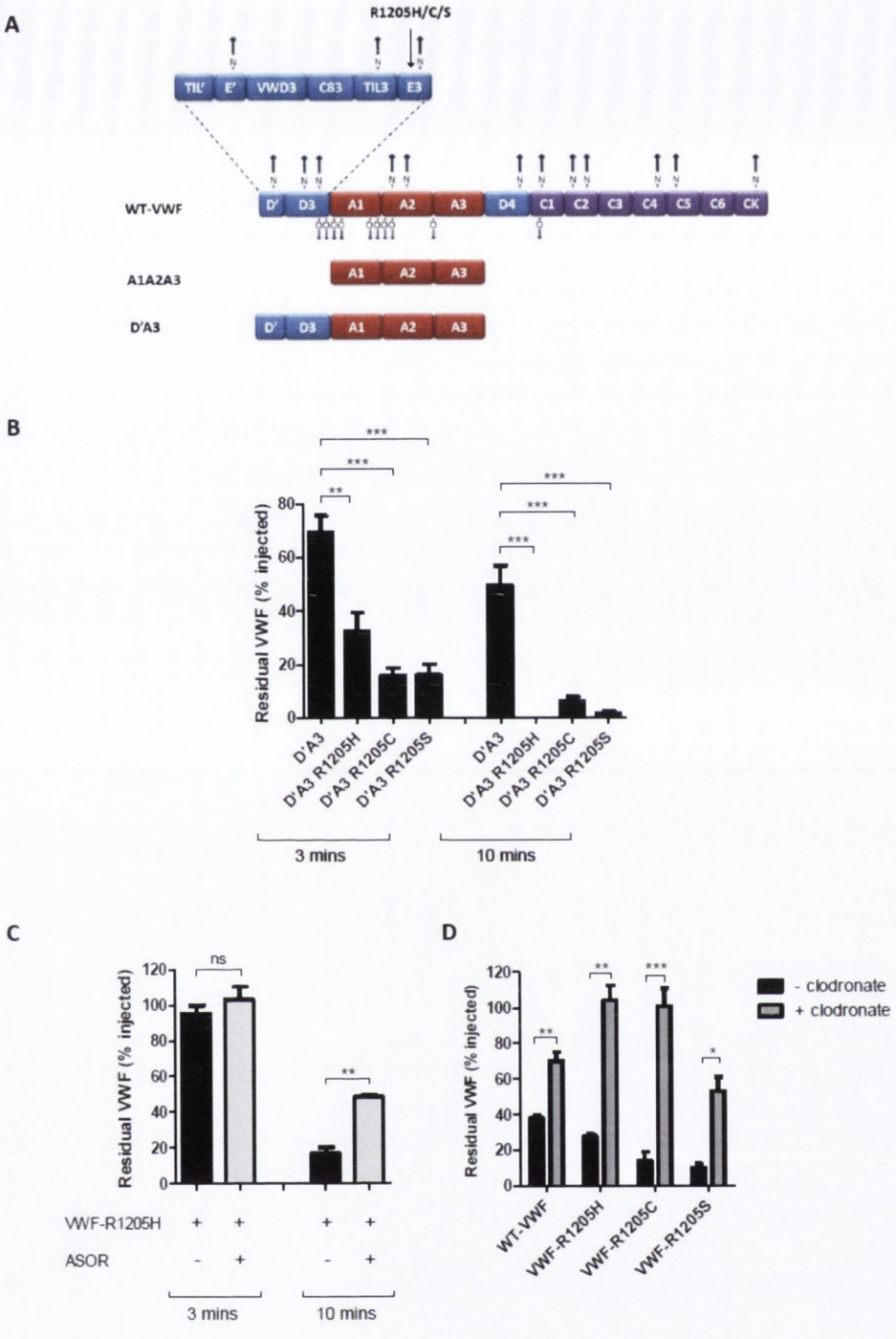


FIGURE 2


A
R1205H/C/S
TIL' E' VWD3 C83 TIL3 E3
WT-VWF
D' D3 A1 A2 A3 D4 C1 C2 C3 C4 C5 C6 CK
A1A2A3
A1 A2 A3
D'A3
D' D3 A1 A2 A3
B
Residual VWF (% injected)
D'A3
D'A3 R1205H
D'A3 R1205C
D'A3 R1205S
3 mins
10 mins
C
Residual VWF (% injected)
ns
VWF-R1205H
ASOR
3 mins
10 mins
D
Residual VWF (% injected)
- clodronate
+ clodronate
WT-VWF
VWF-R1205H
VWF-R1205C
VWF-R1205S

Appendix V: Publications

Elucidating the role of carbohydrate determinants in regulating hemostasis: insights and opportunities

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Recent improvement in modern analytical technologies has stimulated an explosive growth in the study of glycobiology. In turn, this has lead to a richer understanding of the crucial role of *N*- and *O*-linked carbohydrates in dictating the properties of the proteins to which they are attached and, in particular, their centrality in the control of protein synthesis, longevity, and activity. Given their importance, it is unsurprising that both gross and subtle defects in glycosylation often contribute to human disease pathology. In this review, we discuss the accumulating evidence for the significance of glycosylation in mediating the functions of the plasma glycoproteins involved in hemostasis and thrombosis. In particular, the role of naturally occurring coagulation protein glycoforms and inherited defects in carbohydrate attachment in modulating coagulation is considered. Finally, we describe the therapeutic opportunities presented by new insights into the role of attached carbohydrates in shaping coagulation protein function and the promise of carbohydrate modification in the delivery of novel therapeutic biologics with enhanced functional properties for the treatment of hemostatic disorders. (*Blood*. 2013;121(19):3801-3810)

Introduction

Glycan structures are attached to more than half of all known proteins,[1] and genes encoding the molecular apparatus required for glycosylation constitute 1% to 2% of the human genome.[2] Despite the prevalence of glycan attachment to human proteins and lipids, the field of glycobiology has traditionally represented something of a Cinderella subject. However, recent advances in synthetic, and particularly analytic, methodologies have led to heightened awareness regarding the structural and functional significance of carbohydrate structures on proteins. Accumulating data make it clear that the glycan structures expressed on many glycoproteins play critical roles in modulating functional activity. In addition, variation in carbohydrate structures has been implicated in the pathogenesis of a number of human diseases. Moreover, it seems inevitable that evidence regarding the physiological and pathological importance of carbohydrate expression will continue to emerge in the coming years. In this context, it is perhaps unsurprising that regulation of glycan expression on novel recombinant therapeutic glycoproteins is already established as a key quality parameter within the pharmaceutical industry. In this review, we provide an overview of the critical roles played by carbohydrate determinants in regulating human hemostasis and thrombosis. In particular, using exemplar coagulation glycoproteins, we have sought to highlight some of the different molecular mechanisms through which glycan variation can influence glycoprotein biology. Although we have selected specific examples and focused on plasma coagulation glycoproteins, these concepts can nevertheless be considered a paradigm equally applicable to other human secretory glycoproteins.

Protein glycosylation

N-linked glycosylation

N-linked glycans on human glycoproteins are attached to the amide nitrogens of asparagine (Asn) side chains. *N*-linked glycosylation begins in the endoplasmic reticulum (ER),[3,4] where a preassembled oligosaccharide core structure is transferred from a dolichol lipid donor onto specific Asn residues within nascent polypeptide chains.[5] This reaction is catalyzed by the enzyme complex oligosaccharyltransferease, which targets Asn residues located in the consensus sequence Asn-X-serine (Ser)/threonine (Thr) (where X can be any amino acid except proline).[6] Importantly, *N*-linked glycosylation within the ER is actually a cotranslational event occurring on the luminal aspect of the ER membrane. As a consequence, depending on polypeptide folding and conformation, not all Asn residues in a consensus sequence will necessarily be glycosylated. The net effect, therefore, is that polar *N*-linked glycans are typically found on the surfaces of glycoproteins, rather than being buried deep within the protein interior.

The initial 14-sugar core *N*-linked structure attached during protein synthesis in the ER is mannose-rich ($Glc_3Man_9GlcNAc_2$). This core glycan is subsequently remodeled by a series of glycosyltransferases and glycosidases as the protein passes through the ER and onto the Golgi.[7] This process commences in the ER with the removal of 2 terminal glucose moieties by the exoglycosidases glucosidase 1 and 2. Glucose cleavage enables the protein to interact with 2 homologous ER lectins, calnexin (Cnx) and calreticulin (Crt), and thereby engage in a folding cycle.[8] Once properly folded, glycoproteins are subsequently transported to the Golgi, where the

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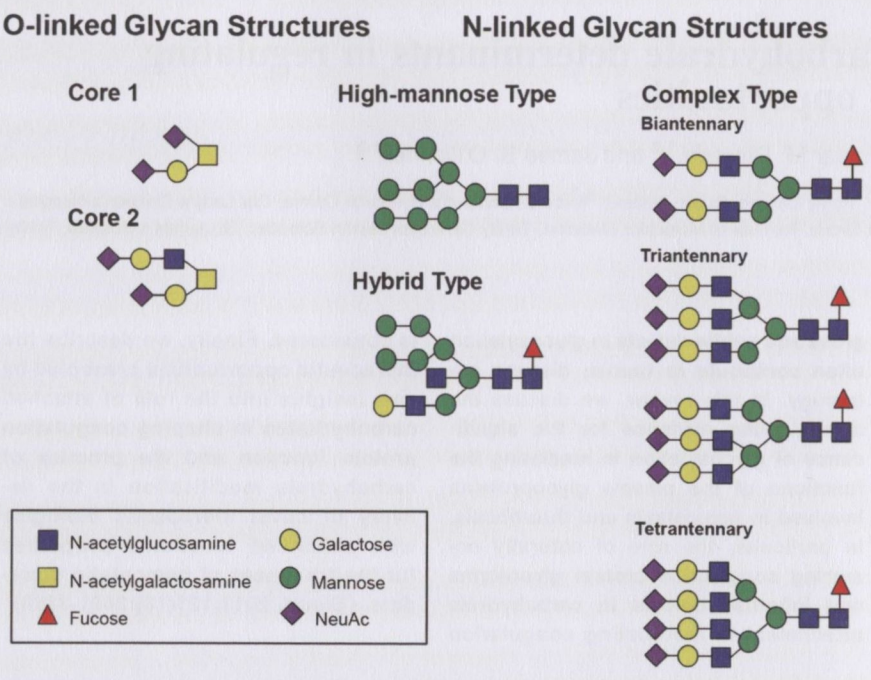


Figure 1. Examples of typical *N*- and *O*-linked glycan structures expressed on human plasma glycoproteins.

N-linked glycans are further modified. Unsurprisingly, given that more than a hundred different glycosyltransferases are encoded by the human genome, the final *N*-linked carbohydrate structures can be complex and heterogeneous in nature. Nevertheless, *N*-linked glycans can be classified into 1 of 3 subgroups: high-mannose, hybrid, or complex (Figure 1). This process is distinct from that of glycation, which refers to the nonenzymatic irreversible attachment of reducing sugars to proteins, and in contrast to glycosylation, it is not enzyme-controlled or dependent on predefined attachment sites.

O-linked glycosylation

O-linked glycans on human glycoproteins are attached to Ser or Thr residues. *O*-linked glycosylation differs from *N*-linked glycosylation in a number of important regards.[9] First, *O*-linked glycosylation is a true posttranslational modification, as *O*-linked carbohydrate structures are only synthesized on proteins as they transit through the Golgi. Second, there is no preassembled *O*-linked oligosaccharide core structure. Rather, *O*-linked carbohydrate synthesis involves sequential addition of monosaccharide units in a stepwise manner. These reactions are catalyzed by a series of specific glycosyltransferases analogous to those required for *N*-linked glycans synthesis. Finally, for *O*-linked glycosylation to occur, Ser or Thr residues do not need to exist as part of a specific consensus sequence. Nevertheless, previous studies have shown that *O*-linked glycosylation of Ser or Thr is more common if Ser/Thr residues are present in clusters or are located in areas rich in proline or alanine residues.[10] Final *O*-linked glycan structures are simpler than complex *N*-linked sugars (Figure 1).

Heterogeneity of glycosylation

In view of the number of distinct human glycosyltransferases and glycosidases already described, it is perhaps not surprising that glycan databases include descriptions of more than 500 different *N*-linked carbohydrate structures. The marked heterogeneity of *N*-linked glycans structures has proven one of the major obstacles to the investigation of the potential physiological and pathological significance of carbohydrate structures. A further level of complexity is added by virtue of the fact that many glycoproteins contain multiple individual *N*- and *O*-linked glycosylation sites. In addition, different types of glycan structures can be expressed on each of these specific Asn residues within the same protein. Importantly, expression levels for the individual glycosyltransferase and glycosidase enzymes vary significantly between different tissues and can also be influenced by disease state or normal aging. As a consequence, a given individual can express various glycoforms of a particular glycoprotein that differ only with respect to their carbohydrate profiles.

Many of the human proteins involved in regulating normal hemostasis circulate as soluble glycoproteins in plasma. Before their secretion, these proteins often undergo complex posttranslational modification, including significant glycosylation. As a result, complex branching carbohydrate structures can account for up to 25% of their final molecular mass. These carbohydrate structures play critical, but often underappreciated, roles in modulating many of the key biological properties of these coagulation proteins.

Role of glycans in modulating intracellular trafficking

Carbohydrate determinants regulate transit through the ER

Secretory glycoproteins, including coagulation factors, are synthesized by ER-bound ribosomes. After processing within the ER, proteins follow an intracellular pathway through the ER–Golgi intermediate compartment (ERGIC) to the Golgi before finally being secreted into the plasma. Within the lumen of the ER, significant folding and modification of newly synthesized proteins occurs. This process is regulated by a series of enzymes and molecular chaperones, including immunoglobulin-binding protein (BiP), Cxn, and Crt. Only correctly folded proteins are allowed to exit the ER. Misfolded proteins either are retained within the ER or are subject to degradation by the ER-associated protein degradation pathway.[4]

Previous studies have clearly defined the critical role played by carbohydrate structures in regulating glycoprotein interaction with ER-resident molecular chaperones. In particular, the 14-sugar core *N*-linked structure on nascent polypeptide chains is a key regulator of these interactions. Cxn and Crt are homologous lectins that bind monoglucosylated polypeptides in concert with the thiol oxidoreductase ER p57, facilitating correct folding and preventing protein aggregation. The fate of the Cxn/Crt-bound polypeptide is ultimately determined by uridine diphosphate–glucose:glycoprotein glucosyltransferase (GT), which acts as a folding sensor that detects characteristic biophysical properties of misfolded proteins. If misfolding is detected, the protein is reglucosylated by GT and reenters the Cxn/Crt cycle, where it can either continue until correctly folded or be transferred for degradation. If properly folded, GT does not reglucosylate high mannoses, and the protein is ready for transport to the Golgi apparatus.

The physiological relevance of carbohydrate determinants in regulating transit through the ER has been highlighted in a series of elegant studies examining biosynthesis of the homologous coagulation glycoproteins factor V (FV) and factor VIII (FVIII), respectively. Once activated, these glycoproteins play crucial roles in the coagulation cascade, acting as cofactors in the prothrombinase and intrinsic tenase complexes. FV and FVIII share identical domain structures (A1-A2-B-A3-C1-C2), and significant structural homology exists between their A and C domains. In contrast, the B domains of FV and FVIII exhibit limited sequence similarity.[11] Nevertheless, both B domains are extensively glycosylated, containing 25 (FV) and 18 (FVIII) potential *N*-linked glycosylation sites, respectively. Moreover, although the amino acid sequence encoding the FVIII B domain has diverged widely between human, porcine, and murine genes, the large number of *N*-linked glycosylation sites has remained strikingly conserved. In spite of their homologous structures, expression studies have demonstrated that FV is secreted from mammalian cells significantly more efficiently than FVIII. Limiting steps in FVIII secretion have been identified and include extended interactions with ER chaperone molecules, which in turn limit its progress to the Golgi and onward to secretion from the cell. In particular, residues within the A1 domain of FVIII have been shown to mediate stable interaction with BiP.[12] In contrast, FV does not associate with BiP.[13] Furthermore, FVIII has also been shown to bind both Crt and Cxn, which also slows its secretion.[14] Unsurprisingly, these interactions are mediated in large part through *N*-linked glycan structures expressed within the FVIII B domain. Although FV can also interact with the chaperone Crt, it does not appear to bind to Cnx.

Carbohydrate determinants regulate transit from ER to Golgi

On successful folding and packaging, new proteins travel from the ER to the Golgi for additional posttranslational modifications before secretion. This is achieved by formation of coat protein complex II (COPII) vesicles, which bud from the ER lumen and migrate to the Golgi apparatus via the ERGIC.[15] Despite their different ER processing, FV and FVIII have a shared prerequisite for specialized ER-to-Golgi transport machinery. In particular, lectin mannose-BiP 1 (LMAN1; also known as ERGIC-53) and multiple coagulation factor deficiency protein 2 (MCFD2) are cargo transporters for ER-to-Golgi traffic of FV and FVIII.[16,17]

N-linked oligosaccharides are key to FV/FVIII interactions with the LMAN1/MCFD2 complex. LMAN1 association with FV/FVIII is enhanced by the presence of fully glucose trimmed mannose 9 structures on B domain–located carbohydrates, as demonstrated using an LMAN1 mutant with defective mannose binding ability and, consequently, severely diminished FVIII-LMAN1 interaction. LMAN1/MCFD2 gene mutations that prevent interaction with FV/FVIII (or each other) have been shown to be the cause of combined FV/FVIII deficiency, an autosomal recessive disorder associated with a mild to moderate bleeding tendency caused by reduced FV and FVIII plasma levels of 5% to 30%.[18,19] Cumulatively, these data serve to emphasize the critical importance of *N*-linked glycan structures in regulating the intracellular trafficking of secretory glycoproteins.

Role of glycans in modulating susceptibility to proteolysis

In addition to modulating intracellular processing, carbohydrate expression on plasma glycoproteins influences a number of their biological properties. In particular, glycans have been shown to modulate susceptibility to proteolysis. The critical importance of sugar structures in this regard has been highlighted through a series of recent studies on the proteolysis of the large, multimeric sialoglycoprotein, von Willebrand Factor (VWF). Before secretion, VWF undergoes extensive posttranslational modification, including significant glycosylation. As a result, each VWF monomer contains 12 potential *N*-linked and 10 potential *O*-linked glycosylation sites with carbohydrate structures.[20] The glycan structures of VWF have been characterized[21] and shown to be highly complex and heterogeneous in nature. The most common *N*-linked structure is a monosialylated biantennary complex chain.[22] In contrast, the majority of the *O*-linked glycans of VWF are composed of the tumor-associated T-antigen.[23] Thus, both the *N*- and *O*-linked VWF glycans are commonly capped by terminal negatively charged sialic acid moieties. Unusually, covalently linked ABO(H) blood group carbohydrate determinants have also been described as terminal sugar residues on a proportion of both the *N*-linked (13%) and *O*-linked (1%) glycans of VWF.[22,24,25]

Plasma VWF multimer composition is a critical determinant of functional activity. High–molecular weight multimers bind both subendothelial collagen and platelet glycoprotein Ibα; with significantly higher affinities than lower–molecular weight forms.[26] Interestingly, the *O*-linked glycans of VWF have recently been reported to modulate the critical interaction with glycoprotein Ibα.[27,28] In normal plasma, the multimeric composition of circulating VWF is tightly controlled by a disintegrin and metalloproteinase with thrombospondin type repeats 13 (ADAMTS13). The physiologic importance of regulating VWF multimer composition is highlighted in type 2A VW disease and thrombotic thrombocytopenic purpura. In type 2A VWD, increased proteolysis is associated with concomitant loss of High–molecular weight multimers and a bleeding phenotype. Conversely, inherited or acquired ADAMTS13 deficiency or dysfunction results in thrombotic thrombocytopenic purpura, characterized by an excess of circulating ultralarge VWF with the subsequent development of platelet-rich thrombi in the microvasculature.[29]

For many years, it has been recognized that glycosylation profiles on circulating glycoproteins play critical roles in modulating susceptibility to proteolysis. In particular, loss of terminal sialic acid expression has been shown to result in increased proteolysis by various different proteases.[30,31] For example, desialylation of VWF significantly enhances sensitivity to proteolysis by plasmin, chymotrypsin, and cathepsin B. In contrast to this accepted paradigm,

however, recent data from our laboratory have shown that depletion of terminal α2-6 linked sialic acid from the *N*-linked glycans of VWF specifically inhibits susceptibility to ADAMTS13 proteolysis.[32] Furthermore, terminal expression of ABO(H) blood group determinants on VWF glycans has also been shown to influence VWF permissiveness to ADAMTS13-mediated proteolysis (in the order O ≥ B ≥ A ≥ AB).[33,34] Site-directed mutagenesis studies have suggested that glycan expression at Asn-1574 in the VWF A2 domain adjacent to the ADAMTS13 cleavage site may be of particular importance in this context.[35] Mutation of this Asn residue with subsequent elimination of the associated glycan chain resulted in markedly enhanced susceptibility of VWF to ADAMTS13 proteolysis. In contrast, mutation of neighboring glycan Asn-1515 had no such effect. On the basis of these findings, therefore, it is clear that variations in carbohydrate expression profiles can critically regulate plasma glycoprotein susceptibility to proteolysis, and thereby influence normal physiology. Moreover, these alterations in carbohydrate structure may involve only subtle changes in terminal glycan determinant expression. In addition, glycan variation at particular *N*-linked sites may also be of specific importance in this regard.

Role of glycans in modulating biological activity

Previous in vitro studies have demonstrated that modification of carbohydrate determinants on plasma glycoproteins, by either exoglycosidase digestion or site-directed mutagenesis, can significantly influence key aspects of biological function. It is important to note, however, that even in normal individuals, some plasma glycoproteins naturally circulate as different glycoforms (Table 1). Although these protein isomers contain identical amino acid compositions, they differ with respect to the number and/or type of their attached glycan structures. As a consequence of altered glycosylation profiles, individual glycoforms may demonstrate clinically relevant differences in their functional properties. Within the coagulation cascade, there are several notable examples of naturally occurring, partially glycosylated plasma glycoforms that exhibit differential functional properties compared with their fully glycosylated counterparts.

Coagulation is initiated in vivo by the exposure of tissue factor (TF) on extravascular cells on vascular injury, which then interacts with activated factor VII (FVIIa) to activate factor X (FX). TF has *N*-linked glycosylation consensus sequences at 3 positions (Asn-11, Asn-124, and Asn-137); however, their contribution to TF procoagulant activity is subject to debate. TF possesses different glycan structures depending on whether it was derived from human placenta or generated via recombinant expression in bacterial or insect cells.[36-38] Deglycosylation of placenta-derived TF resulted in a significant (fourfold) reduction in catalytic rate (k_{cat}) for extrinsic FXase activity, indicating an important role for *N*-linked glycosylation in modulating TF procoagulant function.[38] In contrast, previous studies have reported that recombinant TF expressed in *Escherichia coli* possessed similar procoagulant activity to that expressed in mammalian cells.[39] In addition, recent studies have shown that recombinant TF mutants lacking specific individual *N*-linked glycan consensus sequences also exhibit functional activity similar to that of wild-type TF.[40]

Human coagulation FX is activated in vivo by FIXa and FVIIa in the presence of cofactors FVIIIa and TF, respectively. FX possesses 2 *N*-linked (Asn-39 and Asn-49) and 2 *O*-linked (Thr-17 and Thr-29) glycosylation sites, all of which are contained within the activation peptide of the zymogen protein. Various reports have suggested that FX carbohydrate moieties can modulate FX activation.[41-43] Mutagenesis of FX *N*- and *O*-linked glycan attachment sites significantly increased FX activation by FVIIa or FIXa but exhibited a limited effect on the catalytic efficiency of either the intrinsic (FIXa/FVIIIa) or extrinsic (TF/FVIIa) FXase complexes.[42] Enzymatic desialylation of FX attenuates the rate of activation by either the intrinsic or extrinsic FXase complex, implying an important role for terminal sialic acids in enhancing FXase complex formation.[43] Further to their role in FX activation, *N*-linked (but not *O*-linked) glycans on the FX activation peptide have been proposed to protect FX from rapid clearance via glycan-dependent interactions with macrophages in mice, accounting for its prolonged plasma half-life in comparison with related vitamin K–dependent zymogens.[44,45]

The serine proteinase inhibitor antithrombin constitutes the major plasma inhibitor of thrombin and circulates as a single-chain glycoprotein that possesses 4 *N*-linked glycosylation sites at Asn-96, Asn-135, Asn-155, and Asn-192, respectively. These glycan structures exist predominantly in the form of disialylated biantennary complex chains.[46] Two different plasma glycoforms of antithrombin (α- and β-antithrombin) have been described.[47] Fully glycosylated α-antithrombin accounts for the majority of total plasma antithrombin. In addition, a minor glycoform (β-antithrombin) contributes 10% to 15% of total plasma antithrombin. This glycoform is identical to α-antithrombin but for the absence of any *N*-linked oligosaccharide expression at Asn-135.[48] The loss of this specific glycan chain results in markedly enhanced protease inhibitor activity.[47,49] As a consequence, despite representing only a small minority of plasma antithrombin, β-antithrombin has been suggested to be the principal mediator of antithrombin protease inhibitor activity in vivo.[50] Kinetic and crystallographic analyses of the molecular basis underlying the enhanced activity of β-antithrombin have demonstrated that the presence of the oligosaccharide structure at Asn-135 sterically impedes a conformational change required to activate antithrombin on heparin/heparan binding.[51] Thus, absence of this steric hindrance at Asn-135 in β-antithrombin enables rapid adoption of an active conformation once bound to heparin, thereby enhancing its inhibitory activity.[52]

Protein C (PC), similar to antithrombin, is crucial for the regulation of thrombin generation in vivo. PC circulates in zymogen form and is activated by the thrombin–thrombomodulin complex. After activation by the thrombin–thrombomodulin complex, activated PC (APC) inhibits further thrombin generation by proteolytic degradation of procoagulant-activated cofactors FVa and FVIIIa. PC possesses 4 *N*-linked glycosylation sequons: 1 located within its first epidermal growth factor (Asn-97) and the remaining 3 located in its protease domain (Asn-248, Asn-313, and Asn-329).

In addition, 3 different glycoforms of human PC have been described in normal human plasma: α-PC accounts for 70% of total plasma PC and is characterized by the presence of complex biantennary oligosaccharide chains at all 4 *N*-linked glycosylation sites, β-PC accounts for approximately 25% of total plasma PC and differs from α-PC in that it is not glycosylated at Asn-329,[53] and γ-PC represents only 5% of total plasma PC and lacks oligosaccharide chains attached at both Asn-329 and Asn-248. Several lines of evidence support the hypothesis that these different glycoforms of PC have important biological differences. For example, site-directed mutagenesis studies have suggested that PC activation by the

Table 1. Coagulation glycoproteins—carbohydrate composition and biological relevance

Haemostatic glycoprotein	*N*-linked sites	*O*-linked sites	Functional significance of glycan structures	Physiological and pathological glycoforms
Fibrinogen	4	0	Promotes fibrinogen solubility[93]	Pathological: fibrinogens Lima,[80] Caracas II,[81] Niigata,[94] Pontoise, Asahi,[82] and Kaiserslautern[95]
Prothrombin	3	0	None described	None described
TF	3	ND	Glycans modulate procoagulant activity of TF[36-38]	None described
Factor V	26	ND	Glycans modulate intracellular trafficking from ER to Golgi.[13,14] *N*-linked glycans also influence FVa phospholipid binding affinity and FVa susceptibility to APC mediated proteolysis.[58-60]	Physiological: FVa_1 and FVa_2 Pathological: factor V Liverpool (Ile359Thr)[79]
Factor VII	2	2	Influence hepatic clearance and plasma half-life of recombinant FVIIa. Loss of *O*-linked glycans impairs procoagulant activity of FVIIa in plasma.[96]	None described
Factor VIII	24	7	Glycans influence FVIII folding and intracellular trafficking during biosynthesis.[13,14] *N*-linked glycans also regulate FVIII uptake by dendritic cells[91,97] and clearance by the ASGPR.	Pathological: factor VIII (Met1772Thr) and (Ile566Thr) [25]
Factor IX	2	5	None described	Pathological: factor IX (Arg94Ser) [83]
Factor X	2	2	Both *N*- and *O*-linked glycans are negative modulators of FX zymogen activation.[42] *N*-linked glycans also regulate FX clearance.[45]	None described
Factor XI	5	ND	None described	None described
Factor XII	2	7	None described	None described
Factor XIII	3	ND	None described	None described
VWF	12	10	*N*-linked and *O*-linked glycans influence VWF synthesis, secretion, and biological activity.[27,35,98] Glycan expression also regulates susceptibility to ADAMTS13 proteolysis[34] and is a critical determinant of VWF clearance.[67]	Physiological: ABO blood group–specific glycoforms
PC	4	0	Modulate PC zymogen activation by thrombin thrombomodulin complex.[54] Glycans also influence the anticoagulant and antiinflammatory properties of APC.[55]	Physiological: α-protein C, β-protein C, γ- protein C[53]
Protein S	3	ND	None described	Pathological: protein S Heerlen (Ser460Pro) [84]
Antithrombin	4	0	Glycans influence conformational changes in antithrombin after heparin binding, and thereby regulate serpin activity.[47,51]	Physiological: α-antithrombin and β-antithrombin[47] Pathological: antithrombin (Ile7Asn),[99] antithrombin (Ser82Asn) [100]

ND, not determined.

thrombin–thrombomodulin complex is modulated by the presence of *N*-linked oligosaccharides at Asn-313.[54] APC anticoagulant activity may also be subject to modulation by its glycan structures, but reports on its importance have been conflicting. Specifically, a naturally occurring PC mutation encoding only β-PC (N329T) exhibited mildly reduced anticoagulant activity when purified from plasma, activated, and assayed for its ability to degrade FVa.

In contrast, a recombinant version of β-APC in which glycosylation at Asn-329 was eliminated exhibited approximately twofold increased anticoagulant activity compared with wild-type recombinant APC.[54,55] In addition to its anticoagulant role, APC also possesses potent anti-inflammatory and antiapoptotic activity that is mediated at least in part by activation of protease-activated receptor 1 (PAR1).[56] We have recently demonstrated that a recombinant APC mutant that mimics the glycosylation pattern of β-APC (APC-N329Q) exhibits an increased capacity to maintain endothelial cell barrier integrity and inhibit endothelial cell apoptosis compared with wild-type APC.[55] Interestingly, Asn-329 is located proximal to a putative PAR-1 binding exosite on the surface of the APC protease domain,[57] and recent work has indicated that elimination of the oligosaccharide chain at this position accelerates the rate of PAR1 cleavage by APC, possibly by facilitating increased PAR1 access to the binding

exosite on APC (E. M. Gleeson, J. S. O'Donnell, and R. J. Preston, unpublished data). On the basis of these findings, therefore, it is clear that the different APC glycoforms present in normal human plasma exhibit important differences in their biological activities that are likely to be of physiological and pathological relevance.

Partial *N*-linked glycosylation resulting in the synthesis of heterogeneous glycoforms with distinct biological properties has also been reported for a number of important procoagulant plasma glycoproteins. Human FV is abundantly glycosylated, with both *N*- and *O*-linked carbohydrate structures accounting for 15% to 25% of the total molecular mass. FV is activated by limited specific proteolysis by either thrombin or FXa and then serves as a critical cofactor in the prothrombinase complex. Subsequently, FVa is inactivated by APC-catalyzed proteolysis at Arg-306 and Arg-506. Inactivation of FVa by APC plays a critical role in down-regulating thrombin formation. Two different glycoforms of FV are present in the normal human circulation. As a consequence, activation by thrombin results in the generation of 2 distinct forms of FVa (FVa_1 and FVa_2) that differ only with respect to their glycosylation profiles.[58] Site-directed mutagenesis studies have established that unlike FVa_1, the FVa_2 glycoform appears to result from partial glycosylation at Asn-2181 in the C-terminal C2 domain.[59] Importantly, several reports have shown that this variation in the *N*-linked glycan component of FVa significantly modulates its functional properties.[58-60] For example, the affinity of the human FVa_2 glycoform binding to anionic phospholipids was approximately threefold higher than that of FVa_1.[59] Moreover, FVa_1 and FVa_2 also displayed differential susceptibilities to APC-mediated proteolysis. In particular, at low phospholipid concentrations, FVa_1 was inactivated at a 15-fold slower rate compared with FVa_2.[60] These distinct biological differences serve as a further example of how the relative concentrations of naturally occurring coagulation protein glycoforms have the potential to markedly influence overall thrombin generation at sites of vascular injury.

Role of glycans in modulating clearance

N- and *O*-linked carbohydrate structures play major roles in determining the rate of clearance of many human glycoproteins from plasma. Terminal sialic acids are of critical importance in this regard. The removal of capping sialic acid residues leading to exposure of penultimate Gal and GalNAc moieties typically results in markedly enhanced glycoprotein clearance. In mammals, desialylation is achieved by a family of 4 sialidases (also known as neuraminidases; Neu1-Neu4) that catalytically remove α-glycosidase-linked sialic acid groups from carbohydrate structures.[61] This clearance is mediated primarily via the hepatic lectin asialoglycoprotein receptor (ASGPR or Ashwell receptor). A member of the calcium-dependent (C-type) lectin receptor family abundantly expressed in the liver, ASGPR is composed of 2 homologous *trans*-membrane polypeptides (Asgr-1 and Asgr-2) that assemble into a hetero-oligomer on the cell surface. The C-terminal extracellular domains of Asgr-1 and Asgr-2 form a carbohydrate recognition domain that selectively binds glycoproteins expressing either β-D-galactose (βGal) or *N*-acetyl-D-galactosamine (GalNAc) terminal sugar determinants in a calcium-dependent manner. However, these βGal and GalNAc residues are more typically expressed on plasma glycoproteins as subterminal moieties on oligosaccharide chains capped by sialic acid. If the terminal sialic acid residue is lost, the ASGPR can bind the exposed βGal or GalNAc and mediate endocytosis.

The critical importance of sialic acid expression in determining plasma half-life has been observed for several different coagulation glycoproteins. Enzymatic removal of terminal sialic acid residues from the abundantly sialylated VWF ex vivo markedly reduces plasma half-life in rabbits (240 vs 5 minutes for normal and desialylated VWF, respectively).[62] In keeping with this observation, genetic inactivation of a specific sialyltransferase (ST3Gal-IV) in a transgenic mouse also resulted in significantly reduced plasma VWF levels as a consequence of a twofold increased rate of clearance.[63] The importance of the ASGPR in modulating physiological VWF clearance is further underlined by recent data demonstrating that VWF half-life is significantly increased in ASGPR-1 knockout mice.[64]

In addition to its role in regulating VWF plasma clearance, the ASGPR may also modulate the clearance of a number of other coagulation glycoproteins, including FVIII. As previously described, FVIII is heavily glycosylated, and the *N*-linked glycans of human FVIII are commonly capped by negatively charged sialic acid residues.[65] Surface plasmon resonance studies have demonstrated that FVIII also binds the ASGPR with high affinity (K_d = 2 nM). This interaction is mediated through the *N*-linked carbohydrate structures clustered within the B domain of FVIII. Furthermore, administration of an ASGPR antagonist significantly inhibited FVIII clearance in mice, suggesting that the ASGPR may contribute to normal physiological clearance of FVIII from plasma.[66]

Similar to sialic acid, ABO(H) blood group determinants are also expressed as terminal sugar residues on the carbohydrate structures of both VWF and FVIII. This ABO(H) expression has direct clinical relevance, as ABO blood group is major determinant of plasma VWF levels. Group O individuals have 25% less circulating VWF compared with non-O individuals (group A, B, or AB).[67,68] Moreover, plasma VWF levels are even lower in individuals with the rare Bombay blood group phenotype, in which H antigens are not expressed.[34] The effect of ABO(H) blood group antigens on VWF levels is explained by differences in clearance rates between each blood group. As such, the VWF plasma half-life is significantly shorter in normal group O vs non-O individuals (10.0 vs 25.5 hours, respectively).[67] Nevertheless, the molecular mechanism underlying the enhanced clearance of group O VWF remains unknown. However, given that the ASGPR selectively binds either GalNAc or Gal residues, it seems likely that another clearance receptor is responsible for modulating this phenomenon. A weak effect of the Secretor blood group locus on plasma VWF levels has also been reported.[69] Interestingly, this blood group system is similar to ABO, in that it is characterized by the presence or absence of specific terminal carbohydrate determinants on oligosaccharide structures. To date, it remains unclear whether this Secretor influence is also modulated through an effect on VWF clearance.

In addition to the ASGPR, a variety of other lectin receptors has been characterized. These lectins typically contain a carbohydrate recognition domain that has binding specificity for particular terminal glycans moieties expressed on *N*- and/or *O*-linked carbohydrate structures. Examples of other lectins that have been implicated in modulating glycoprotein clearance include Mac-1 ($\alpha_M\beta_2$), the macrophage galactose lectin, and the scavenger receptor C-type lectin.[70] The relative contribution of these individual receptors in mediating clearance of individual plasma coagulation glycoproteins has not yet been defined. However, recent data have demonstrated that macrophage-mediated endocytosis may be important in the physiological clearance of both VWF and FVIII.[71] Furthermore, data from our laboratory have shown that the rate of VWF clearance by

macrophages is markedly influenced by VWF glycan expression.[72] Recent data have also demonstrated that galectin 1, galectin 3, and siglec 5 can also all bind to human VWF.[73,74] In addition, other putative lectin-like receptors that may be involved in determining plasma levels of the VWF-FVIII complex have been identified through genome-wide association studies and include C-type lectin domain family 4 member M and stabilin 2.[75] Thus, although the molecular mechanisms responsible for modulating the clearance of glycoproteins from plasma remain poorly understood, carbohydrate expression is of critical importance in regulating the rate of clearance.

Aberrant glycosylation can cause human disease

Although rare, almost 50 different congenital disorders of glycosylation have been identified.[76] These disorders typically involve defects in *N*-linked glycosylation and are associated with severe multiorgan clinical phenotypes including skeletal and neurological abnormalities. Significant coagulopathies have also been observed in children with congenital disorders of glycosylation.[76-78] In particular, factor XI, PC, antithrombin, and protein S are commonly deficient. The molecular mechanism or mechanisms responsible for the reduced plasma levels of these specific coagulation glycoproteins remains unclear. Nevertheless, significant thrombotic and bleeding complications are well recognized as constituting important clinical features of these conditions.[77,78]

Aberrant glycosylation of specific proteins, including coagulation factors, has also been implicated in the etiology of human pathology. Point mutations that result in the introduction of novel *N*-linked glycosylation sites are of particular importance. For example, the amino acid substitution Ile359Thr within the heavy chain of FV (FV Liverpool) creates a new *N*-linked glycosylation consensus sequence, such that an additional glycan chain is expressed at Asn-357.[79] As a result, the FVa-Ile359Thr molecule is resistant to APC-mediated proteolysis, and consequently, FV Liverpool is associated with a prothrombotic phenotype. A number of different amino acid substitutions that introduce additional *N*-linked glycosylation sites have also been described in patients with congenital dysfibrinogenemia. These include fibrinogens Lima (Aα Arg141Ser),[80] Caracas II (Aα Ser434Asn),[81] Asahi (γ Met310Thr),[82] and Kaiserslauten (γ Lys380Asn). In each of these cases, the attachment of an extra *N*-linked glycan causes impaired functional activity and a consequent bleeding tendency. Similarly, an FIX gene mutation that results in an extra glycosylation site has been identified in a family with hemophilia B.[83] Interestingly, the Arg94Ser substitution actually leads to the introduction of a new *O*-linked glycosylation site in the second epidermal growth factor-like domain of FIX, which in turn markedly attenuates activation by FXIa.

In contrast, mutations leading to the loss of a single specific *N*-linked glycosylation site have also been implicated in disease pathology. Protein S is a plasma glycoprotein that is important in regulating thrombin generation in vivo. First, protein S functions as a nonenzymatic cofactor for APC inactivation of FVa and FVIIIa. In addition, protein S may also regulate hemostasis by APC-independent inhibition mechanisms. Protein S Heerlen is found in approximately 0.5% of the population and is characterized by a Ser to Pro substitution at position 460.[84] This change results in the loss of *N*-linked glycosylation at Asn-458 and has been associated with an increased risk for venous thromboembolism.[85] The clinical phenotype relates in part to the fact that the Ser460Pro substitution results in reduced plasma protein S levels because of an enhanced clearance.[86] In addition, protein S Heerlen demonstrates reduced cofactor activity for APC-inactivation of FVIIIa.[85]

Glycan modification: therapeutic implications and opportunities

As summarized in this review, carbohydrate structures on human coagulation proteins play essential roles in determining stability, circulatory half-life, and biological activity. As a consequence, in the production of biopharmaceuticals, glycosylation is of critical importance. In particular, for the synthesis of recombinant glycoprotein therapeutics, it is well established that glycosylation profiles can vary significantly, depending on the cell line chosen for expression. Moreover, recombinant proteins generated in vitro can also demonstrate significant heterogeneity in terms of their glycan profiles. This obviously has major implications, given that many studies of coagulation protein structure and function have used recombinant proteins that may express carbohydrate determinants that differ markedly to those expressed on the native human proteins. Unsurprisingly, these glycan variations also can have important therapeutic sequelae. For example, recombinant FVIIa (rFVIIa; NovoSeven) used for the treatment of patients with hemophilia with inhibitors contains 2 *N*-linked and 2 *O*-linked glycans and is expressed in baby hamster kidney (BHK) cells.[87] Although all 4 sites are glycosylated in the purified rFVIIa molecule, approximately 10% of rFVIIa molecules from BHK cells have *N*-linked glycans lacking terminal sialylation. Moreover, a further 30% of the rFVIIa possesses significantly reduced *N*-linked sialic acid expression. This variable sialylation has important consequences in determining the plasma half-life of therapeutic rFVIIa, as hyposialylated rFVIIa is rapidly cleared from the circulation through the hepatic ASGPR.[88]

Patients with hemophilia A can be treated using either plasma-derived or recombinant FVIII products. Unsurprisingly, glycan expression differs significantly between plasma-derived and recombinant FVIII.[89] Moreover, glycosylation variation has also been described between different commercial recombinant FVIII products that have been synthesized in different mammalian cell lines (including Chinese hamster ovary and BHK).[89] For example, rFVIII from Chinese hamster ovary cells express the NeuGc epitope, which accounts for 0.5% of total sialic acid.[90] In contrast, Gal-α(1,3)Gal structures have been identified on ~3% of BHK-expressed rFVIII. Importantly, high levels of antibodies against NeuGc and Gal-α(1,3)Gal both occur naturally in most humans. Interestingly, recent studies have also demonstrated that specific glycan chains on FVIII may influence dendritic cell uptake mediated through the macrophage mannose receptor (CD206).[91] Thus, removal of the mannosylated sugars at Asn-239 (A1 domain) or Asn-2118 (C1 domain) abrogated dendritic cell endocytosis of FVIII and presentation to $CD4^+$ T-cells. Importantly, these data raise the possibility that variations in glycan expression on recombinant FVIII products may influence immunogenicity and, consequently, risk for inhibitor development in patients with hemophilia.[92]

As our understanding of the critical role played by glycan structures in regulating the biological activity and half-life of plasma glycoproteins continues to further develop, it seems likely that significant opportunities for the development of novel therapeutic

agents will arise. In particular, glycoengineering involving targeted selective carbohydrate modification may enable the development of recombinant glycoprotein therapies with improved clinical efficacy. From the data presented in this review, the wide spectrum of coagulation factor properties that can be influenced by protein glycosylation are readily apparent. Manipulation of carbohydrate structures may be useful in prolonging plasma half-life of recombinant clotting factor concentrates; for example, through hypersialylation. Alternatively, glycoengineering may be useful in reducing the immunogenicity of recombinant therapeutic glycoproteins. Clearly, even minor glycan modification of terminal sugar moieties, or indeed the loss or introduction of a specific glycosylation site, may be enough to develop a glycoform with enhanced therapeutic properties.

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Authorship

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