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**Defining the roles of von Willebrand Factor and Factor VIII
glycosylation in regulating lectin interaction and modulating *in*
vivo clearance.**

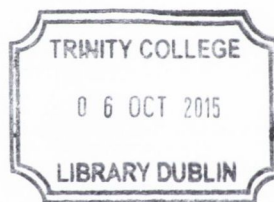


A thesis submitted to the University of Dublin, Trinity College,
for the Degree of Doctor of Philosophy in the School of Medicine.

Jamie O' Sullivan

Submitted March 2015

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Thesis 10868

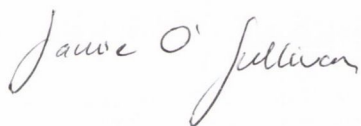
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Abstracts leading to oral/poster presentations

- **N-linked glycans within A1A2A3 domains of VWF play a critical role in modulating macrophage-mediated clearance.**

Alain Chion, Jamie O' Sullivan, Gudmundur Bergsson, Sean Keyes, Orla Rawley, Vince Jenkins, Tom A. McKinnon, Mike Laffan, Teresa Brophy, Jame S. O' Donnell.

56th American Society of Haematology Annual Meeting, 2014.

Oral presentation

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

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

8th Bari Interaction Conference, 2014.

Poster presentation

- **N-linked glycans of VWF play a critical role in modulating clearance via macrophages.**

Jamie O' Sullivan, Alain Chion, Gudmundur Bergsson, Orla Rawley, Niamh O' Regan, Sean Keyes, Teresa Brophy, James S. O' Donnell.

Haematology Association Ireland, Annual Meeting 2014.

Oral presentation

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

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

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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
- **Identification of galectin-1 and galectin-3 as novel binding partners for factor VIII.**
Jamie O' Sullivan, Orla Rawley, P. Vince Jenkins, Alain Chion, Teresa Brophy and James S. O' Donnell.
Haematology Association Ireland, Annual Meeting 2013.
Oral poster presentation
- **Defining the molecular mechanism underlying the physiological interaction between VWF and Galectin-3 in normal plasma.**
 Orla Rawley, Jamie O'Sullivan, Niamh O'Regan, Alain Chion, Teresa Brophy, P. Vincent Jenkins, James S. O'Donnell.
Haematology Association of Ireland, Annual Meeting 2012.
Oral presentation
- **Galectin-1 Binding to VWF in Normal Plasma: Critical Roles for Specific Terminal Glycan Determinants and Amino Acid Residues Adjacent to the ADAMTS13 Cleavage Site.**
Jamie O'Sullivan, Orla Rawley, Niamh O'Regan, Alain Chion, Teresa Brophy, P. Vincent Jenkins, James S. O'Donnell.
Haematology Association of Ireland, Annual Meeting 2012.
Oral poster 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 VWF 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, James S. O'Donnell.

Haematology Association of Ireland, Annual Meeting 2010.

Oral poster presentation

Publications

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

Rachel McGrath, Martje van den Biglaar, Barry Byrne, Jamie M. O' Sullivan, Orla Rawley, Richard O' Kennedy, Jan Voorberg, Roger Preston, James S. O'Donnell.
Blood 2013;122(25):4107-10.

- **Arginine 1205 substitutions results in accelerated macrophage-dependent clearance *in vivo*.**

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.

Journal of Thrombosis and Haemostasis 2015 May;13(5):821-6.

Summary

von Willebrand Factor (VWF) is a large multimeric sialoglycoprotein which mediates critical roles in normal haemostasis. VWF tethers platelets at sites of vascular injury and also serves as a chaperone for coagulation factor VIII (FVIII). Both VWF and FVIII are extensively glycosylated, with N- and O-linked carbohydrates structures constituting approximately 20% of their molecular mass. Importantly, these glycans have been shown to influence VWF and FVIII biology. However the molecular mechanisms through which these glycan structures serve to modulate VWF and FVIII physiology remains poorly understood. In this context, the ability of VWF and FVIII to interact with lectins is likely to play an important role. Recently VWF has been shown to circulate in plasma complexed with specific lectins galectin-1 and galectin-3. Moreover, this interaction was found to directly modulate VWF-dependent early thrombus formation *in vivo*. On this basis, we sought to define the molecular basis underlying the VWF-galectin-1 interaction.

Galectin-1 was expressed and purified from *E.coli* using nickel affinity chromatography. Plasma-derived and blood group specific VWF was purified by ethanol cryoprecipitation and gel filtration chromatography. To characterize the specific VWF glycan determinants involved in mediated galectin-1 binding, the glycosylation profile of VWF was modified via exoglycosidase digestion.

Interestingly, the N-linked VWF glycan structures were shown to critically mediate galectin-1 binding. Furthermore specific roles for terminal sialic acid and ABO(H) blood group determinants expressed on VWF glycans were observed. Utilising domain truncations of VWF, an important role for the VWF A domains was identified. In support of this finding, site-directed mutagenesis of the two N-linked glycans in the A domains, (N1515 and N1574), significantly attenuated galectin binding.

The glycan profiles of FVIII and VWF share significant similarities. Consequently we hypothesised that galectins may also constitute a novel lectin family capable of binding human

FVIII. Interestingly we observed that both galectin-1 and galectin-3 bound to rFVIII in a dose-dependent and N-glycan dependent manner. Importantly, in keeping with the qualitative and quantitative differences in glycosylation between different commercial rFVIII concentrates both galectin-1 and galectin-3 bound with distinct affinities. Importantly, the FVIII-galectin interaction served to directly modulate FVIII activity. In particular, *in vitro* studies demonstrated a specific role for galectin-1 in negatively regulating FVIII cofactor activity.

VWF glycosylation is known to critically regulate its plasma half-life. Moreover VWF is also a key factor in determining the survival of FVIII. Despite their physiological relevance, the molecular mechanisms through which the VWF glycans modulate its survival remain poorly understood.

To investigate the role of VWF glycans and specific VWF domains in regulating VWF clearance, we expressed and purified a series of recombinant VWF variants and truncations with/without specific glycan sites. Subsequently, VWF variants and glycoforms thereof were intravenously injected into VWF^{-/-} mice, and plasma VWF clearance rates determined by ELISA. VWF-macrophage interactions were also quantified *in vitro* using THP-1 cells.

We observed an important role for VWF A domains in influencing VWF clearance in a macrophage-mediated manner in VWF^{-/-} mice. The A domains of VWF contain two complex-type N-linked glycans at N1515 and N1574. Site-directed mutagenesis of N1515 resulted in rapid VWF clearance *in vivo* and enhanced macrophage binding *in vitro*. Interestingly macrophage depletion served to significantly prolong the half-life of this VWF glycan variant. Collectively these data suggests that specific removal of the VWF glycan at N1515 enhances macrophage-mediated clearance of VWF via critical sites localised within the VWF A domains. Interestingly the presence of an intra-chain disulphide bridge within the A2 domain, to restrict its unfolding in a manner homologous to the A1 and A3 domains of VWF, normalised the rapid clearance associated with the glycan mutation N1515Q. In conclusion, our novel data identify an important role for VWF A domains in regulating macrophage-mediated VWF clearance. In

addition, we further demonstrate that the N-linked glycans structures located at within the A2 domain play specific roles in protecting VWF against *in vivo* clearance by macrophages. Our data suggests that VWF glycans serve to shield critical macrophage interactive sites within VWF A2 domain. Given the role of enhanced clearance of VWF in patients with VWD, these findings are of direct clinical importance.

Abbreviations

ADAMTS13: A Disintegrin and Metalloprotease with Thrombospondin repeats

$\alpha_M\beta_2$: alpha-M beta-2 integrin; CD11b/CD18; Mac-1

ASGPR: Asialoglycoprotein Receptor

ASOR: Asialo-orosomucoid protein

β_2 -GP1: β_2 -glycoprotein 1

BDD-FVIII : B Domain Deleted Factor VIII

BSA: Bovine Serum Albumin

BHK: Baby Hamster Kidney cells

Ca^{2+} : Calcium

cAMP: Cyclic adenosine monophosphate

CHO: Chinese Hamster Ovary cells

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 immunosorbant assay

ER: Endoplasmic reticulum

Fuc: Fucose

FVIII: Factor VIII

FVIIIa: Activated FVIII

FIX: Factor IX

FIXa : Activated Factor IX

FX: Factor x

FXa: Activated Factor x

Gal: Galactose

GalNAc: N-acetylgalactosamine

GdCl₃: Gadolinium chloride

GlcNAc: N-acetylglucosamine

GPIb α : Platelet glycoprotein Ib α

GPIIb-IIIa: Platelet glycoprotein IIb/IIIa

HEK: Human Embryonic Kidney cell

HMWM: High Molecular Weight Multimers

HUVECs: Human Umbilical Vein Endothelial cells

K_{on} : Association rate constant

K_{off} : Dissociation rate constant

LacNAc: N-acetylglucosamine

LSEC: Liver Sinusoidal Endothelial cells

LDL: Low Density Lipoprotein

LLG: Leucine - Leucine - Glycine adhesion motif
 LMWM: Low Molecular Weight Multimers
 LRP: Low density lipoprotein related receptor
 MRT: Mean Residence Time
 NeuAc: N-acetylneuraminic acid; sialic acid
 NIF: Neutrophil Inhibitory Factor
 OPG: Osteoprotegerin
 Pd-FVIII: Plasma-derived FVIII
 Pd-VWF: Plasma-derived VWF
 Plt-VWF: Platelet VWF
 PMA: Phorbol Myristate Acetate
 PNG: Peptide-*N*-Glycosidase F
 PSGL-1: P-selectin glycoprotein ligand 1
 RAP: Receptor Associated Protein
 RGD: Arginine - Glycine - Aspartic Acid motif
 rFVIII : Recombinant Factor VIII
 rVWF : Recombinant VWF
 SEM: Standard error of the mean
 Siglec: Sialic acid binding immunoglobulin-like lectin
 SPR: Surface Plasmon Resonance
 TGF- β : Transforming Growth Factor
 THP-1: Human acute monocytic leukaemia cell
 TRAIL: Tumour necrosis factor-related apoptosis-inducing ligand
 TSP-1: Thrombospondin-1
 TTP : Thrombocytopenia Purpura
 UL-VWF: Ultra-large VWF
 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
 VWF^{-/-} mice: VWF deficient mice
 WPB: Weibel-Palade Bodies
 WT: Wild-type

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1 Introduction

1.1 von Willebrand Factor

von Willebrand factor (VWF) is a large multimeric adhesive glycoprotein that exerts critical functions in primary haemostasis. First, VWF serves as a molecular bridge mediating platelet tethering to exposed subendothelial collagen at sites of vascular injury (figure 1.1). Second, VWF also acts as a carrier molecule for procoagulant factor VIII (FVIII). This interaction prolongs the survival of FVIII in circulation from ~2 hours in the absence of VWF, to 12 hours when FVIII circulates in complex with VWF (Lollar, 1991).

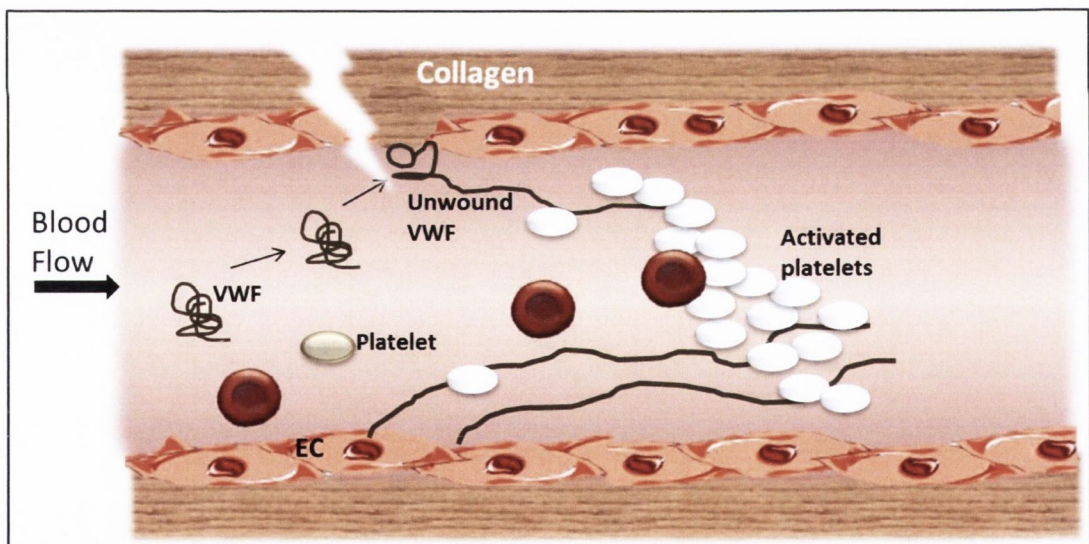


Figure 1.1 The role of VWF in haemostasis

Within the normal population there is significant variation in plasma VWF antigen levels (5-20 μ g/mL). von Willebrand disease (VWD) is the most common inherited bleeding disorder and results from qualitative and/or quantitative VWF

deficiency (Sadler *et al*, 2006). VWD affects up to 1% of the general population, and can be classified into three major groups. Type 1 involves a partial quantitative deficiency of VWF. Type 2 VWD is caused by a variety of qualitative defects in the VWF glycoprotein. Finally, type 3 VWD is rare but involves a complete absence of VWF. Due to the variety of functional defects involved, VWD type 2 can be further sub-grouped. Type 2A includes variants with decreased platelet adhesion caused by reduction in high-molecular-weight VWF multimers (HMWM). Type 2B includes VWF variants with gain of function mutations for platelet glycoprotein 1b (GPIb α). Type 2M VWF mutations result in defective adhesion of VWF to platelets and collagen despite normal multimer distribution. Finally, type 2N VWD is characterised by decreased affinity for FVIII. Table 1.1 summarises the classification of VWF based on the Scientific and Standardization Committee on von Willebrand factor of the International Society of Thrombosis and Haemostasis (Sadler *et al*, 2006).

Type	Description
1	Partial quantitative VWF deficiency
2	Qualitative VWF defects
2A	Decreased VWF-dependent platelet adhesion and a reduction in HMWM
2B	Increased GPIb α affinity, reduction in HMWM
2M	Defects in VWF-platelet and collagen interactions with normal multimers
2N	Reduced FVIII affinity
3	Virtually complete deficiency of VWF (less than 0.05 IU/mL)

Table 1.1 Classification of VWD

Conversely, pathologically elevated VWF plasma levels have also been associated with disease. Several studies have demonstrated that increased plasma

VWF is associated with an enhanced risk of venous thromboembolism (Nossent *et al*, 2006), myocardial infarction (Crawley *et al*, 2008) and stroke (Bongers *et al*, 2006).

1.2 VWF Biosynthesis

1.2.1 VWF gene and tissue expression

The *VWF* gene is located on the short arm of chromosome 12 (12p13.2), spanning 178kb of genomic DNA with 52 exons (Ginsburg *et al*, 1985). The primary translational product, pre-pro-VWF encodes 2813 amino acids comprised of a 22 residue signal peptide, a 741 residue propeptide and a 2050 residue mature protein (Titani *et al*, 1986). In addition a *VWF* pseudogene is located on the long arm of chromosome 22 (22q11-13). The pseudogene corresponds to exons 23-34 of the *VWF* gene. This pseudogene does not produce a functional transcript due to the presence of nonsense mutations (Mancuso *et al*, 1991).

In vivo biosynthesis of VWF is limited to endothelial cells (EC) and megakaryocytes (Jaffe *et al*, 1974; Nachman *et al*, 1977). Moreover, Yamamoto *et al* described significant differences in VWF expression across organ-specific vascular beds (Yamamoto *et al*, 1998). The highest mRNA VWF levels were detected in lung and brain EC, while relatively low levels were found in liver and kidney microvessels. Overall, highest VWF expression was demonstrated in venous EC compared to EC of an arterial origin. This finding was confirmed by immunohistochemical studies in the porcine vascular system where VWF was found to be expressed in all venous EC but absent from arterial EC (Rand *et al*, 1987).

1.3 VWF domain structure

The VWF translational protein is composed of a series of repeated homologous domains (A-D). Historically the VWF domain structure was given as D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-COOH (N- to C-terminal, figure 1.2A). Recently however, electron microscopy as well as sequence alignment analysis has provided further details on the structure of VWF resulting in a re-annotation of the domains (figure 1.2B) (Zhou *et al*, 2012).

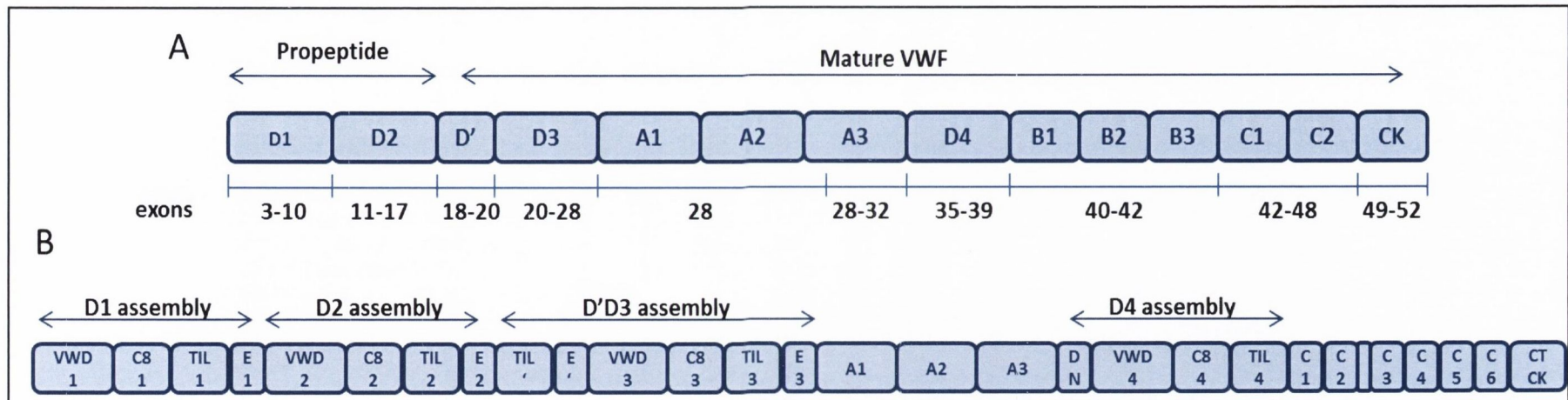


Figure 1.2 Domain structure of VWF

(A) The historical domain illustration of VWF

(B) The re-annotation of VWF domains in accordance with Zhou *et al*

1.3.1 The D domains

The VWF propeptide is comprised of the D1 and D2 domains. The mature VWF subunit includes a truncated D' domain as well as complete D3 and D4 domains. Zhou *et al* have recently sub-divided the D domains based on structure into von Willebrand D (VWD), 8-cysteine (C8), trypsin inhibitor-like domain (TIL), fibronectin type 1-like domains (E) and a unique D4N module in D4 (Zhou *et al*, 2012). D' is a truncated D assembly containing only a TIL and an E subdomain. In contrast the D1, D2 and D3 assemblies comprise VWD, C8, TIL and E subdomains, while the D4 assembly lacks the E subdomain but carries D4N module (figure 1.2B).

1.3.2 The A domains

Each monomer of VWF contains three large A domains. These von Willebrand A (VWA) domains are found in a number of other plasma proteins including complement factors, the I-domain of integrins and collagens (Perkins *et al*, 1994). The crystal structures of the A1, A2 and A3 domains have been determined (Emsley *et al*, 1998; Zhang *et al*, 2009; Huizinga *et al*, 1997). A feature of all three A domains is an intra-chain disulphide bond. Both A1 and A3 contain long range disulphide bridges which result in a ~186 amino acid loop. The A2 domain is unique in that it contains a vicinal disulphide bond which is important in modulating stability (Luken *et al*, 2010). Furthermore, the A2 domain is further characterised by a calcium binding site at its core which is also important in determining stability of the domain (Zhou *et al*, 2011a).

1.3.3 The C domains

The former VWF B and C domains have recently been re-annotated as 6 tandem von Willebrand C (VWC) and VWC-like domains (Zhou *et al*, 2012). The VWF

C1, C3 and C5 domains are classified as VWC domains on the basis of their sequence homology. The intervening domains C2, C4 and C6 are designated VWC-like domains. A number of other human proteins share similarities with VWC domains. These include glycosylated mucins and matrix proteins that modulate growth factor responses such as the TGF- β family (Bork, 1993) .

1.3.4 The CK domain

The C-terminal ~90 residues comprises the CK domain or cysteine knot (CTCK). This motif contains six conserved cysteines arranged in a knot-like topology. It is a common structural motif found at the C-terminus of growth factors, such as nerve growth factor, transforming growth factor and platelet-derived growth factor (Meitinger *et al*, 1993). These proteins display a similar tendency to dimerise via disulphide bonds formation. The VWF CK domain contains 11 cysteine residues, 8 of which have been shown to be involved in intra-molecular disulphide bond formation (Katsumi *et al*. 2000).

1.4 Post-translational modification

Within EC, the pre-pro-VWF primary translational protein product undergoes extensive post-translational modification prior to secretion (Wagner, 1990). This includes signal peptide cleavage, C-terminal dimerization, N- and O-glycosylation, sulfation, N-terminal multimerisation and proteolytic cleavage of the propeptide (figure 1.3).

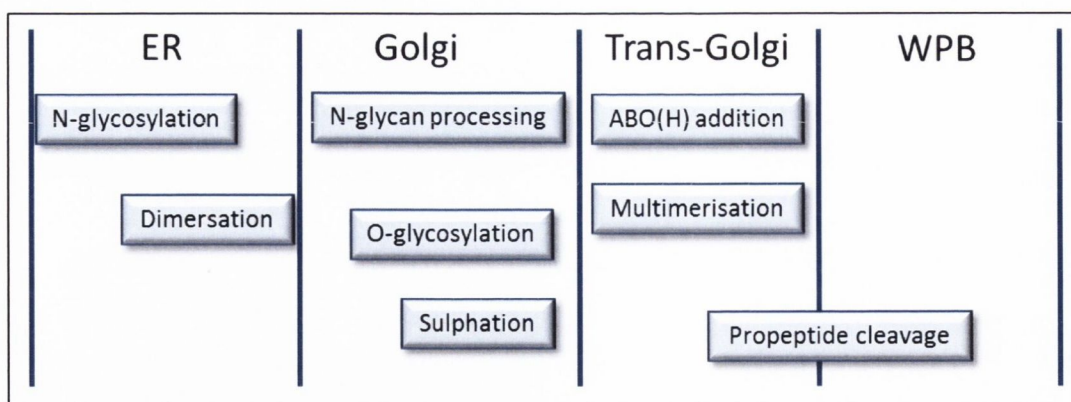


Figure 1.3 Post-translational modifications of VWF.
The intracellular compartments important for VWF post-translational events.

1.4.1 N-linked glycosylation

Following synthesis, the 2813 amino acid pre-pro-VWF product is transferred to the endoplasmic reticulum (ER). Here signal peptide cleavage occurs to form pro-VWF. Within the ER, high mannose oligosaccharides are also coupled to the nascent polypeptide chain. This occurs as a co-translational event. The high mannose oligosaccharides are attached at consensus N-linked glycan sites (NXS/T, where X can be any amino acid except proline). A total of 12 of these potential sites exist within each mature VWF subunit, and a further 4 sites are present within the propeptide region (Titani *et al*, 1986). Titani *et al* also report the presence of a rare N-glycan sequence, NXC within the D3 domain. Figure 1.4 depicts the N-linked glycan map of VWF.

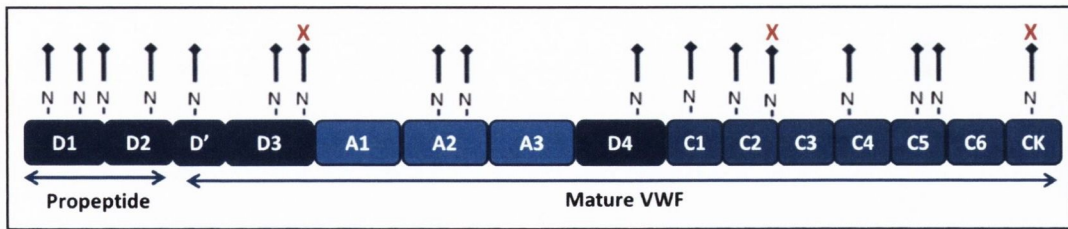


Figure 1.4 VWF N-linked glycan sites

Potential N-linked sites are located at N99, N156, N257, N666 in the propeptide and N857, N1147, N1231, N1515, N1574, N2223, N2290, N2357, N2400, N2546, N2585, N2635 and N2790 in the mature VWF subunit. The red X indicates N-linked sites whose glycosylation has not yet been confirmed (N1231, N2400 and N2790).

The primary 14-sugar core N-linked glycan structure is subsequently remodelled by a series of glycosyltransferases and glycosidases as the protein passes through the ER and Golgi (Schwarz & Aepli, 2011). Within the ER exoglycosidases glucosidase 1 and 2 mediate sequential glucose trimming of the pre-assembled N-glycans.

1.4.2 Dimerization

In the ER pro-VWF monomers form “tail to tail” dimers via C-terminal disulphide bond formation. Attachment of core N-linked glycan structures is a prerequisite for efficient dimerization (Wagner *et al*, 1986). Previous studies have also demonstrated that only dimeric VWF molecules can exit the ER. Dimerization is dependent on the last 150 residues of the C-terminus of VWF including the cysteine knot domain (Marti *et al*, 1987).

1.4.3 Processing of N-linked glycans

After dimerization in the ER, the pro-VWF is transported to the Golgi where the N-linked glycans are further processed and O-glycosylation takes place. The primary N-linked high-mannose oligosaccharides are modified by the addition of galactose (Gal)

and N-acetylglucosamine (GlcNAc) residues resulting in the formation of complex-type glycan structures. Moreover, sialyltransferases mediate the addition of capping sialic acid (NeuAc) onto the N-linked glycans of VWF. HPLC studies have shown that almost 80% of the total sialic acid content on VWF is expressed on N-linked complex-type glycans where it is usually α 2-6 linked (McGrath *et al*, 2010). In contrast α 2-3 linked sialic acid is predominantly found on the O-linked glycans of VWF.

The glycans of VWF have been recently characterised by mass spectrometry. The N-linked glycans are extremely heterogeneous in nature. Less than 1% of the N-linked glycans are present as high mannose structures (Canis *et al*, 2012). The most common N-linked structure detected on plasma-derived VWF (pd-VWF) was an α 2-6 linked monosialylated biantennary complex-chain (Matsui *et al*, 1992; Canis *et al*, 2012) (figure 1.5). Other features include tri- and tetra-antennary chains, core fucosylation and poly-lactosamine extensions. The variety of modification gives rise to the presence of a heterogeneous population of glycan chains expressed on circulating plasma VWF (figure 1.5). VWF glycans within the D3 domain are also reported to be further modified by the addition of sulfate (Carew *et al*, 1990). The process of sulfation occurs in the trans-Golgi network and can occur on sialylated glycans (Canis *et al*, 2012).

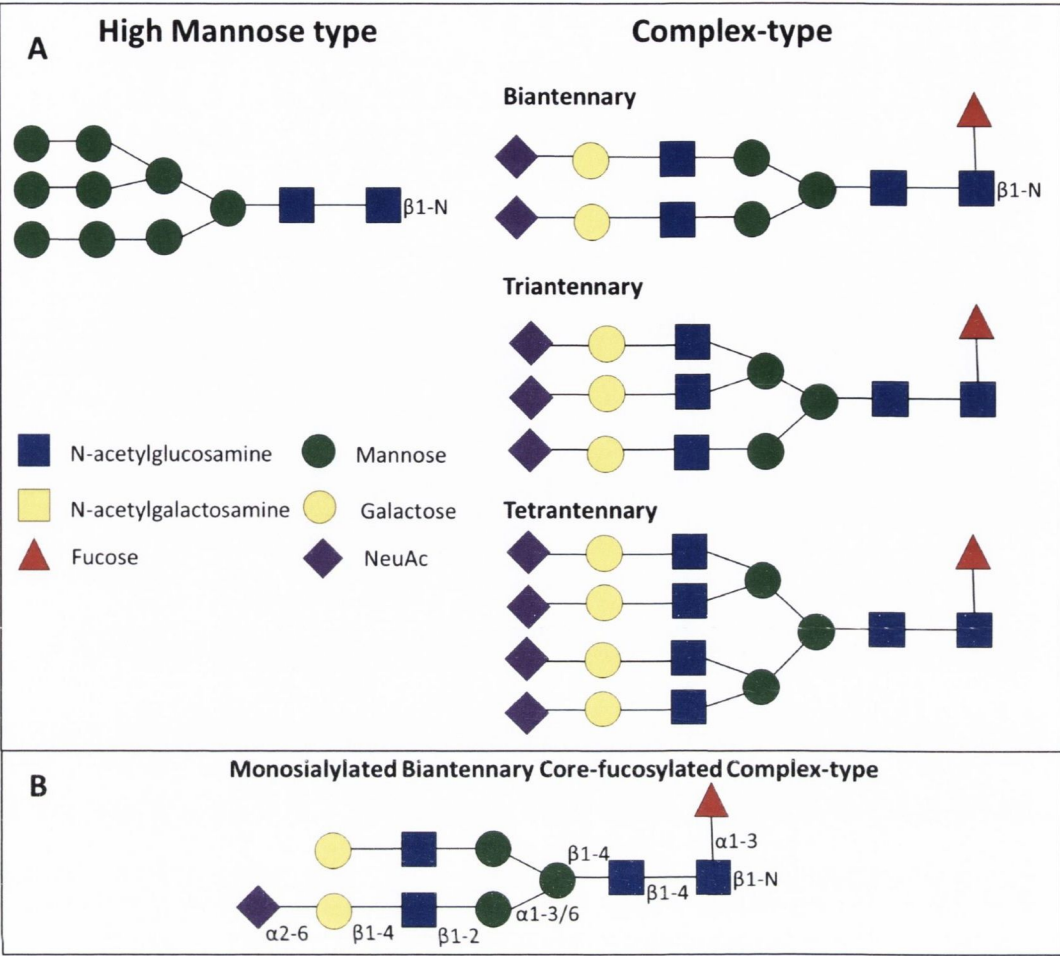


Figure 1.5 N-glycome of VWF
(A) The heterogeneous variety of major glycans described for VWF, including both complex-type and high mannose oligosaccharides.
(B) The most common N-linked glycan structure (~78%) in the N-glycome of VWF.

1.4.4 Addition of ABO(H) blood group antigens

The N-linked glycans of VWF are unusual in that they express covalently linked terminal ABO(H) blood group determinants. (Matsui et al. 1992). Within EC, fucosyltransferase (FUT1) competes with $\alpha 2-6$ sialyltransferases in order to incorporate terminal $\alpha 1-2$ fucose. Consequently, fucosylation is restricted to non-sialylated termini, or those carrying $\alpha 2-3$ sialylation. The addition of $\alpha 1-2$ fucose to the N-glycans termini creates the H-antigen [Fuc ($\alpha 1-2$) Gal ($\beta 1-4$) GlcNAc ($\beta 1-$)] which is

the terminal glycan expressed in blood group O individuals (figure 1.6). This H-antigen serves as an essential precursor for the subsequent development of blood group A or B antigens. The A-antigen (N-acetylgalactosamine) and the B-antigen (galactose) are generated by specific A and B glycosyltransferase enzymes further modifying the H antigen (Yamamoto, 2000). Individuals with the rare Bombay blood group do not express the H antigen (Bhende *et al*, 2008) and consequently cannot express terminal A or B antigens regardless of their genotype.

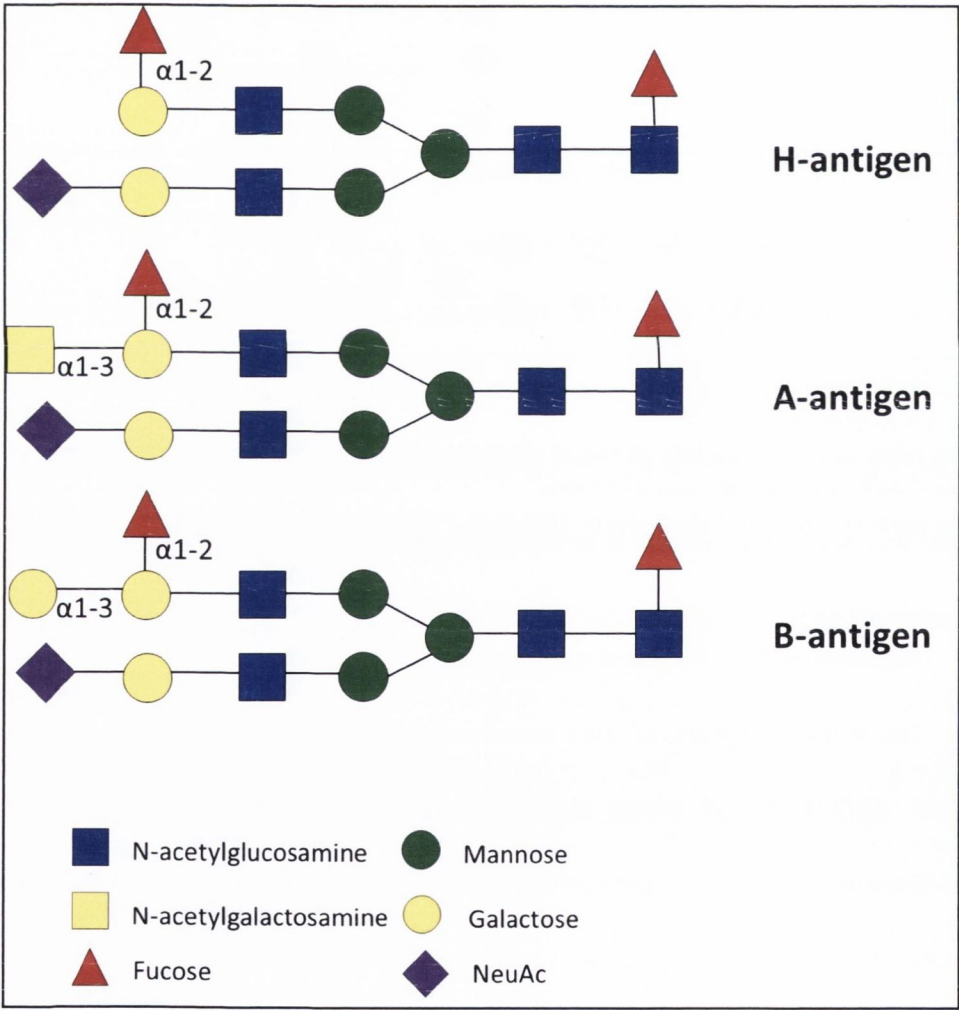


Figure 1.6 Structure of VWF blood group antigens on VWF N-linked glycans

ABO(H) antigens are found on ~13% of VWF N-linked glycans (Matsui *et al*, 1992). Mass spectrometry analysis recently confirmed that ABO(H) structures are present on all but one of the N-glycan sites of VWF (N1147) (Canis *et al*, 2012). However the relative abundance of ABO(H) expression at each glycan site varies. Significantly, these data suggests that the addition of α 1-2 fucose is not a site-specific process. The most heavily fucosylated site is N2635 in the C5 domain with H-antigen expression levels of approximately 16%. In contrast, N857, N2290 and N2585 represent poorly fucosylated sites, containing only 1-3% H-antigen (figure 1.7).

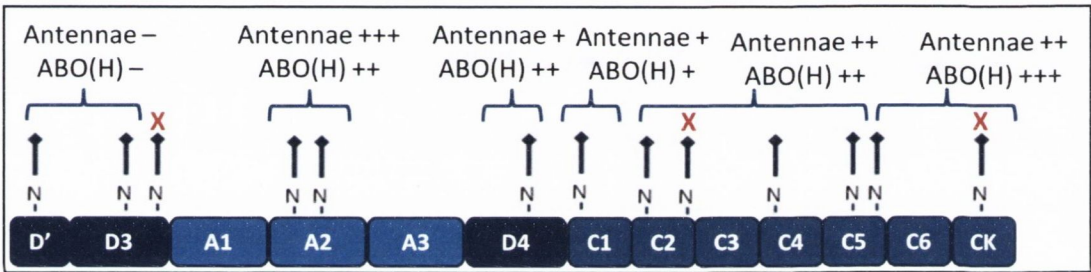


Figure 1.7 N-glycan sites and expression ABO(H) antigens
Glycan specific illustration of relative ABO(H) expression at N-linked glycan sites. The most heavily fucosylated site is in the C5 domain while the two glycans in A2 demonstrate the highest level of branching.

1.4.5 O-linked glycosylation

O-linked glycosylation occurs in the Golgi and is characterised by the addition of GalNac to the hydroxyl group of either serine or threonine residues. This is followed by the addition of β 1-3 Gal creating core 1 O-glycan. This structure is capped with two sialic acid residues and is termed the sialylated T-antigen; [NeuAc (α 2-3)] Gal (β 1-3) [NeuAc(α 2-6)]GalNAc (Samor *et al*, 1989). This structure accounts for 75% of the total O-linked glycan population expressed on VWF (figure 1.8). Additionally, a minor portion of approximately 10% of O-glycans are characterised as core 2 structures

(Canis *et al*, 2010). Mass spectrometry analysis has reported the presence ABO(H) blood group antigens on a proportion of these core 2 structures accounting for approximately 1% of the total VWF O-glycan population (Canis *et al*, 2010). The O-linked glycans of VWF are extensive sialylated. For example, nearly 40% of core 1 structures on VWF were shown to express unusual disialosyl motifs, in which 2 sialic acid moieties are combined via an α 2-8 linkage (figure 1.8).

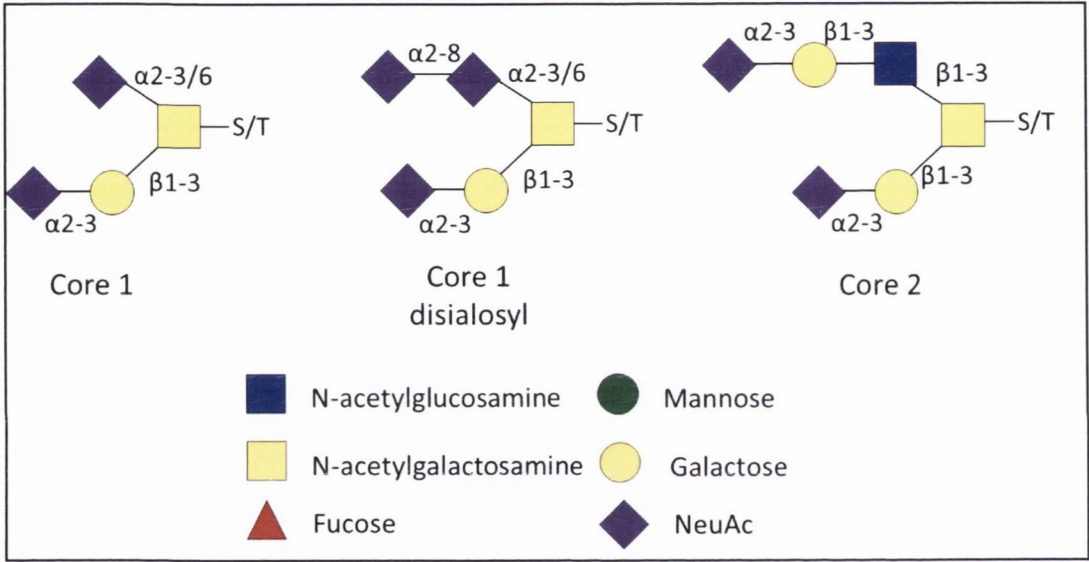


Figure 1.8 O-glycome of VWF

VWF contains ten putative O-glycan sites (figure 1.9). Eight of these sites cluster in groups of four at the N-and C-terminal of the A1 domain. The remaining two O-glycans sites are in the A3 and D4 domains (Samor *et al*, 1989).

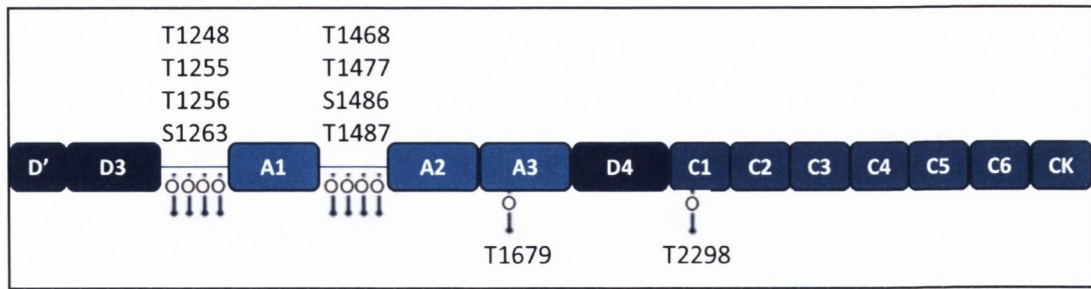


Figure 1.9 O-linked glycan sites in VWF
 Potential O-linked sites are located at T1248, T1255, T1256 and Ser1263 at the N-terminal A1 flanking region and T1468, T1477, S1486 and T1487 at the C-terminal A1 flanking region. Single O-linked glycan sites are found at S1679 in A3 and S2298 in C1.

1.4.6 Multimerisation

Mature VWF circulates as a series of large heterogeneous multimers. VWF polymerisation is initiated by C-terminal dimer formation within the ER. However in the trans-Golgi, VWF dimers undergo further “head-to-head” multimerisation via additional disulphide bond formation in the N-terminal region (figure 1.10). In particular site-directed mutagenesis has identified that C1099 and C1142 represent critical residues essential for VWF multimerisation (Purvis *et al*, 2007). In keeping with the acidic environment of the Golgi, VWF multimerisation is highly pH-dependent. *In vitro* treatment of EC to increase the pH ablates VWF multimerisation (Wagner *et al*, 1986). Recent electron microscopy studies have demonstrated that the acidic pH of the trans-Golgi (pH 6.2) enables VWF dimers to form dimeric bouquets (Zhou *et al*, 2011b).

The VWF propeptide (D1D2) is essential for normal VWF multimerisation (Mayadas & Wagner, 1989). The propeptide displays intrinsic protein disulphide isomerase (PDI) activity, and thus functions as a chaperone for VWF multimerisation within the Golgi. Vicinal cysteines within the propeptide enable this region to catalyse

thiol protein disulphide exchange between adjacent VWF dimers (Mayadas & Wagner, 1992).

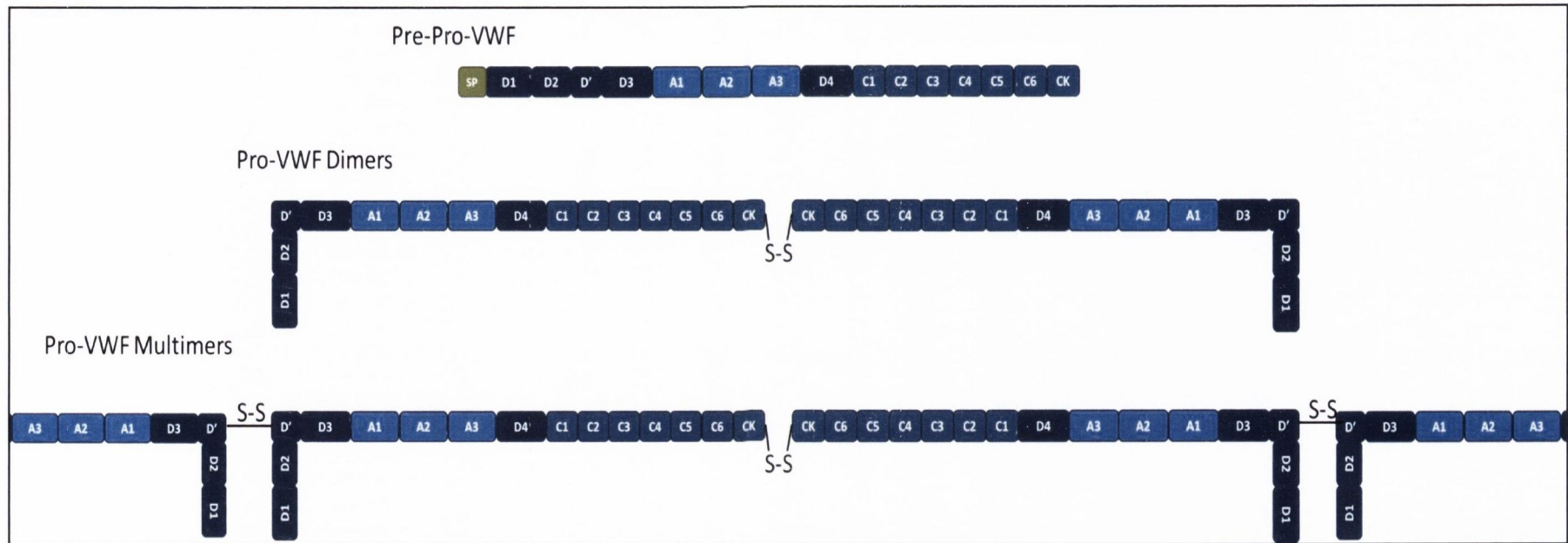


Figure 1.10 VWF polymerisation

1.4.7 Intracellular Packaging

Within EC, VWF is partitioned between two pathways in the Golgi. The majority of VWF undergoes constitutive secretion into the plasma. The remainder (approximately 5%) is stored in cytoplasmic granules called Weibel-Palade bodies (WPB) (Sporn *et al*, 1989). Within WPB, VWF multimers are assembled into ordered tubules for storage. This ordered packaging facilitates subsequent unwinding and secretion of VWF multimers upon stimulation. Tubular assembly requires low pH and calcium ions presence within the trans-Golgi. Additionally, VWF propeptide and D'D3 domains are required for VWF tubular packaging (Huang *et al*, 2008).

1.4.8 Propeptide cleavage

Cleavage of the propeptide region of VWF occurs in a calcium-dependent manner mediated by intracellular furin. This final step in VWF biosynthesis occurs in both the trans-Golgi and within the storage granules. Furin cleaves at S763 following the dibasic amino acid pair K761 and R762 within the C-terminal of D2 (Rehemtulla and Kaufman, 1992). Removal of the propeptide is essential for efficient FVIII binding (Wise *et al*, 1991). After cleavage, the propeptide remains non-covalently associated with VWF. Thus a 1:1 stoichiometry of mature VWF subunit to VWF propeptide is observed in normal plasma (Wagner *et al*, 1987). However, VWF propeptide clearance occurs independently of VWF. Importantly, the VWF propeptide has a significantly shorter half-life of just 2-3 hours compared with a half-life of 8-14 hours for mature VWF (Borchiellini *et al*, 1996).

1.5 VWF storage and secretion

VWF is stored in endothelial WPB and in platelet α -granules. Both organelles store high molecular weight multimeric (HMWM) VWF which is secreted through a regulated pathway following stimulation.

1.5.1 Weibel Palade Bodies

Weibel-Palade bodies (WPB) are rod-shaped, membrane-bound organelles within EC (Weibel and Palade, 1964). They are approximately 4 μ M in length and 0.1 μ M in diameter. They contain bundles of tubules, approximately 150Å thick, embedded in a dense matrix and orientated parallel to the long axis of the rod body (Wagner *et al*, 1991). Formation of these storage bodies is dependent on VWF synthesis. Expression of VWF in non-EC results in the formation of pseudo-WPB, which share their characteristic rod shape and contain VWF (Voorberg *et al*, 1993). WPB store VWF multimers and VWF propeptide as their major constituents. In addition WPB also contain a number of other components including P-selectin (Bonfanti *et al*, 1989), oestoprotegrin (Zannettino *et al*, 2005) and angiopoietin-2 (Fiedler *et al*, 2004). WPB release their constituents by means of regulated exocytosis. Secretagogues including vasopressin analog 1-desamino-8-Darginine vasopressin (DDAVP) and adrenaline activate cAMP-dependent signalling causing a rise intracellular calcium stimulating exocytosis of WPB (Kaufmann *et al*, 2000).

1.5.2 Platelet α granules

In contrast to EC, VWF biosynthesis within megakaryocytes is poorly understood. Platelet VWF is stored in α -granules within platelets. Like WPB, α -granules arise from the trans-Golgi network and VWF multimers can be visualised by electron

microscopy as longitudinal arrays (Cramer *et al*, 1985). These α -granules appear during megakaryocytic maturation and also store a number of other molecules including fibrinogen, thrombospondin, Factor V and platelet factor-4 (Harrison & Cramer, 1993).

1.6 VWF structure and function

1.6.1 FVIII binding

FVIII circulates in non-covalent complex with VWF in plasma. VWF binds to the N-terminal light chain of FVIII with a critical role for acidic amino acid residues 1669-1689 in the a3 domain of the FVIII light chain (Lollar *et al*, 1988). An additional role for the C2 domain of the light chain in FVIII has been described in mediated VWF binding (Saenko & Scandella, 1997). VWF serves to protect FVIII from premature proteolytic inactivation by activated protein C (APC), stabilises its heterodimer structure, and prolongs its survival in plasma (Weiss *et al*, 1977; Fay *et al*, 1991). The critical physiological relevance of the VWF-FVIII interaction is highlighted by the markedly reduced FVIII plasma levels in patients with undetectable VWF levels (VWD type 3), and in patients with a defect in the FVIII-interactive site of VWF (VWD type 2N). Following activation by thrombin, FVIII is cleaved at R1689 causing it to dissociate from VWF and interact with phospholipids and Factor IXa to participate in the tenase complex.

The FVIII binding region within VWF has been localized to residues S764-R1035 within the N-terminus of the D'D3 domains (Foster *et al*, 1987). Moreover a number of VWD type 2N mutations cluster within this region. Recently, S764 and K773 have been highlighted as critical residues for modulating FVIII binding (Castro-Núñez *et al*, 2013).

Each monomer of multimeric VWF contains a single FVIII binding site. However within normal plasma the FVIII:VWF stoichiometry is approximately 1:50 (Vlot *et al*, 1995).

1.6.2 Subendothelium matrix binding

At sites of vascular injury, VWF mediates platelet adhesion to the vascular subendothelium by binding to collagen. Fibrillar collagens type I and III are most important for VWF binding (Pareti *et al*, 1987). Collagen binding is mediated via the A1 and A3 domains of VWF (Cruz *et al*, 1995). Deletion of the A1 domain does not alter collagen binding compared to wild-type (Sixma *et al*, 1991). However deletion of the A3 domain significantly attenuated collagen III binding. Furthermore site-directed mutagenesis has revealed a critical role for the H1786 residue in the A3 of domain of VWF in modulating collagen III binding (Romijn *et al*, 2001). The physiological relevance of this mutation has been highlighted in a mouse vascular injury model. Mice expressing H1786A collagen binding mutant demonstrated significantly reduced thrombosis in a ferric-chloride injury model (Marx *et al*, 2008). Consequently, the A3 domain is considered the major physiological collagen III binding region. The VWF A1 domain has been shown to mediate binding to nonfibrillar collagen VI, which is abundant in arterial subendothelium (Hoylaerts *et al*, 1997).

1.6.3 Platelet binding

Following vessel damage, VWF binds to subendothelial collagen and subsequently tethers platelets and supports platelet aggregation to facilitate thrombus formation. Platelets express two distinct receptors for VWF: the GPIb-IX-V complex and the GPIIb-IIIa complex. VWF binding to the GPIb α receptor on non-activated platelets is important in the initial tethering of platelets to the subendothelium (figure

1.11). Once platelets become activated, the GPIIb-IIIa complex becomes an additional high affinity ligand for VWF. This promotes platelet spreading and aggregation, and ultimately the formation of a stable platelet plug. Binding of VWF to platelet receptors is highly dependent on the rate of shear within the vasculature. In the absence of shear stress, VWF adopts a globular, condensed configuration which is non-permissive to GPIIb binding. However shear stress promotes VWF unwinding to form an extended chain that enables interaction with platelet receptors (Siedlecki *et al*, 1996; Ikeda *et al*, 1991). At low rates of shear stress (less than 600 s^{-1}) platelet GPIIb-IIIa can mediate platelet tethering to fibrinogen in a process independent of VWF *in vitro* (Savage *et al*, 1996). In contrast, at higher shear stress VWF is an essential component in platelet tethering. (Savage *et al*, 1996, 1998).

Mohri *et al* identified important regions for mediating GPIIb binding residing within the VWF A1 domain, on either side of the disulphide loop (Mohri *et al*, 1988). This finding was confirmed by Girma *et al* who defined a critical role for residues 1237-1251 and 1458-1471 within the A1 domain (Girma *et al*, 1990). Co-crystal structures of the VWF A1 domain with GPIIb has revealed this critical interface (Dumas *et al*, 2004). In addition, a number of VWD type 2M mutations cluster within this VWF binding site for GPIIb. Moreover, Yago *et al* have further demonstrated that specific VWD type 2B mutations can overcome the need for high shear in order for VWF to be able to bind GPIIb (Yago *et al*, 2008). An additional regulatory region for GPIIb binding within the A1 N-terminal flanking region has also been identified. Auton *et al* determined that structural association between the N-terminal flanking region of the A1 domain (amino acids Q1238-E1260) and the A1 domain negatively regulates GPIIb binding under shear (Auton *et al*, 2012).

GPIIb-IIIa binding to VWF is mediated via a site within the C-terminus of VWF. The amino acid sequence RDGS which constitutes a typical integrin binding motif, has been identified in the C4 domain of VWF. This site is critical in mediating GPIIb-IIIa binding. Beacham *et al* demonstrated that amino acid substitutions into the RGDS site ablated VWF binding to GPIIb-IIIa (Beacham *et al*, 1992). On resting platelets, GPIIb-IIIa does not interact with VWF. However upon platelet activation GPIIb-IIIa displays high affinity binding with VWF via the RDGS sequence.

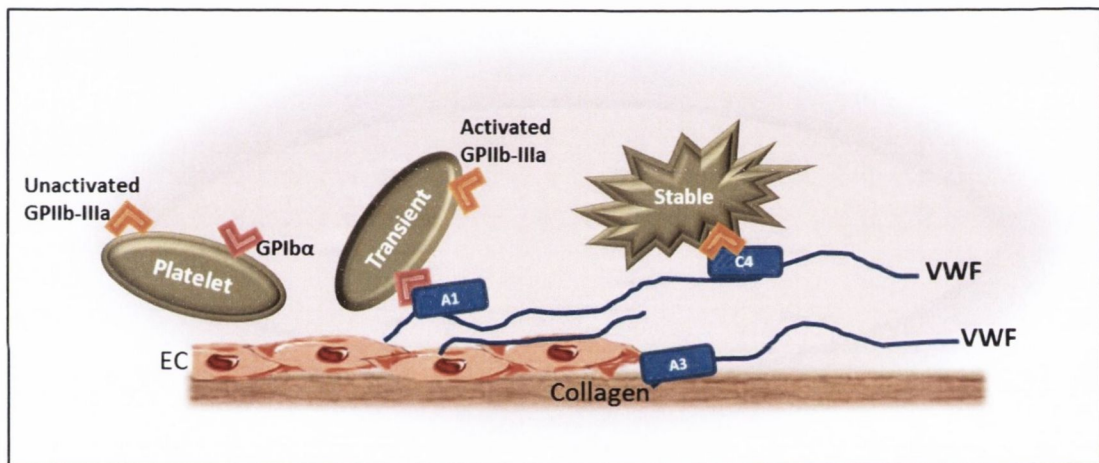


Figure 1.11 Model of VWF-dependent platelet adhesion

1.6.4 Additional interactions

Recent studies have shown that VWF is capable of interacting with a variety of binding partners in normal plasma (Lenting *et al*, 2012). Figure 1.12 illustrates some of binding partners of VWF and the specific domains that regulate their interaction.

P-selectin is a WPB constituent that interacts with VWF, via the D'-D3 domains (Michaux *et al*, 2006). Consequently absence of VWF results in reduced P-selectin expression, and secondary defects in leukocyte recruitment (Denis *et al*, 2001). In

addition, Padilla *et al* reported a role for P-selectin expressed on the endothelium in tethering newly secreted VWF strings (Padilla *et al*, 2004). However subsequently this role for P-selectin was disputed by Huang *et al* who found that the integrin $\alpha V\beta 3$ was more important in tethering VWF strings to EC surface (Huang *et al*, 2009). Like P-selectin, osteoprotegrin (OPG) is another WPB components that has been shown to form a complex with VWF in normal plasma (Zannettino *et al*, 2005). OPG interacts specifically with the VWF A1 domain (Shahbazi *et al*, 2007). The physiological relevance of this interaction has yet to be elucidated. However OPG is a well-established anti-osteoclastic modulator and recent work has suggested that the FVIII-VWF-OPG complex may inhibit the anti-apoptotic effect of OPG on TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) (Baud'huin *et al*, 2009). VWF can also bind to the adhesive matrix glycoprotein thrombospondin-1 (TSP1) via the A3 domain. Interestingly, this interaction serves to competitively inhibit ADAMTS13-mediated proteolysis of ultra-large VWF strings (Wang *et al*, 2010). Conversely, complement factor H binding to the VWF A2 domain acts to enhance ADAMTS13-mediated cleavage of VWF (Feng *et al*, 2013).

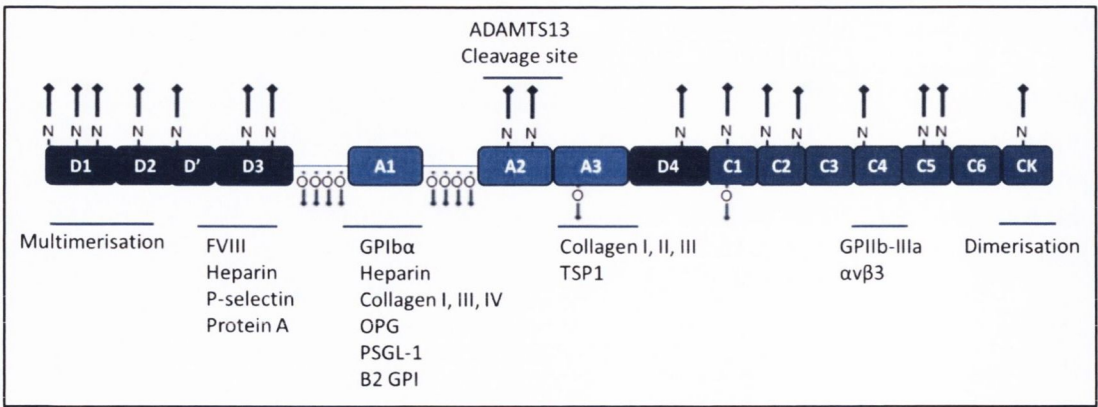


Figure 1.12 Structure-function of VWF

1.7 Platelet VWF

While steady state plasma VWF is primarily derived from EC, VWF stored within platelet α - granules actually accounts for 10-25% of the total circulating VWF (Howard *et al*, 1974). A number of differences exist between VWF of EC origin (plasma-derived, pd-VWF) and platelet-derived VWF (plt-VWF). Plt-VWF is enriched in UL-VWF multimers (Fernandez *et al*, 1982). Similar collagen binding activities have been reported for plt- and pd-VWF. In contrast, plt-VWF has a significantly reduced affinity for platelet receptor GPIb α , despite the fact that it is enriched in UL-VWF multimers (Williams *et al*, 1994). Importantly, plt-VWF has significant differences in glycosylation profile compared to pd-VWF (Williams *et al*, 1994; Kagami *et al*, 2000). In particular plt-VWF has a 2-fold reduction in both sialic acid and sub-terminal galactose expression. Additionally, while the H-antigen is expressed on plt-VWF glycans, no A- or B- blood group antigens have been detected (McGrath *et al*, 2013). Recently the physiological importance of plt-VWF has been defined in studies using chimeric mice (Kanaji *et al*, 2012). Plt-VWF was showed to partially correct bleeding time and significantly decreased blood loss volume compared with VWF deficient mice.

1.8 VWF glycosylation influences its function

Mature VWF expresses 12 N-linked glycans and a further 10 O-linked glycans. Consequently, these carbohydrates constitute approximately 20% of the monomeric mass of the glycoprotein. Moreover, VWF glycan structures have been shown to directly modulate its biosynthesis, susceptibility to ADAMTS13 proteolysis and influence its circulatory survival (table 1.2).

1.8.1 Biosynthesis

Treatment of EC *in vitro* with tunicamycin, which inhibits the addition of the precursor mannose-containing oligosaccharide within the ER, serves to block the secretion of VWF (Wagner *et al*, 1986). McKinnon *et al* further demonstrated that tunicamycin treatment not only reduced VWF secretion, but also targeted the non-glycosylated VWF for degradation within the cells. (McKinnon *et al*, 2010). Moreover EC treatment with castanospermine, to inhibit glucose trimming in the ER, also abolished VWF secretion. To investigate the role of specific glycan sites in regulated VWF synthesis and secretion, site-directed mutagenesis was used to eliminate each individual site. This work revealed a critical role for four specific N-glycan sites in regulating VWF production (McKinnon *et al*, 2010).

1.8.2 Proteolysis

ADAMTS13-mediated proteolysis of VWF is essential in regulating its multimeric distribution and haemostatic activity (Zheng *et al*, 2001). Terminal sialic acid expressed on VWF glycans is important in protecting VWF against proteolytic degradation by serine proteases and plasmin (Federici *et al*, 1984). In contrast however recent data has highlighted a role for sialic acid in specifically targeting VWF for ADAMTS13-mediated proteolysis (McGrath *et al*, 2010). Proteolysis of VWF by ADAMTS13 is also blood-group dependent, such that blood group O VWF is cleaved more rapidly than non-O VWF *in vitro* (Bowen, 2003). Individuals with the rare Bombay blood group demonstrate a further increase in susceptibility to ADAMTS13 cleavage (O'Donnell *et al*, 2005). The importance of VWF glycans in regulating ADAMTS13 proteolysis was further highlighted by site-directed mutagenesis studies. McKinnon *et al* specifically mutated the N-glycan at N1574 within the A2 domain adjacent to the ADAMTS13

cleavage site. This served to significantly enhance ADAMTS13 susceptibility compared to wild-type VWF (McKinnon *et al*, 2008). More recently, specific O-linked glycans have also been shown to regulate ADAMTS13 proteolysis. In a shear based assay, loss of O-linked glycosylation at S1486, or complete removal of cluster 2 O-linked glycans, was also found to enhance proteolysis. In contrast mutation of the O-linked glycans in cluster 1 had no effect on ADAMTS13-mediated proteolysis (Nowak *et al*, 2014).

1.8.3 Platelet and collagen binding

Removal of the O-linked glycans within cluster 1 at the N-terminal of the A1 domain enhances the VWF-GPIb α interaction (Nowak *et al*, 2012). This observation is consistent with additional reports demonstrating that the linker region between D3 and A1 negatively regulates GPIb α binding (Tischer *et al*, 2013). In addition, loss of the cluster 1 O-linked glycans also enhanced adhesion to collagen under shear (Nowak *et al*, 2012). Interestingly mutation of the cluster 2 O-linked glycans did not alter collagen binding or GPIb α interaction (Nowak *et al*, 2014). Collectively the findings suggest that loss of the O-linked glycans at cluster 1 serves to enhance flexibility within the hinge region of D3 and A1 domains influencing both platelet binding and adhesion to collagen.

1.8.4 Plasma half-life

A number of animal models have clearly demonstrated a direct link between variation in VWF glycosylation and altered rates of clearance. Moreover in humans, variation in VWF glycosylation is also an important determinant of half-life. Almost one third of the normal variation in VWF plasma levels is due to the influence of ABO blood group. Blood group O individuals have significantly lower circulating VWF than non-

group O. Accumulating evidence indicates that this variation in plasma level is due to an effect of blood group on VWF clearance (Gallinaro *et al*, 2008).

Process	Effect of VWF glycan variant
Biosynthesis	Decreased with N-less VWF or N-linked glycan mutants N99Q, N857Q, N2400Q and N2789Q
ADAMTS13 Proteolysis	Reduced with asialo-VWF, Enhanced with N1574Q-VWF, Decreased for AB v. O-VWF
Platelet binding	Increased with cluster 1 O-less VWF
Collagen type III binding	Reduced with cluster 1 O-less VWF
Plasma half-life	Reduced with asialo-VWF, Increased with AB v. O-VWF, Reduced with T1255A/T1256A VWF

Table 1.2 Involvement of VWF glycans in its biology

1.9 VWF clearance

Although VWF structure and function have been well characterised the biological basis underlying VWF clearance remains poorly defined. Potential clearance pathways include receptor-mediated endocytosis, proteolytic degradation, renal excretion and extravasation. Given the large molecular weight of VWF the latter two options appear unlikely. Furthermore, studies in animal models have demonstrated that VWF clearance is not dependent on ADAMTS13-mediated proteolysis. For example, the clearance rate of type 2A VWF was not significantly different to wild-type VWF in rats (Stoddart *et al*, 1996). Additionally the plasma half-life of murine VWF was not different in ADAMTS13 deficient mice compared to wild-type (Badirou *et al*, 2010). Currently, receptor-mediated clearance of VWF is therefore considered the most likely mechanism responsible for its circulatory removal.

1.9.1 VWF clearance pathways

Biodistribution studies with radio-labelled human VWF have highlighted that the liver and spleen play a dominant role in clearance of VWF infused in both mice (van Schooten *et al*, 2008) and rabbits (Sodetz *et al*, 1977).

1.9.1.1 Asialoglycoprotein receptor

The hepatic lectin asialoglycoprotein clearance receptor (ASGPR) was the first described VWF clearance receptor (figure 1.13) (Grewal *et al*, 2008). ASGPR is a member of the calcium-dependent (C-type) lectin family of receptors and shares homology with the human macrophage lectin expressed on macrophages and dendritic cells (Valladeau *et al*, 2001; Li *et al*, 1990). It is encoded by two separate genes (*ASGR1* and *ASGR2*) on chromosome 17. ASGPR exists as a hetero-oligomeric receptor composed of two homologous transmembrane polypeptides (Spiess & Lodish, 1985). ASGPR is abundantly expressed on hepatocytes (Steer & Ashwell, 1980). Glycoproteins are endocytosed via a clathrin-coated pit pathway, dissociate from the ASGPR intracellularly in a pH-dependent manner, and are then targeted for lysosomal degradation (Bridges *et al*, 1982). Several factors regulate avidity of ligand binding including sugar specificity (60-fold greater affinity for GalNAc > Gal) (Baenziger & Maynard, 1980). However, ASGPR can also recognise particular sialylated structures including terminal α 2-6 linked sialic acid which is abundantly expressed on the N-linked glycans of VWF (Park *et al*, 2005). Ligand valency is also important, as clustering of terminal sugars results in increased occupancy of each ASGPR subunit. Consequently branched glycan chains are cleared by ASGPR more efficiently (tetra-antennary > tri > bi > mono) (Connolly *et al*, 1982). A high degree of ASGPR conservation suggests a critical role for this lectin in the clearance of abnormally glycosylated glycoproteins.

However to date only two endogenous ligands (VWF and platelets) have been described for ASGPR (Grewal *et al*, 2008). Mice with a genetic inactivation of ASGPR-1 subunit have a 1.5-fold increase in circulating VWF plasma levels (Grewal *et al*, 2008).

1.9.1.2 Macrophages

Immunohistochemical staining studies performed following VWF infusion revealed localization of VWF within the macrophage-rich regions of both liver and spleen (van Schooten *et al*, 2008). Moreover VWF specifically colocalized with CD68+ Kupffer cells in the liver, and with F4/80 macrophages in the red pulp of the spleen. Subsequent *in vivo* studies demonstrated that chemical inactivation of macrophages via gadolinium chloride treatment significantly prolonged VWF survival in VWF^{-/-} mice. These data suggest that hepatic macrophages or Kupffer cells may play a critical role in regulating VWF clearance *in vivo* (van Schooten *et al*, 2008).

1.9.1.3 Low density lipoprotein receptor-related protein

Kupffer cells express a variety of potential clearance receptors, including C-type lectins, β 2-integrins and scavenger receptors. Interestingly, previous studies have suggested that plasma FVIII and VWF levels may be associated with polymorphisms within the *LRP1* gene (Morange *et al*, 2005). Specifically, a D2080N polymorphism in *LRP1* was associated with significantly reduced plasma FVIII and VWF (Morange *et al*, 2005). Low density lipoprotein receptor-related protein 1 (*LRP1*) is a ubiquitously expressed scavenger receptor that mediates diverse roles in various biological processes including lipoprotein metabolism, degradation of proteases, and intracellular signalling (Lillis *et al*, 2008). In addition *LRP1* can also function as an endocytic clearance receptor regulating FVIII plasma levels in mice (Saenko *et al*, 1999;

Lenting *et al*, 1999). Interestingly, Bovenschen *et al* reported that conditional liver-specific LRP1 deficient mice have modest increases in both plasma FVIII and VWF (Bovenschen *et al*, 2003). A role for LRP1 in regulating VWF clearance was supported by further studies in a mouse model deficient specifically in macrophage-LRP1 (macLRP1⁻) (Rastegarlar *et al*, 2012). These MacLRP⁻ mice had a 1.6-fold increased plasma VWF levels compared to macLRP⁺ mice. Since hydrodynamic expression of LRP1 antagonist RAP in macLRP1⁻ mice did not serve to further increase VWF plasma levels, it is likely that macrophage-specific LRP1 represents the sole LRP1 receptor involved in VWF clearance. Although no binding of VWF to LRP1 was observed under static conditions, significant binding was demonstrated under conditions involving shear stress *in vitro* (Castro-Núñez *et al*, 2012).

1.9.1.4 β 2 Integrins

Mac-1 (α M β 2-integrin) is a leukocyte specific integrin important in modulating binding to EC. Mac-1 is also capable of mediating phagocytosis. Mac-1 recognises a diverse array of ligands including ICAM-1, fibrinogen and coagulation Factor X (Diamond *et al*, 1990; Wright *et al*, 1988; Altieri *et al*, 1988). The α M component of Mac-1 has a cation-dependent binding site and a non-cation dependent lectin domain. The lectin domain of human Mac-1 demonstrates affinity for glycoproteins terminating in glucosamine or N-acetylglucosamine (Thornton *et al*, 1996). Importantly hepatic macrophages expressing Mac-1 have been shown to be critical in the clearance of rapidly chilled platelets (Simon *et al*, 2000). Cold platelet storage exposed N-acetylglucosamine residues on the N-linked glycan on platelet GPIb α , which were then recognised by Mac-1 resulting in rapid phagocytosis (Hoffmeister *et al*, 2003). To regulate their adhesive properties, β 2-integrins are typically maintained in a low-

affinity resting state. However upon activation, they undergo conformational changes and transition to high-affinity states. Various stimuli promote this transition, including manganese ions, cytokines or receptor cross-linking. More recently, LRP1 has also been highlighted as a novel activator of $\beta 2$ -integrins. Using purified proteins, Spijkers *et al* demonstrated that LRP1 could associate with the I domain of αM . Moreover this interaction served to promote leukocyte adhesion to EC (Spijkers *et al*, 2005). Given this novel association between $\beta 2$ and LRP1, the role of these integrins in influencing VWF clearance has also been examined. Rastegarlar *et al* found that hydrodynamic expression of NIF, a Mac-1 antagonist, served to increase plasma VWF and FVIII levels in wild-type mice. However no effect of NIF was observed in macLRP⁻ mice. In keeping with Spijkers *et al* these data therefore suggest that $\alpha M\beta 2$ -integrin may modulate VWF clearance, in an LRP1-dependent manner (Rastegarlar *et al*, 2012).

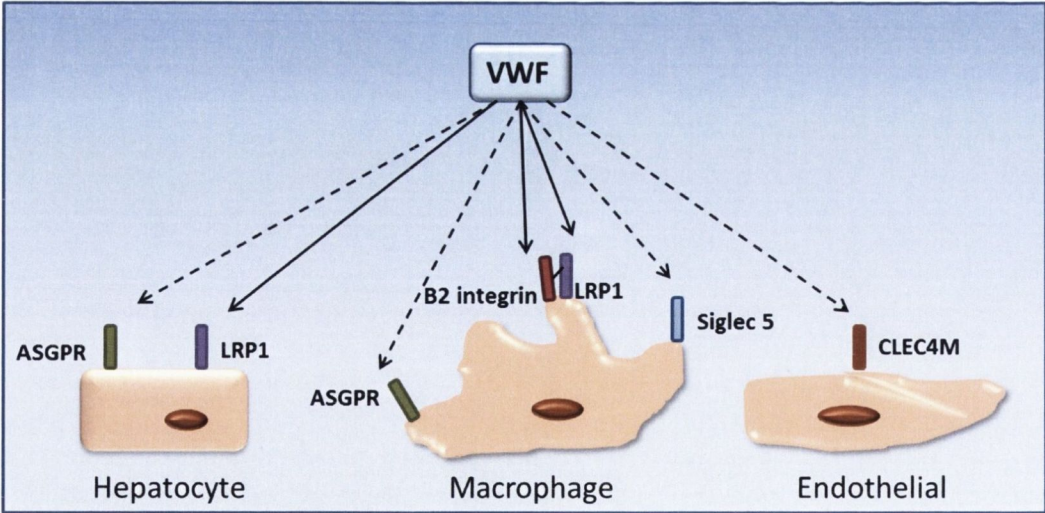


Figure 1.13 Potential clearance pathways for VWF
Cellular and receptor basis for VWF clearance, the dashed arrows highlight glycan-dependent interactions with lectin receptors and the solid arrows depict protein-protein interactions.

1.9.2 The role of VWF clearance in VWD

Reduced survival of VWF as a pathogenic mechanism in VWD was first highlighted in 1985. A subset of patients with VWD were observed to have a markedly reduced VWF half-life following DDAVP administration (De la Fuente *et al*, 1985). Subsequent studies have shown that a number of different mutations within VWF are associated with enhanced clearance (figure 1.14). Consequently, enhanced clearance has led to a proposed subdivision of type 1 VWD that has been termed type 1C (Haberichter *et al*, 2006). Identifying these patients has direct clinical importance in that DDAVP treatment may be less useful given the reduced half-life of endogenous VWF. The steady-state ratio of VWF propeptide to VWF antigen has been used as a surrogate marker to identify patients with enhanced clearance VWF. The VWF propeptide and mature antigen are secreted from EC in a 1:1 ratio. However VWF propeptide clearance occurs independently of VWF antigen. In addition the VWF propeptide has a significantly shorter half-life of just 2-3 hours, compared to 8-14 hours for mature VWF (Borchiellini *et al*, 1996; Dobrkovska *et al*, 1998). Consequently, an elevated VWF propeptide to VWF antigen ratio is suggestive of an accelerated VWF clearance rate (Haberichter *et al*, 2006). The archetypal VWD 1C mutation is VWD-Vicenza which is caused by the point mutation R1205H (Casonato *et al*, 2002). VWD Vicenza is characterised by markedly low VWF plasma levels (~10% of normal) and significantly reduced survival of VWF following DDAVP (Castaman *et al*, 2008). Casonato *et al* have also demonstrated that accelerated clearance can also be important in the aetiology of VWD type 2B (Casonato *et al*, 2010). Some patients with type 2B VWD have increased VWF propeptide to antigen ratios and demonstrate reduced plasma VWF survival following DDAVP (Casonato *et al*, 2010).

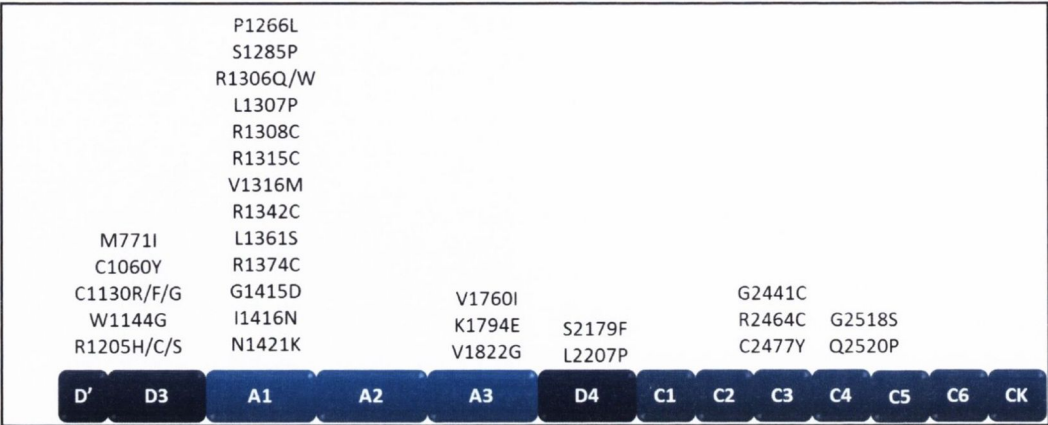


Figure 1.14 Location of potential VWF clearance mutations
Examining the VWF propeptide to antigen ratio, more than 20 different VWF mutations have been highlighted as potential fast clearance mutations. Of note the mutations are dispersed over the whole mature VWF subunit, however a significant cluster appears around D3-A1 domains.

1.9.3 VWF glycosylation and clearance

Previous studies have suggested that abnormalities in VWF glycosylation may lead to a VWD phenotype through enhanced clearance. Gralnick *et al* described abnormal VWF glycosylation in three patients with reduced PAS (Periodic Acid Schiff) reactivity. Importantly, PAS reactivity is influenced by sialic acid expression, suggesting that quantitative reduction in sialic acid in these VWD patients may have been important in the pathophysiology of their reduced VWF levels (Gralnick *et al*, 1976). However a subsequent study reported that reduced VWF PAS-reactivity was not common in patients with VWD (Zimmerman *et al*, 1979).

In addition, a number of animal models have also suggested that variation in VWF glycosylation may directly influence VWF clearance. Human pd-VWF displayed a biphasic clearance pattern in New Zealand White rabbits (Sodetz *et al*, 1977). Following desialylation of VWF (asialo-VWF), clearance was significantly enhanced, such that 92% the infused VWF accumulated in the liver within 10 minutes. This

enhanced hepatic clearance was competitively inhibited by simultaneous infusion of asialo- α 1-acid glycoprotein, suggesting a role for hepatic ASGPR in mediating this process. Clearance of recombinant VWF devoid of the O-linked glycans has also been studied in Sprague-Dewley rats. This VWF glycoform was cleared from plasma significantly faster than wild-type VWF (Stoddart *et al*, 1996). This finding was recently confirmed by Badirou *et al* following hydrodynamic VWF expression studies in VWF^{-/-} mice. Mice expressing the VWF variant T1255A/T1256A which has lost two specific O-linked glycans, was cleared significantly faster than wild-type VWF (Badirou *et al*, 2012). The molecular mechanism responsible for the enhanced clearance of this O-glycan modified VWF remains to be determined.

RIIS/J mice demonstrate prolonged bleeding times and have significantly reduced VWF levels (Sweeney *et al*, 1990). Subsequent studies revealed the genetic defect responsible for the reduced VWF levels in these mice was distinct from the murine *VWF* locus on chromosome 11 (Mohlke *et al*, 1996). In further studies, it was shown that aberrant glycosyltransferase expression in EC in RIIS/J mice causes aberrant glycosylation of VWF in these mice and consequently results in enhanced clearance (Mohlke *et al*, 1999). This mouse model provides clear evidence that genetic loci distinct from the *VWF* gene may be responsible for modulating VWF clearance. The importance of VWF glycosylation profile in regulating clearance has also been observed in mice deficient in the sialyltransferase ST3Gal-IV enzyme. This enzyme catalyses the addition of α 2-3 linked sialic acid to a penultimate Gal or GalNAc residue. ST3Gal-IV^{-/-} mice also display decreased plasma VWF:Ag levels due to enhanced clearance. This rapid clearance was competitively inhibited by the co-injection of asialo-fetuin again suggesting a key role for the ASGPR (Ellies *et al*, 2002). Importantly

Ellies *et al* also reported a small cohort of individuals with abnormally low VWF plasma levels, who also demonstrated increased terminal galactose or N-acetylgalactosamine expression. Importantly variation in VWF glycosylation can also occur during bacterial sepsis. For example, VWF desialylation has been associated with *Streptococcus pneumoniae* infection (Grewal *et al*, 2008).

1.10 VWF lectin interactions

The role of VWF glycosylation in regulating its clearance suggests that interaction with lectins may be important. A number of different genome wide association studies (GWAS) have also reported that specific lectins may be important determinants of plasma VWF:Ag levels. For example Smith *et al* identified eight candidate loci significantly associated with plasma VWF:Ag levels. These loci included members of scavenger clearance receptor families, *SCARA5*, *STAB2*, and *CLEC4M* (Smith *et al*, 2010). Interestingly *CLEC4M* (L-SIGN) is a lectin receptor expressed in liver sinusoidal EC and lymph nodes. *CLEC4M* displays significant homology to DC-SIGN expressed on dendritic cells. Recent studies have shown that *CLEC4M* can function as an endocytic receptor for VWF (Rydz *et al*, 2013). *In vitro* binding studies demonstrated that pd-VWF bound to *CLEC4M* in a mannose-dependent manner. In addition HEK293 cells with stably expressed *CLEC4M* were able to bind and internalise pd-VWF.

Siglec-5 (sialic acid Ig-like lectin 5) is another lectin that has been shown to bind VWF. Siglec-5 is classified as an I-type lectin and contains four extracellular Ig-like domains (Cornish *et al*, 1998). Ligand preferences include α 2-3 and α 2-6 linked sialic acid (Brinkman-Van Der Linden & Varki, 2000). Siglec-5 is expressed on monocytes, macrophages, and neutrophils. Given the role of macrophages in mediating VWF

clearance, the potential role of siglec-5 as an endocytic receptor was investigated (Pegon *et al*, 2012). First, *in vitro* binding assays revealed that both VWF and FVIII bound immobilized Siglec-5 in a dose-dependent and reversible manner. This binding of siglec-5 to VWF was sialic acid-dependent. Second, immunofluorescent studies demonstrated that VWF and FVIII could both bind to HEK293 cells expressing siglec-5. Moreover cell binding assays performed at 37°C resulted in the internalisation of VWF and FVIII. Finally, expression of human siglec-5 in murine hepatocytes following hydrodynamic injection resulted in a 70% reduction in plasma levels VWF. Collectively these recent publications highlight that VWF is capable of interacting with a number of novel lectin families.

1.11 Galectins

The galectins constitute another family of lectins that have recently been reported as putative novel binding partners for VWF (Saint-Lu *et al*, 2012). Galectins are a family of soluble lectins which share homology within their Carbohydrate Recognition Domain (CRD) (figure 1.15). The galectins display binding specificities for β -galactoside expressing glycans.

Galectin-1 and galectin-3 are of particular interest. Both are expressed in EC and have been shown to interact with VWF (Saint-Lu *et al*, 2012). Initially, SPR analysis demonstrated that both galectin-1 and galectin-3 bound to VWF in a dose-dependent and glycan-dependent manner. Furthermore, *in vitro* studies showed that galectin-1 and galectin-3 were colocalised with VWF in EC. Moreover after EC stimulation, typical VWF strings were shown to co-stain for galectin-1. Finally, within normal plasma, galectin-1 and galectin-3 were shown to circulate in complex with VWF. This

interaction is critical for galectin survival, since VWF^{-/-} mice were shown to have a 2-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 suggesting that VWF serves as a molecular chaperone for galectin-1 and galectin-3 in plasma. Importantly, galectin binding was also shown to directly influence VWF function. *In vitro*, EC were stimulated to secrete VWF multimers and platelet adhesion was measured. In the presence of the galectin inhibitor lactose, the number of VWF multimer strings was significantly increased, and more platelets were bound per string. Cumulatively, these data suggest a novel role for the galectins as negative modulators of VWF-platelet string formation at the EC surface. These findings were supported by *in vivo* studies that demonstrated increased platelet-VWF string formation in the mesenteric venules of galectin-1/galectin-3 dual knockout mice compared to wild-type mice upon endothelial stimulation. Most critically, Saint-Lu et al found that galectins modulate VWF-dependent early thrombus formation *in vivo*. In a ferric-chloride injury model galectin-1/galectin-3 dual knockout mice displayed enhanced thrombi formation compared to wild-type mice.

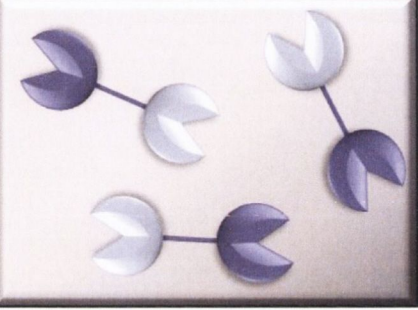
Classification	Structure
Proto-type Single CRD that dimerises <i>Galectin-1, -2, -5, -7, 10, -11, -13, -14 and -15</i>	
Tandem repeat-type Two distinct CRDs <i>Galectin-4, -6, -8, -9 and -12</i>	
Chimera-type one CRD and a non-lectin tail that oligomerises <i>Galectin-3</i>	

Figure 1.15 Structural illustrations for the galectins

1.12 FVIII glycosylation

Like VWF, FVIII is also heavily glycosylated (figure 1.16), containing 25 consensus sites for N-linked glycosylation. Mass spectrometry analysis has demonstrated that 21 N-linked glycan are occupied, with 17 sites located within the B domain (Hironaka *et al*, 1992). The N-glycome of human FVIII has been characterised. The primary N-linked glycan structure is a complex-type biantennary oligosaccharide. Additionally, tri- and tetra-antennary structures were also reported. Human FVIII is also extensively sialylated with the majority of N-glycans carrying at least one sialic acid residue. FVIII

also expresses blood group determinants on its complex-type N-glycans. In addition to these complex type glycans, high mannose oligosaccharides were also a feature of the FVIII N-glycome (Hironaka *et al*, 1992; Kaufman *et al*, 1988). Finally seven O-linked glycans were also identified on FVIII, all of which are localised to the B domain.

A number of important differences in glycosylation have been noted between plasma-derived FVIII (pd-FVIII) and recombinant FVIII (rFVIII). First, rFVIII does not express ABO(H) blood group determinants. Second, rFVIII can also express a number of non-human glyco-epitopes dependent upon the cell-line used for expression. These abnormal glyco-epitopes include galactose in an α 1-3 linkage to the sub-terminal galactose (Gal α 1-3Gal), and an N-glycolylneuraminic acid (Neu5Gc) (Hironaka *et al*, 1992; Varki, 2009).

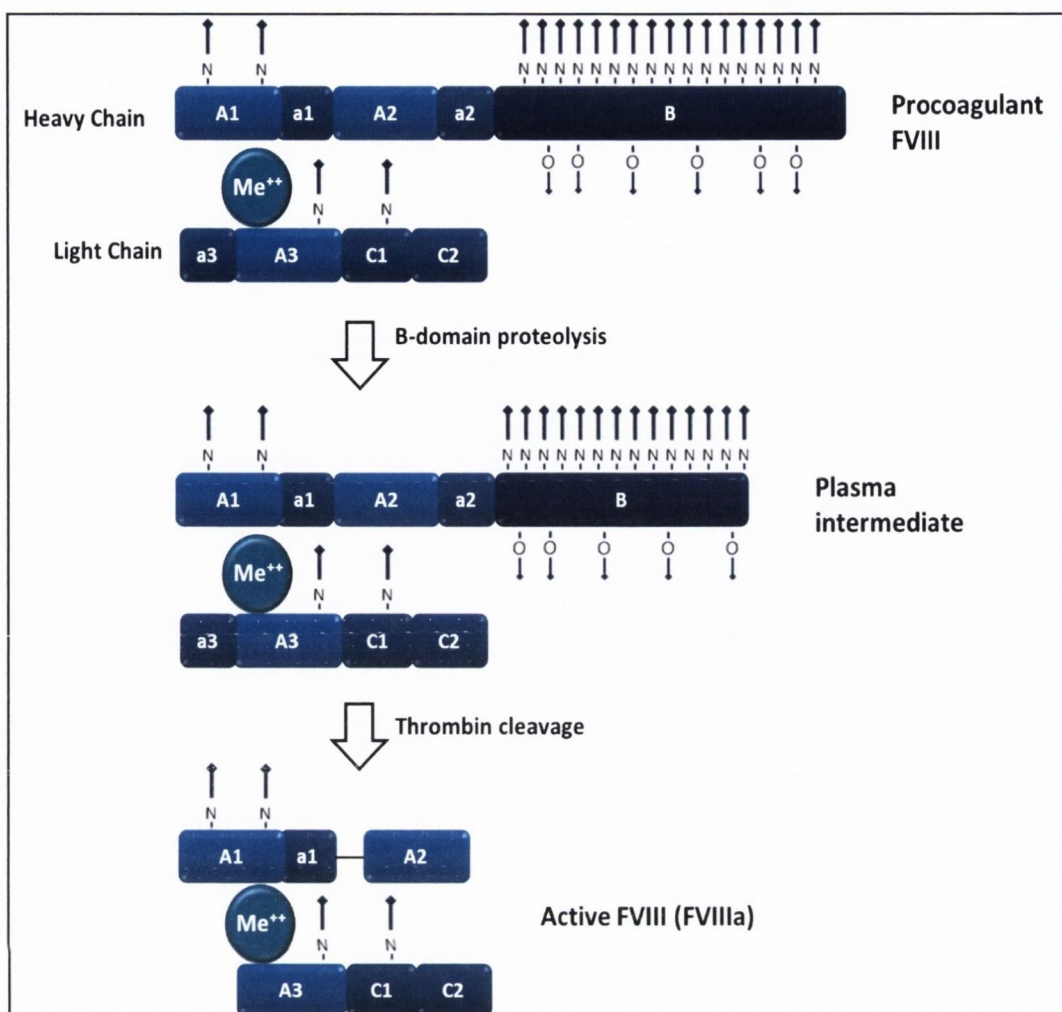


Figure 1.16 FVIII domains and the associated glycans

N-linked sites within FVIII at N42 and N239 in the A1 domain. N757, N784, N828, N900, N963, N1001, N1005, N1066, N1185, N1255, N1259, N1282, N1300, N1412 and N1442 in the B domain. N1810 in A3 and N2118 in C1. Additionally 6 O-linked glycan are found in the B domain.

1.13 FVIII lectin interactions

1.13.1 Biosynthesis

Like VWF, the glycosylation of FVIII is important in modulating its biology. The intracellular processing and trafficking of FVIII is regulated by the ER lectins calnexin and calreticulin, as well as the Golgi lectin LMAN1 (also known as ERGIC-53). FVIII interaction with ER folding chaperones calnexin and calreticulin is mediated by FVIII N-

linked glycans within the B domain (Pipe *et al*, 1998). In particular, the primary monoglucosylated N-linked oligosaccharides added to FVIII in the ER serve as ligands for calnexin and calreticulin binding. Consequently inhibition of glucose trimming with castanospermine ablates interaction of FVIII with both calnexin and calreticulin, and results in reduced FVIII secretion. FVIII transition from the ER to the Golgi is mediated by interaction with the LMAN1 lectin. LMAN1 association with FVIII is regulated by oligosaccharides within the B domain (Moussalli *et al*, 1999). The physiological importance of the FVIII-LMAN1 interaction is illustrated in patients with mutations in LMAN1, (or its associated protein MCFD2), which result in combined FV/FVIII deficiency (Nichols *et al*, 1998).

1.13.2 FVIII activity

To date, limited data suggest that FVIII glycans are important in regulating its biological activity. *In vitro* studies by Kosloski *et al* demonstrated that FVIII activity was reduced 2-fold upon enzymatic removal of its N-linked glycans. Additionally, deglycosylated rFVIII also displayed reduced affinity for phosphatidylserine containing membranes (Kosloski *et al*, 2009). Given that the heavily glycosylated B domain is dispensable for FVIII activity the potential role of FVIII glycans in influencing FVIII activity remains to be fully determined (Toole *et al*, 1986).

In contrast, N-glycan mutations have been reported in haemophilia A patients. Two patients with severe haemophilia were found to have mutations resulting in the creation of novel N-linked glycan sites (Aly *et al*, 1992). Both patients had normal plasma FVIII antigen levels, but FVIII activity levels were reduced (<1%). The first patient mutation M1772T, created a new consensus sequence for an N-linked glycan at

N1770 in the light chain of FVIII. The second patient mutation, I566T, created an N-glycosylation site at N564 in the A2 domain (Aly *et al*, 1992).

1.13.3 FVIII clearance

FVIII can interact with a number of other lectins, including ASGPR, which has been implicated as a potential FVIII clearance receptor. Bovenschen *et al* demonstrated that FVIII bound to ASGPR in dose-dependent and glycan-dependent manner. Importantly B domain deleted FVIII (BDD-FVIII) demonstrated markedly reduced binding to ASGPR, suggesting that the high density of N-linked glycans in FVIII B domain modulates ASGPR binding. *In vivo* clearance experiments in mice confirmed that FVIII survival was prolonged by the presence of ASGPR antagonist ASOR (Bovenschen *et al*, 2005).

1.13.4 FVIII immunogenicity

FVIII glycosylation has also been suggested to regulate its uptake by antigen presenting cells, and subsequent activation of T-cells. FVIII bound C-type lectin, CD206, expressed on the surface of dendritic cells. Critically this interaction was mediated by FVIII mannose-containing glycans outside the B domain (Dasgupta *et al*, 2007). FVIII expresses two high mannose glycan structures at positions N239 and N2118. Interestingly Dasgupta *et al* reported that removal of the mannose glycan chain N2118 in the C1 domain of FVIII attenuated FVIII-mediated dendritic cell activation.

1.14 Study objectives

Both VWF and FVIII are heavily glycosylated proteins with glycans constituting approximately 20% of their total molecular mass. This glycosylation significantly influences the biology of both VWF and FVIII. The molecular mechanisms through which these glycan effects are mediated remain poorly understood. Accumulating data suggest that the ability of VWF and FVIII to interact with specific lectins may be important in this regard. Recent evidence has demonstrated that galectin-1 and -3 constitute novel binding partners for VWF, and that these galectin interactions modulate VWF-mediated thrombus formation *in vivo*. In light of this, the first objective of this study was to define the biochemical basis through which VWF interacts with galectin-1. Since the glycan determinants expressed on human FVIII are similar in structure to those expressed on VWF, we further hypothesised that galectins may also constitute novel binding partners for FVIII. This hypothesis was addressed as a second aim of this study.

VWF glycosylation is an established regulator of plasma VWF levels. Several *in vivo* studies have shown that variation in VWF glycosylation, including the presence of ABO(H) determinants and sialic acid, directly modulate VWF survival. However the biological mechanisms through which glycans regulate VWF circulatory survival remain elusive. Consequently the final objective of this thesis was to define the role played by specific VWF domains, and VWF glycans in determining *in vivo* clearance.

2 Materials and Methods

2.1 Purification of human plasma-derived VWF using fast protein liquid chromatography

2.1.1 Purification of VWF from Haemate P®

Plasma-derived (pd-VWF) was purified from the commercial concentrate Haemate P®. Haemate P® (ZLB Behring, Germany) is a 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 pd-VWF from Haemate P®.

2.1.2 Gel Filtration

The principle of this technique relies on the fact that larger proteins elute from the column at a faster rate than smaller proteins. Consequently gel filtration was used to separate high, intermediate and low molecular weight multimeric VWF fractions. 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 buffer (20mM Tris, 10mM sodium citrate, pH 7.4). 5-10mL of reconstituted Haemate P® (or blood group specific cryoprecipitate) was applied to the column at a flow rate of 0.5mL/min and eluted in the same buffer at a flow rate of 1mL/min (figure 2.1). Fractions were collected and VWF concentration was determined by VWF antigen enzyme-linked immunosorbant assay (ELISA). VWF multimeric composition was determined by collagen binding assay and multimer gel analysis and purity assessed by silver staining (figure 2.2).

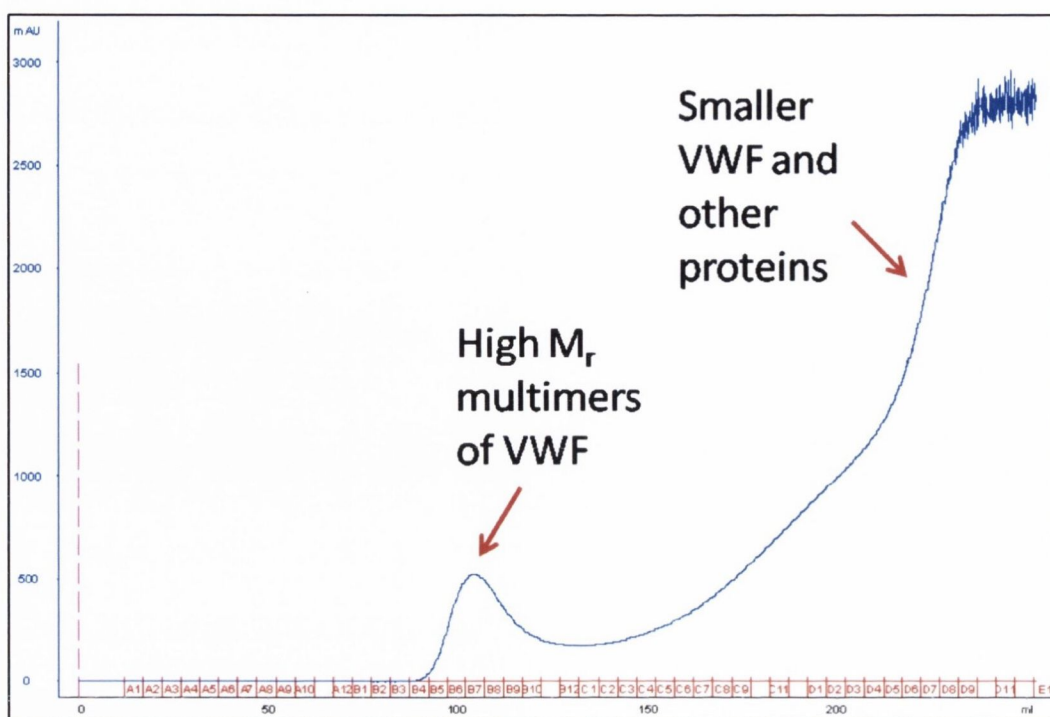


Figure 2.1 Gel filtration of Haemate P® pd-VWF

High purity, high molecular weight multimeric VWF is eluted in the first peak, followed by smaller VWF multimers and contaminating plasma proteins.

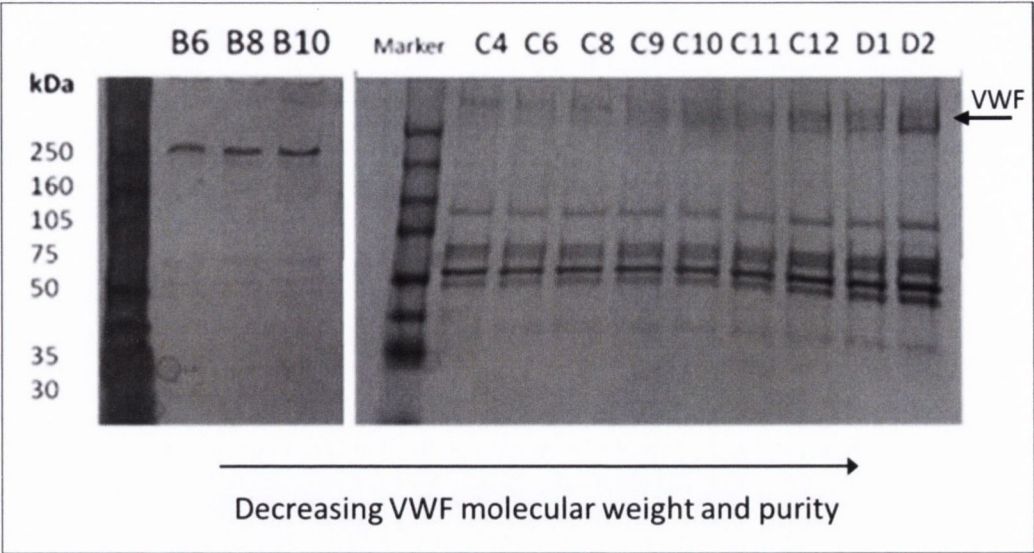


Figure 2.2 SDS-PAGE and silver stain assessment of gel filtered VWF elutes
Purity of VWF fractions collected after gel filtration chromatography was analysed by SDS-PAGE followed by silver staining. The purity of VWF, present at ~250kDa decreases with increasing fraction number.

2.1.3 Purification of blood-group specific plasma-derived VWF

Blood group specific VWF (group O, group AB and Bombay respectively) was purified from plasma by cryoprecipitation. Cryoprecipitation results in a subset of circulating proteins (including VWF) precipitating 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 slowly stirred. Ethanol (50% v/v) pre-chilled to 4°C was added drop wise to the stirring plasma to a final concentration of 8% (v/v). The mixture was left stirring 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 Tris-Citrate buffer and centrifuged at 3500rpm for 30 minutes at room temperature. The VWF-containing supernatant was removed and VWF was further purified by gel

filtration chromatography. VWF concentration was subsequently determined by VWF antigen ELISA and purity assessed by silver staining (figure 2.3).

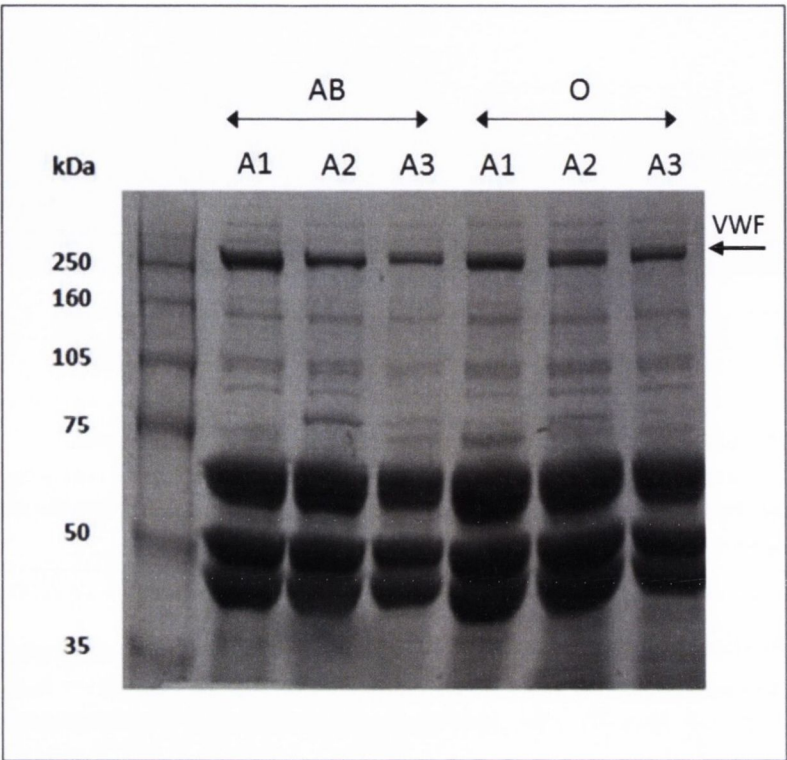


Figure 2.3 SDS-PAGE and Coomassie assessment of blood group specific VWF
Purity of blood group-AB fractions A1, A2, A3 and blood group-O fractions A1, A2, A3 collected after cryoprecipitation and gel filtration were assessed by SDS-PAGE and Coomassie staining. VWF is visible as a band at ~250kDa.

2.2 Purification of human platelet-derived VWF

Platelets surplus to requirement were obtained from the Irish Blood Transfusion Service (St James's Hospital, Dublin). Two units of leukocyte-depleted pooled platelets were decanted into 50mL tubes and centrifuged at 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 NaHCO₃, 6mM dextrose, 10mM trisodium citrate, 0.9mM MgCl₂, 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 (500 μ M) and Aprotinin (150nM); E-64 (1 μ M) and Leupeptin (1 μ M); 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.

2.2.1 Platelet lysis by snap freeze-thawing

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

2.2.2 Purification of platelet-VWF by immunoaffinity chromatography

Immunoaffinity chromatography was carried out in collaboration with Dr. Jan Voorberg and Dr. Maartje van den Biggelaar (Department of Plasma Proteins, Sanquin Research, Amsterdam, The Netherlands). 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 and 1M KSCN pH 7.4. VWF containing fractions were dialyzed against 20mM Tris, pH 7.4 and stored at -80°C. VWF concentration was subsequently determined by VWF antigen ELISA.

2.3 Analysis of VWF

2.3.1 VWF:Ag ELISA

VWF antigen (VWF:Ag) was measured by ELISA. Maxisorp plates (Nunc, Denmark) were coated with rabbit polyclonal anti-human VWF antibody (Dako, Denmark) 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-Aldrich, 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) was used to generate a standard curve. All samples were incubated on the plate for 2 hours at 37°C wells after which the plates were washed three times with PBS-T. Following washing, 100µl of anti-VWF-HRP (DAKO, Denmark) diluted 1:1000 in PBS-T was added and incubated for 1 hour at 37°C. Following another three PBS-T washes, 100µl of horseradish peroxidase (HRP) substrate 3,3',5,5'-Tetramethylbenzidine (TMB; Substrate Reagent Pack, R&D Systems, UK) was added to the wells. The reaction was stopped with 50µL 1M H₂SO₄ and absorbance read at 450nm 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.

2.3.2 VWF collagen binding assay

Collagen binding activity (VWF:CBA) can provide a sensitive measure of VWF multimeric composition and functional activity. High molecular weight VWF multimers bind to collagen with significantly greater affinity than low molecular weight forms. VWF:CBA 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 Na₂CO₃, 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) was again 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 1/1000 in PBS-T was added to the wells and incubated for 1 hour at 37°C. Wells were washed and colour was developed as described before (section 2.3.1). A standard curve and log-log plot were constructed as for VWF:Ag ELISA and VWF:CBA 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.

2.3.3 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 Cu²⁺ ions to Cu⁺ by the peptides bonds of proteins. The amount of Cu²⁺ 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 25 μL of each dilution was added to a 96 well plate in duplicate. To construct a standard curve, BSA was diluted over a range of 20-2000 $\mu\text{g}/\text{mL}$ and 25 μL 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). 200 μL 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 ($\mu\text{g}/\text{mL}$). 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).

2.3.4 VWF multimer gel

The multimeric pattern of VWF can be visualised by electrophoresis of VWF using non-reducing agarose gels. VWF multimer analysis was performed using using a Bio-Rad mini gel casting system (Fannin LTD, UK). Biorad glass plates were cleaned thoroughly with methanol before pre-heating at 37°C. 1.8% agarose gels (1.5mm diameter) were prepared by dissolving 0.36g SeaKem[®] HGT(P) Agarose, Lonza, ME, USA in 20mL of separating buffer (200mM Tris, 100mM glycine and 0.1% SDS, pH 9.0). The agarose was dissolved by extensive heating ~80°C before pouring 8mL per/gel into the pre-heated plates. To facilitate gel setting, the gels were cooled at 4°C for 10 minutes. Stacking gels (0.75% agarose) were prepared by heating 0.15g SeaKem[®] HGT(P) Agarose in 20mL of stacking buffer (70mM Tris, 5mM EDTA, 0.1% SDS pH 6.7). Once

dissolved, 4mL of stacking gel were poured on top of the cooled separating gels in the casting unit. A 10 well comb (1.5mm diameter) was inserted immediately before incubating the gels at 4°C to allow them to set.

VWF samples containing ~2ng VWF mixed with NuPAGE® LDS Sample Buffer (4X; Life Technologies, UK) and incubated at 70°C for 10 minutes before loading onto the gels. The gels were run using Bio-Rad mini-gel electrophoresis tank. The outer chamber of the tank was filled with running buffer (50mM Tris, 75mM glycine) while the inner chamber was filled with (100mM Tris, 150mM glycine 0.1% SDS). The gels were electrophorised for 150 minutes at 50 volts.

2.3.5 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Pre-cast gradient gels (4-12% NuPAGE® Bis-Tris Gels; and 3-8% NuPAGE® Tris-acetate Gels Life Technologies, UK) were used for analysis of VWF following purification. Samples containing ~50ng VWF (for western blot) or ~1-2µg (for Coomassie Blue staining) were mixed with NuPAGE® LDS Sample Buffer (4X; Life Technologies, UK) and incubated at 70°C for 10 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 175V for approximately 45 minutes in NuPAGE® MOPS SDS running buffer for the Bis-Tris gels and NuPAGE® Tris-Acetate buffer for the Tris-acetate gels (Life Technologies, UK). Detection of proteins was performed by both coomassie blue staining and western blotting.

2.3.6 Coomassie Staining

Coomassie[®] (Coomassie[®] Brilliant Blue, Sigma-Aldrich, Ireland) blue staining was used for the non-specific detection of proteins. After SDS-PAGE, gels were washed with dH₂O and incubated in Coomassie[®] blue staining solution (0.1% (w/v) brilliant blue (Sigma-Aldrich, Ireland), 20% (v/v) methanol, 10% (v/v) acetic acid) for 40 minutes. Gels were then rinsed in dH₂O and incubated in destain (50% (v/v) methanol, 10% (v/v) acetic acid) for 1 hour with gentle agitation.

2.4 Western blotting

Following electrophoresis, proteins were transferred to a PVDF membrane (Immobilon-P; Millipore, Ireland) using an electroblot system (Trans-Blot Electrophoretic Transfer Cell, Bio-Rad, Fannin LTD, UK). 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, 0.2% SDS and 20% (v/v) methanol). Following protein transfer, the membrane was blocked for non-specific binding with 3% solution BSA 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).

2.5 Glycosidase Digestions

Modification of pd-VWF and FVIII glycan structures was performed using a series of specific exoglycosidase enzymes. Table 2.1 summarises the properties of the different glycosidases Digestions were carried out overnight under non-denaturing conditions at 37°C. Glycan changes in VWF and FVIII following glycosidase digestion were assessed by lectin ELISA and compared to untreated controls.

Enzyme (Source)	Substrate	Optimised Concentration	Supplier
Peptide N Glycosidase F (<i>Flavobacterium meningosepticum</i>)	N-glycans	312U/µg protein	New England Biolabs, UK
O-glycosidase (<i>S. pneumoniae</i>)	O-glycans	0.5mU/µg protein	Sigma-Aldrich, Ireland
α2,3,6,8,9 neuraminidase (<i>Arthrobacter ureafaciens</i>)	Total sialic acid	1.25mU/µg protein	Calbiochem, Merck, UK
α2-3 neuraminidase (<i>Streptococcus pneumonia</i>)	α2-3 sialic acid	0.6mU/µg protein	Sigma-Aldrich, Ireland
β1-4 galactosidase (<i>Streptococcus pneumonia</i>)	β1-4 galactose	0.5mU/µg protein	Calbiochem, Merck, UK
Endoglycosidase H (<i>Streptomyces picatus</i>)	High mannose glycans	500U/ µg protein	New England Biolabs, UK

Table 2.1 Exoglycosidase enzymes

2.5.1 Lectin ELISA

Control untreated pd-VWF (or FVIII) and glycan modified VWF (or FVIII) 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 appropriate biotinylated lectins (Table 2.2; 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 H₂SO₄, 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	Substrate
Concanavalin A (Con A)	N-glycans
Jacalin	O-glycans
<i>Sambucus nigra</i>	Terminal α2-6 and to a lesser degree α2-3 linked sialic acid
<i>Maackia amurensis</i> II	α2-3 sialic acid
<i>Ricinus communis</i> agglutinin (RCA)	D galactose
<i>Galanthus Nivalis</i>	High mannose glycans

Table 2.2 Biotinylated Lectins

2.6 FVIII materials

A number of commercially available purified recombinant FVIII preparations were used including full-length FVIII expressed in Chinese Hamster Ovary (rFVIII-CHO) cell line (Advate-Baxter Biosciences); full-length FVIII expressed in Baby Hamster Kidney cells (rFVIII-BHK), (Helixate-Bayer); and B domain deleted FVIII (BDD-FVIII) manufactured in CHO cell lines (Refacto-Wyeth).

2.7 Galectin expression

pET-Galectin-1 *E. coli* expression plasmid was constructed by inserting the galectin-1 cDNA (Origene, MD, USA) into the pET 303/CT-His expression vector

(Invitrogen, UK). This vector contains a C-terminal polyhistidine tag to facilitate galectin purification.

2.7.1 pET 303/CT-His[®] bacterial expression vector

pET 303/CT-His commercial vector from Invitrogen utilises a T7/*lac* promoter for high expression in *E. coli* cells. This vector encodes a multiple cloning site at the N-terminus of the histidine-tag to enable simple cloning of the digested galectin-1 sequence. The vector also includes an ampicillin resistance gene for selection in bacterial cells. The plasmid contains a pBR322 origin of replication for low copy number replication and maintenance of the plasmid in *E. coli* cells.

2.7.2 Amplification of galectin-1 cDNA

The galectin-1 cDNA was amplified utilising primers (Table 2.3) which contained the recognition sequence for restriction enzymes *Xba*I and *Xho*I to allow the galectin-1 sequence to be subsequently ligated into the multiple cloning site of the pET303 expression vector. The product was amplified using Bio-taq polymerase™, as per manufacturers guidelines, (Bioline, UK). This polymerase possesses 5'-3' exonuclease activity and thus results in an "A" nucleotide overhang on the PCR product. The overhangs allows for ease of hybridization of the PCR product into TA cloning vectors, which possess a complementary 3' T overhang. pGEM[®]-T Easy (Promega, UK) is linearized vector with a single 3'-terminal T nucleotide at both ends thus the galectin-1 PCR product was ligated into this vector without the need for restriction enzymes.

Primer	Primer sequence (5'→3')
Forward <i>Xba</i> I Gal-1	AGTTTACACACCGGAAACTGAGCTCGTGGTG
Reverse <i>Xho</i> I Gal-1	GCTGGTCTGGTGTTCCGTAAGATCTGGAGGA

Table 2.3 PCR primers designed for galectin-1 amplification
 Highlighted in red are the sequences corresponding to the restriction enzyme site.

Reactions were performed using the MJ Research PTC 200 Thermo Cycler with heated lid (GMI, MN, USA). The PCR protocol is outlined in table 2.4 below.

Step	Temperature	Time
	95°C	2 minutes
Denaturation	95°C	20 seconds
Annealing	53°C	10 seconds
Extension	70°C	15 seconds/kb 30 cycles

Table 2.4 PCR program for galectin-1 amplification with BioTaq™ polymerase

2.7.3 Agarose gel purification

PCR amplification product was purified via agarose gel electrophoresis followed by gel extraction. 0.7% agarose (w/v) was dissolved in Tris Borate EDTA (TBE) buffer (90mM Tris, 90mM Boric acid, 2mM EDTA, pH 8.3) by heating. The gels were poured into a horizontal gel electrophoresis tank (BRL 58 Horizontal Gel Electrophoresis Apparatus minitank; Gibco, UK). Ethidium bromide which intercalates within the DNA base-pairs was used to visualise the DNA bands by exposure to ultraviolet light. DNA samples were prepared with 10x Loading Dye 4g sucrose, 25mg Orange G; Sigma-Aldrich, Ireland, in 10 mLs dH2O). The samples were run in parallel with 1kb DNA ladder (New England BioLabs, UK) at 100V for approximately 45 minutes. The gel was visualised under UV light in a UVP BioSpectrum GelDoc Imaging system.



Figure 2.4 Agarose gel electrophoresis of amplified galectin-1 cDNA
Lane 1: 1kb ladder (NEB, UK)
Lane 2: Purified galectin-1 cDNA

2.7.4 Gel extraction

A scalpel was used to excise, the band of interest which was subsequently 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. After a series of wash steps DNA is then eluted from the membrane using a low salt buffer. The kit was used according to the manufacturer’s instructions.

2.7.5 Ligation of the galectin-1 into pGEM-T shuttle vector

After gel purifying the galectin-1 PCR product, the DNA concentration was measured using Nanodrop 2000 microvolume spectrophotometer (Thermo Scientific, UK). Galectin-1 PCR product was ligated into shuttle vector pGEM-T using TA cloning. A

vector to insert ratio of 1:1 was used with 50ng of vector in keeping with manufacturer's recommendations.

2.7.6 Transformation of competent cells

50µl of NEB 10-beta competent *E.coli* cells (New England Biolabs, UK) were thawed on ice. 2µL of the pGEM-T ligation reaction was added to the cells, and incubated on ice for 30 minutes. Cells were heat shocked at 42°C for 30 seconds and then placed on ice for 2 minutes. 80µL pre-heated SOC medium (super optimal broth with catabolite repression; Promega, UK) was then added to the cells and incubated at 37°C for 40 minutes with vigorous shaking (~150rpm). The mixture was subsequently inoculated onto a LB-ampicillin agar plate which had been previously seeded with IPTG (100mM) and X-Gal (1mg) and 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).

2.7.7 Purification of plasmid DNA and confirmation of PCR insert

In order to isolate plasmid DNA for subsequent cloning into the expression vector GeneJet Plasmid Miniprep Kit (Thermo Scientific, UK) was employed. This kit procedure was followed according to manufacturer's guidelines. The presence of the galectin-1 insert was confirmed initially by a shift in size of the plasmid. The pGEM-T Easy® is a linearized vector consisting of 3015bp. Insertion of the galectin-1 cDNA (~600bp) via TA cloning creates an expression plasmid of ~3615bp in length creating a visible shift in migration upon agarose gel electrophoresis.

2.7.8 Subcloning of galectin-1 *Xba*I-*Xho*I fragment into pET303/CT-His vector

In order to introduce the galectin-1 fragment into the pET303/CT-His expression vector, subcloning of the *Xba*I-*Xho*I fragment, containing galectin-1 insert, from pGEM-T to pET303/CT-His was carried out. The *Xba*I-*Xho*I fragment was released via restriction digest with *Xba*I and *Xho*I enzymes. pGEM-T-Gal-1 (~1µg) was digested using restriction enzymes *Xba*I and *Xho*I in NEBCut Smart™ buffer (New England Biolabs, UK) for 1 hour at 37°C. The sample was run out on a 0.7% agarose gel. This digest liberated the ~600bp galectin-1 fragment which was then gel purified.

In addition, a *Xba*I-*Xho*I restriction digest was carried out on pET303/CT-His expression vector in parallel to facilitate insertion of the galectin-1 fragment. Importantly Heat Inactivated Alkaline Phosphatase (Invitrogen, UK), which targets 5' phosphates for removal, was included to prevent self-ligation of the vector. This *Xba*I-*Xho*I fragment of pET303/CT-His vector was then gel purified quantified.

2.7.9 Ligation of galectin-1 *Xba*I-*Xho*I fragment into pET303/CT-His vector

Ligation of the *Xba*I-*Xho*I fragment containing galectin-1 was ligated into the pET303/CT-His vector using the LigaFast™ Rapid DNA Ligation System (Promega, WI, USA). Ligation reactions were used to transform competent *E.coli* cells on LB agar plates 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 and screened for the presence of the *Xba*I-*Xho*I fragment using restriction enzymes (Figure 2.5). Correct insertion of the galectin-1 fragment was confirmed by sequencing.

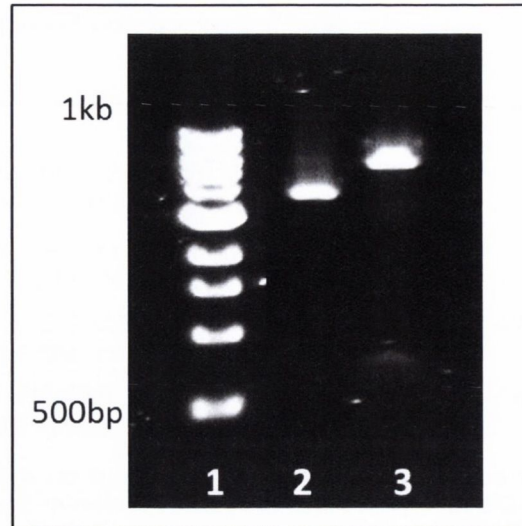


Figure 2.5 Agarose gel electrophoresis of pET-Galectin-1

Lane 1: 1kb ladder (NEB, UK),

lane 2: Purified pET-Galectin-1 uncut,

lane 3: pET-Galectin-1 cut with *XbaI-XhoI* restriction enzyme, resulting in the release of the galectin-1 subcloned fragment at ~600bp.

2.8 Expression of recombinant galectin-1

E.coli BL21 (DE3) expression cells (Invitrogen, UK) were transformed with pET303-galectin 1 and grown overnight at 37°C on LB agar with ampicillin. Two colonies were selected and inoculated into separate two 5mL LB ampicillin cultures and grown overnight at 37° with shaking. These cultures were then used to inoculate a 200mL culture of commercial Overnight Express™ Instant TB Medium (Novagen, UK) which was incubated at 37°C with shaking. Bacterial cells were harvested by centrifugation at 4000rpm for 25 minutes. The cell pellet was resuspended in binding buffer (20mM Tris, 500mM NaCl, 20mM Imidazole, 0.5mM DTT, pH 7.4) in a final volume of 20mL. The cell suspension was aliquoted into 15mL tubes (4mL maximum each) and sonicated (Soniprep 150, MSE, UK) on ice at amplitude 10 microns for 30 seconds following by 30 seconds rest. This cycle was repeated 3 times for each tube. The solution was then centrifuged for 20 minutes at 20,000g twice and the resulting

supernatant was filtered through syringe driven filter units (0.22µm pore, Millipore, Ireland). Recombinant galectin-1 was purified from the filtrate via nickel affinity chromatography (figure 2.6).

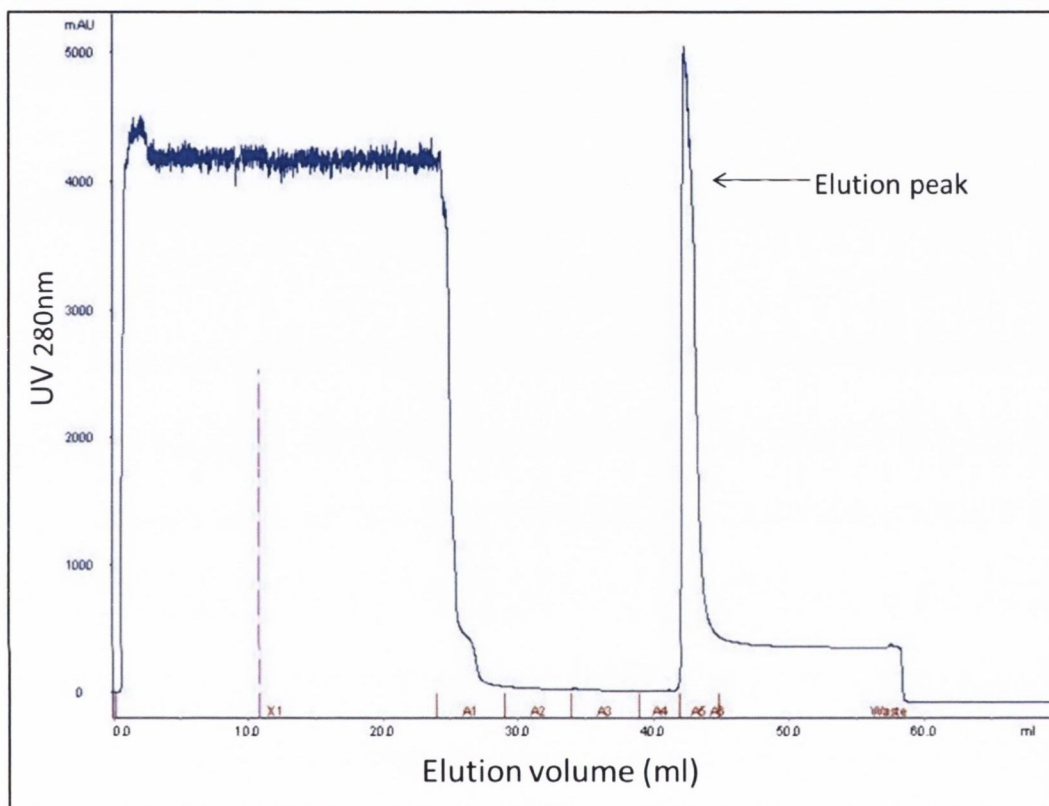


Figure 2.6 Nickel affinity chromatography of galectin-1

2.9 Purification of recombinant galectin-1

A 1 mL HiTrap Chelating Column (GE Healthcare, UK) that was charged with 0.1M NiCl_2 . The galectin filtrate (~20ml) from section 2.8 was loaded onto the column 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 binding buffer at a flow rate of 1mL/min. Galectin-1 protein was eluted in elution buffer (20mM Tris, 500mM NaCl, 500mM Imidazole, 0.5mM DTT, pH 7.4) at a flow rate of

0.5mL/min (Figure 2.6). The galectin containing fraction was then dialyzed against 20mM Tris containing 0.5mM DTT overnight at 4°C. Purity was assessed by Coomassie® Blue staining (Figure 2.7) and galectin was quantified by BCA (section 2.3.3).

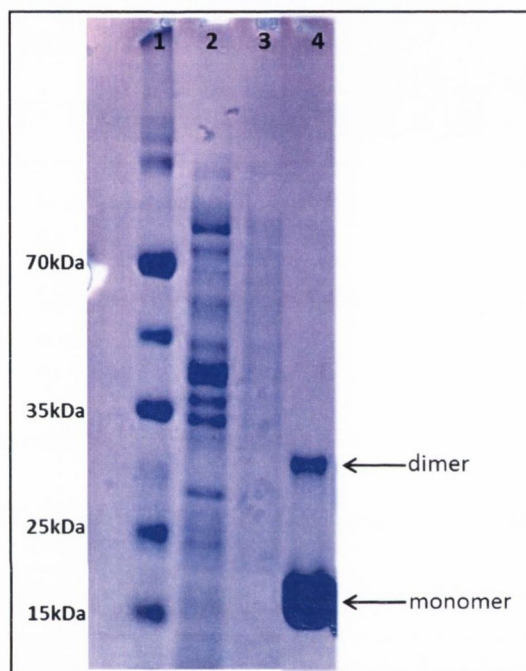


Figure 2.7 Galectin-1 elute was assessed for purity using Coomassie® blue staining
Lane 1: PageRuler Ladder
Lane 2: flow-through from HiTrap Chelating Column
Lane 3: the wash fraction from the column
Lane 4: reduced galectin-1 bands represented at ~15kDa (monomer) and ~30kDa (dimer).

2.10 VWF expression vectors

2.10.1.1 pcDNA VWF expression vector

The VWF expression vector pcDNA VWF was a kind gift from Dr. McKinnon (Imperial College London, UK) and was previously described in (McKinnon *et al*, 2008). This vector encodes an ampicillin resistance gene to allow for selection in bacterial cells and a PUC origin to drive high-copy number replication in *E.coli*. For expression in mammalian cells the vector contains a cytomegalovirus (CMV) immediate-early promoter to ensure high-level gene expression. Additionally, this vector encodes a neomycin resistance gene facilitates selection in mammalian cells and a SV40 origin gene to drive episomal replication in cells expressing the SV40 large T antigen.

2.10.1.2 pEXPR A1A2A3 VWF

The pEXPR-IBA-A1A2A3 VWF expression vector was designed and constructed by Dr. Alain Chion. The pEXPER-IBA 42 (IBA, Germany) vector encodes a CMV promoter, to ensure high level expression in mammalian cells, a polyhistidine tag for fusion at the C-terminal end of the inserted recombinant protein, an ampicillin gene for selection in *E.coli* cells and neomycin for selection in mammalian cells. A1A2A3 VWF coding region spans amino acid residues 1260-1874 and after expression using the pEXPER-IBA vector the protein carries a C-terminal polyhistidine tag. This tag is used for purification and detection purposes.

2.11 Recombinant VWF variants

A number of full-length (table 2.5) and truncated (table 2.6) VWF mutants used in this study have been previously developed in our laboratory by Dr. Alain Chion and Dr. Orla Rawley (Haemostasis Research Group).

VWF construct	Details
pcDNA VWF R1450E	VWD type 2B mimic mutation
pcDNA VWF N1515Q	Removal of the N-glycan at 1515 (cDNA a kind gift from Dr. McKinnon)
pcDNA VWF N1574Q	Removal of the N-glycan at 1574 (cDNA a kind gift from Dr. McKinnon)
pcDNA VWF Cysteine Clamp	Structurally constraint A2 (N1493C and C1670S)
pcDNA VWF Cysteine Clamp N1515Q	Structurally constraint A2 with the N-glycan at 1515 removed
pcDNA ΔA2 VWF	A2 domain deletion (residues 1464-1670)

Table 2.5 Outline of various full length VWF constructs used in this study

VWF construct	Details
pEXPR A1A2A3 VWF	A1A2A3 domains (residues 1260-1874)
pEXPR A1A2A3 VWF N1515Q	Removal of the glycan at 1515 in A1A2A3
pcDNA D'A3 VWF	D'A3 domains (residues 786-1874)
pcDNA A1CK VWF	A1-CK domains (residues 1260-2813)
pEXPR A1A2A3 VWF Cysteine Clamp	Structurally constraint A2 (N1493C and C1670S) within A1A2A3
pEXPR A1A2A3 VWF Cysteine Clamp N1515Q	Structurally constraint A2 with the N-glycan at 1515 removed

Table 2.6 Outline of various truncated VWF constructs used in this study

2.12 Cell culture

All recombinant VWF variants were produced via transient transfection in Human Embryonic Kidney (HEK 293T) cells (ATCC, LGC Standards UK). HEK 293T were maintained in Minimal Essential Media alpha (MEM α ; 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 (Nunc, UK) and passaged at ~80% confluence.

2.12.1 Transient transfection of HEK 293T cells

Transient transfection of HEK 293T cells was performed in serum free medium. T-175 flasks were seeded with HEK 293T and grown in supplemented MEM α until ~90% confluent. Before transfecting, 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 employed as a transfection reagent. A ratio of bPEI to DNA 2:1 was used. Plasmid DNA was diluted to a final concentration of 2 μ g/mL in 150mM NaCl and bPEI was diluted to a final concentration of 1 μ g/mL in 150mM NaCl. The bPEI was then added to the diluted plasmid DNA in a drop-wise manner and incubated for 20 minutes at room temperature to allow complex formation. Subsequently 2mL of the bPEI:DNA complex was added to each flask. Flasks were incubated for 72 hours before conditioned media was harvested. The media was centrifugation at 4,000rpm for 30 minutes and stored at -80°C for purification.

2.13 Concentration and purification of recombinant VWF

2.13.1 Anion exchange chromatography

Condition medium containing full length recombinant VWF protein was concentrated using anion-exchange chromatography. Conditioned medium was loaded onto a HiTrap Q HP column (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 low salt buffer, 20mM Tris, 100mM NaCl, pH 7.4, at a flow rate of 2mL/min. VWF was then eluted in high salt buffer, 20mM Tris, 500mM NaCl, pH 7.4, at a flow rate of 0.5mL/min (Figure 2.8). The VWF containing fraction (~5mL) was dialyzed against 20mM Tris, pH 7.4 at 4°C overnight with at least 2 buffer changes.

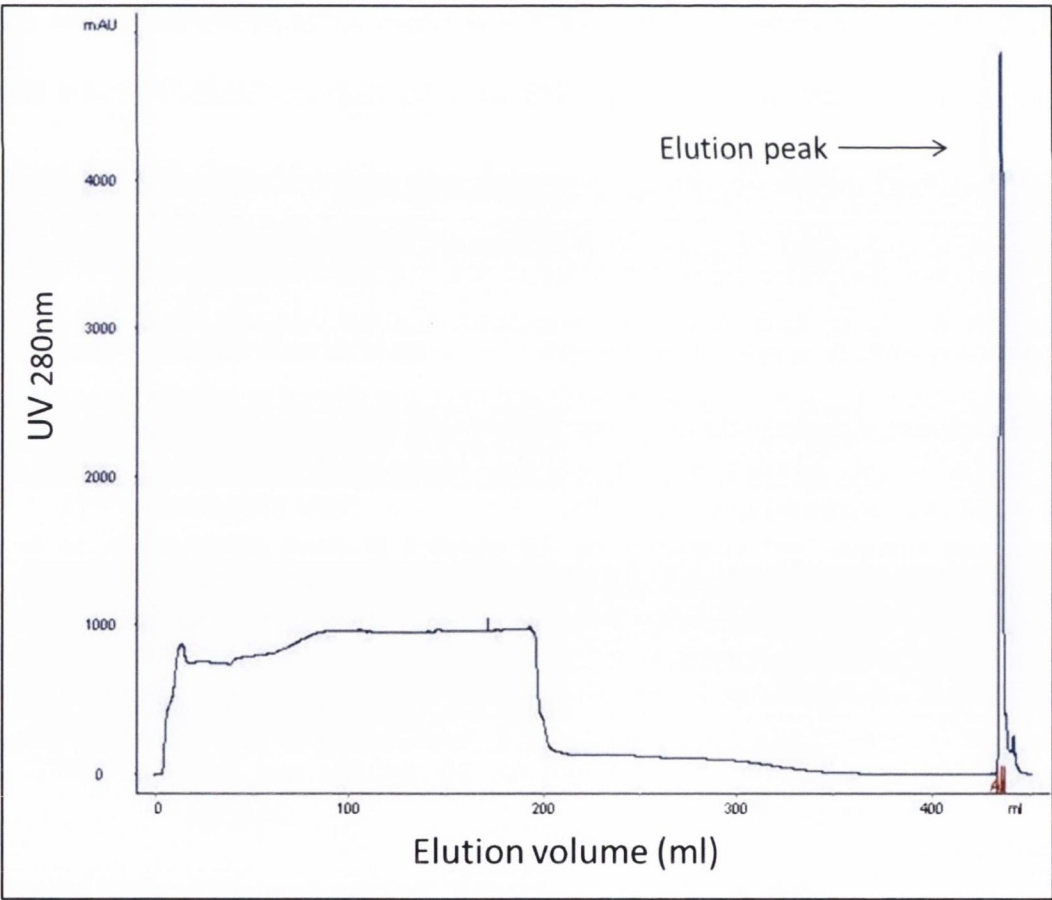


Figure 2.8 Anion exchange chromatography of recombinant VWF

2.13.2 Spin column concentration

Full-length recombinant VWF variants were further concentrated to final volume of ~1mL via centrifugation at 4,000g using Amicon Ultra-15 100K MWCO devices (Millipore, Ireland). VWF was then quantified by ELISA (section 2.3.1) and analysed by western blotting.

2.13.3 Purification via metal affinity chromatography

Truncated A1A2A3-VWF, constructs were expressed with polyhistidine tags to allow for further purification. These variants were concentrated via anion exchange chromatography (section 2.13.1), and then purified via metal ion affinity chromatography. A 1mL HiTrap Chelating Column (GE Healthcare, UK) was charged with 0.1M NiCl₂. The dialyzed anion exchange elute was loaded onto the column at a flow rate of 0.5mL/min in 20mM Tris, 150mM NaCl, 5mM Imidazole, pH 7.4. The column was washed at a flow rate of 1mL/min. Recombinant truncated VWF was eluted from the column with 20mM Tris, 150mM NaCl, 300mM Imidazole, pH 7.4. The ~1mL elute fraction was dialyzed against 20mM Tris, pH 7.4 at 4°C overnight and VWF concentration was then quantified by BCA (section 2.3.3). VWF purity was analysed by Coomassie® Blue staining and western blotting (section 2.3.6 and 2.4).

2.14 *In vitro* binding assays

2.14.1 THP-1 cell culture

THP-1 cells are a human monocytic suspension cell line derived from a patient with acute monocytic leukaemia. These cells were maintained in growth media consisting of RPMI 1640 (Bio-Sciences, Ireland) supplemented with 100U/mL penicillin,

100µg/mL streptomycin, 0.05mM β-mercaptoethanol; (Biosciences, Ireland) and 5% Foetal Calf Serum (FCS; Bio-Sciences, Ireland). For experimental use, cells were seeded on Nunc™ microwell plates with Nunclon™ Delta surface (Fisher Scientific, Ireland) at a density of 5×10^6 cells/mL in growth medium supplemented with 20ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Ireland) which promote differentiation and adherence of the cells so that they adopt a macrophage-like phenotype. After 3 days growth in the presence of PMA, fresh growth medium was added to the cells, which were then rested for an additional 4 days before use. Importantly this THP-1 protocol has been shown to produce cells which closely resemble the phenotype of primary human monocyte-derived macrophages (Daigneault *et al*, 2010).

2.14.2 THP-1 binding assay

Before binding assays were performed, THP-1 macrophages were serum starved in serum free growth medium for 30 minutes at 37°C. The plate of cells were then cooled on an ice bath and all further steps were performed with the cells maintained at 4°C to prevent endocytosis. Recombinant VWF variants were diluted in ice-cold serum free growth medium in the presence of 1mM MnCl₂ and 1mg/mL ristocetin and incubated with the cells for 1 hour on ice. THP-1 macrophages were then washed with 150µL ice cold PBS twice and fixed with 4% paraformaldehyde (Sigma-Aldrich, Ireland) in PBS. The 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) for full length VWF variants. Truncated A1A2A3-VWF variants were detected by Penta-His Alexa Fluor 488 conjugate (QIAGEN, UK).

2.14.3 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) as well as a bright-field view. Eight fields of view were imaged per well at a magnification of 20x, equating to the analysis of approximately 1000 cells/well.

2.14.4 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. Nuclei were identified on the basis of Hoechst staining using the Top Hat segmentation method. FITC-labelled VWF was quantified and reported as total area in association with cells (figure 2.9). Data were graphed as percentage fluorescent area/cell relative to WT-VWF binding (mean $\pm$ SEM).

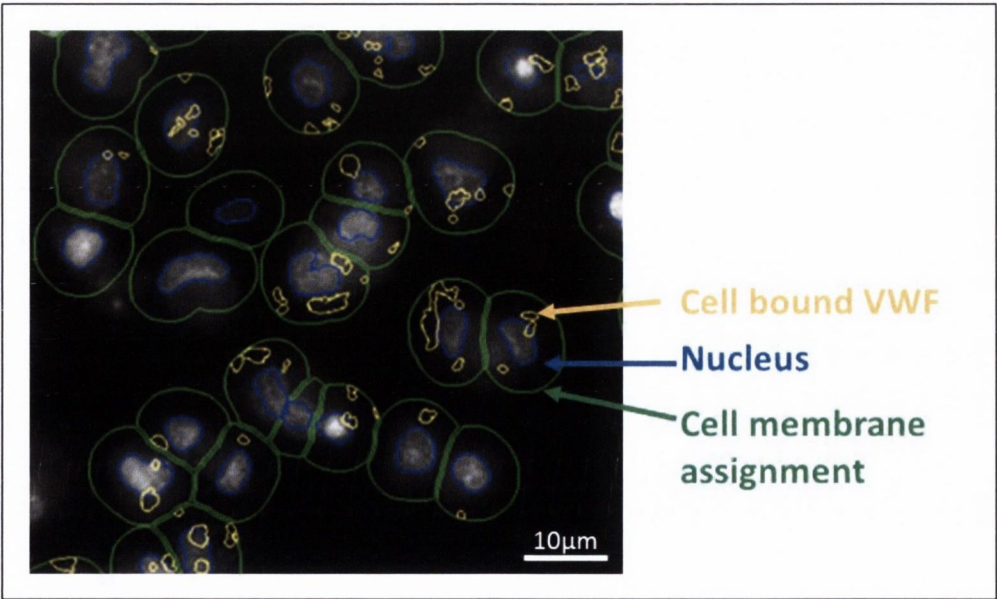


Figure 2.9 A representative images from the IN Cell image analysis THP-1 nuclei are depicted with a blue outline. The software assigns a cell membrane (green) and the cell bound VWF, labelled with a FITC antibody (yellow), is quantified.

2.15 VWF-galectin-1 *in vitro* binding assays

An ELISA was used to examine galectin-VWF interaction. Galectin-1, 330nM, was immobilized onto a 96-well microtiter Maxisorb plate (Nunc, UK), in 50mM sodium carbonate buffer (pH 9.6) overnight at 4°C. The plate was washed in triplicate in PBS-T and blocked using Protein-Free Blocking Solution (Thermo Fisher Scientific, Belgium) for 1 hour at room temperature. After washing, 100µl of VWF (plasma-derived, recombinant or glycan-modified) from 0 to 20nM was incubated on the plate for 2 hours at 37°C. Bound VWF was subsequently detected using 100µL of rabbit polyclonal anti-VWF-HRP (DAKO, Denmark) diluted 1:500 in PBS-T incubated for 1 hour at 37°C. After washing, HRP-activity was measured by addition of 190µl of o-phenylenediamine substrate (Sigma-Aldrich, Ireland) and stopped with 100µl of 1M H₂SO₄. Binding of galectin-1 to glycan-modified pd-VWF was expressed as a percentage of untreated pd-VWF at 2.5nM (non-saturating section of the binding curve) while binding to recombinant VWF variants was expressed as a percentage of recombinant wild-type VWF at 5nM. All binding assays were performed in triplicate.

2.16 FVIII-galectin *in vitro* binding assays

Modified ELISA assays were used to determine FVIII-galectin interactions. FVIII (plasma-derived, recombinant or glycan-modified) were immobilized onto a 96-well PolySorb microtiter plate (Nunc, UK), at concentrations of 2nM - 0.15nM in 50mM sodium carbonate buffer (pH 9.6) overnight at 4°C. The wells were washed three times in PBS-T and blocked using 0.5% (w/v) Polyvinylpyrrolidone (PVP) solution in PBS-T. Galectin-1 (160nM) or galectin-3 (500nM) were incubated on the plate for 2 hours at 37°C. Bound galectin-1/-3 were detected using specific mouse monoclonal antibodies,

anti-galectin-1 (Santa Cruz Biotechnology, USA) and anti-galectin-3 (R&D Systems, MN, USA) and goat anti-mouse HRP-conjugated antibody (Santa Cruz Biotechnology, USA). After washing HRP-activity was measured by addition of 190µl of o-phenylenediamine substrate (Sigma-Aldrich, Ireland) and stopped with 100µl of 1M H₂SO₄. Binding of galectin-1 and -3 to glycan-modified rFVIII was expressed as a percentage of untreated rFVIII at 0.5nM (non-saturating section of the binding curve). Binding of galectin-1 and -3 with equi-molar concentrations of commercial FVIII products was expressed as a percentage of rFVIII-CHO at 0.5nM. All binding assays were performed in triplicate.

2.17 Surface Plasmon Resonance (SPR)

SPR is a phenomenon that occurs in thin conducting films at an interface between media of differing refractive index. Within the Biacore™ system, these media are the glass surface of the sensor chip and the sample solution. Changes in solute concentration at the surface of the sensor chip results in changes in the refractive index of the solution which is measured as an SPR reading. Binding kinetics of FVIII with galectin-1 and -3 were determined using SPR analysis on a BIAcore 3000™ system (GE Healthcare, UK). This system employs glass surface sensor chips covered with a thin gold layer creating optimal conditions for generating an SPR signal. The gold surface is further modified with a monolayer of carboxymethyl dextran to allow biomolecule attachment. The system consists of a series of microfluidic channels to deliver a continuous flow of buffer and sample upon injection. Each chip contains two flow cell surfaces for sample immobilisation and analysis. Flow cell 1 is used as the reference cell while flow cell 2 contains the molecule of interest.

2.17.1 rFVIII immobilisation on sensor chip surface

In our studies rFVIII was covalently immobilized onto flow cell 2 of a CM5 sensor chip using an Amine Coupling Kit (GE Healthcare, UK). Amine coupling chemistry employs a mixture of 400mM ethyl-N’-(3’dimethylaminopropyl)-carbodiimide hydrochloride (EDC) 100mM NHS to activate carboxyl groups on the chip surface. rFVIII was the covalently coupled at a fixed density of 1000 RU in 10mM sodium acetate buffer pH 5.0 (Figure 2.10). This corresponds to a concentration of 1ng/mm² of protein on the chip surface. Un-reacted succinimide esters were subsequently deactivated by capping with 1M ethanolamine (pH 8.5). Flow cell one was activated with the EDC/NHS mixture as previous and capped using ethanolamine and used a reference cell.

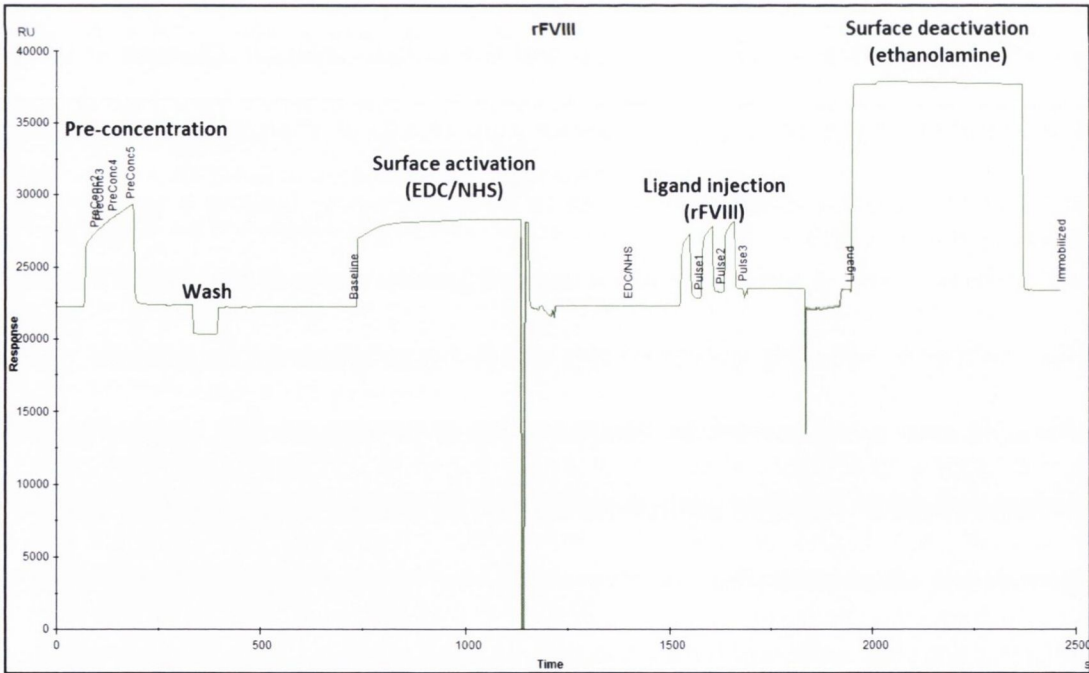


Figure 2.10 Sensorgram of rFVIII immobilisation at targeted RU 1000 level

2.17.2 Regeneration optimisation

To optimise conditions for regeneration of the chip surface after galectin binding, regeneration scouting was performed. The goal of regeneration is to remove bound galectin protein whilst leaving the rFVIII coated surface undamaged. Three standard regeneration conditions were examined, 10mM glycine pH 3.0, 10mM glycine pH 2.0 and 10mM NaOH pH 12. Galectin-1 was injected over the surface in triplet and each regeneration condition tested (figure 2.11). We observed that 10mM NaOH was optimal, with stable baseline measurements (blue line) and reproducible sample binding response (cycle 8, 9, 10).

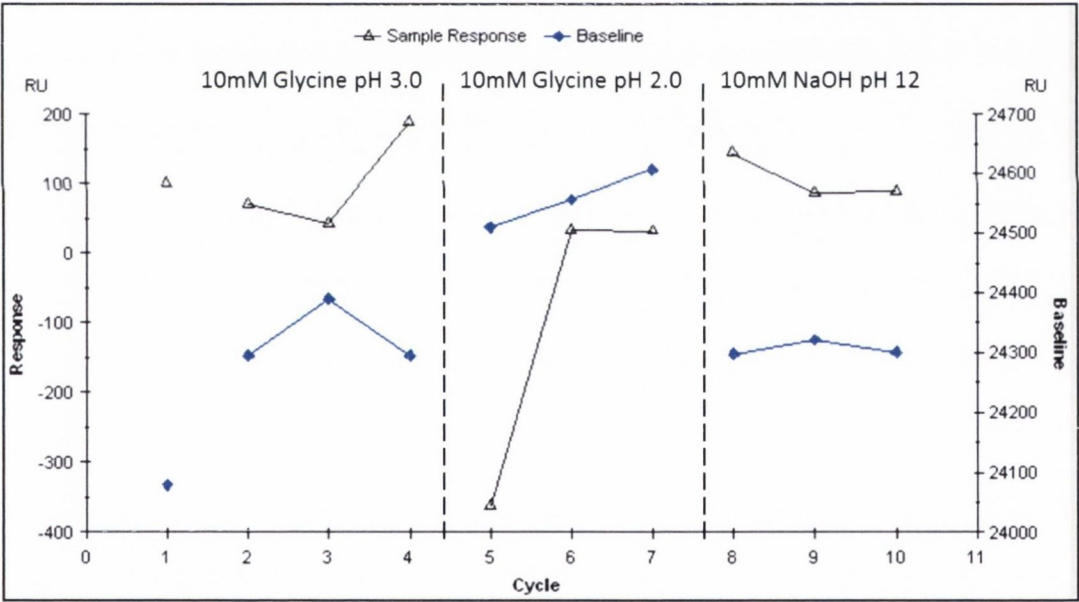


Figure 2.11 Regeneration optimisation
Baseline and sample response (galectin-1 binding) was measured in triplicate for each regeneration condition.

2.17.3 Galectin binding

For binding analysis, increasing concentrations of purified galectin-1/3 were passed over the surface of the chip at a flow rate of 30 μ L/min in HEPES buffer (10mM

HEPES, 3mM EDTA, 150mM NaCl and 0.005% (v/v) Tween 20, pH 7.4 with a contact time of 120 seconds. Dissociation was allowed for 10 minutes and the chip surface was regenerated with 10mM NaOH. The resulting sensorgrams were fit globally to a heterogeneous ligand model using BIAevaluation software (GE Healthcare, UK).

2.18 *In vitro* pull-down assay

Briefly, 100 µg of purified His-tagged recombinant galectin-1 or galectin-3 were incubated with 200µl of cobalt chelate beads (Talon, Clontech, France) in binding buffer (50mM Sodium phosphate, 300mM sodium chloride and 20 mM imidazole pH 8.0) for 1 hour at room temperature. Subsequently the beads were washed thoroughly in PBS-T, separating the beads from the supernatant each time by centrifugation at 4000rpm for 1 minute. Following this, 20µl of washed galectin-coated beads were incubated with 0.5mL of plasma from VWF^{-/-} mice for 1 hour at room temperature. Following incubation, the beads were separated from plasma by centrifugation as before and washed in triplicate with PBS-T. One final wash step was performed in reduced-tween PBS-T (0.01% v/v tween-20). The samples were analysed by SDS-PAGE immunoblotting (section 2.3.5) using rabbit polyclonal anti-FVIII heavy chain antibody at 0.4µg/mL (Santa Cruz Biotechnology, USA), and rabbit polyclonal anti-galectin-1 at 0.25µg/mL (Zymed, Invitrogen, USA) or rabbit polyclonal anti-galectin-3 at 0.7µg/mL (Abcam Cambridge, UK). All antibodies were diluted in 3% BSA in PBS-T and incubated for 1 hour at 37°C. Blots were washed thoroughly in PBS-T before applying secondary HRP-conjugated antibody (goat anti-rabbit IgG-HRP; Santa Cruz Biotechnology, USA).

2.19 FVIII Activity

FVIII activity was determined using a one-stage clotting assay. Galectin-1 or galectin-3 were incubated, at increasing concentrations, with 1nM rFVIII. These samples were subsequently mixed with FVIII-deficient plasma (Langanbach, Ireland) and phospholipids along with a negatively charged surface activator (kaolin, cephalin; C.K. Prest. Stago, Beijing, China) were added and incubated at 37°C for 3 minutes. This results in activation of the contact factors and leads to the generation of FXIa. Upon addition of 0.2mM CaCl_2 , FIX is activated by FXIa and FXa is then generated by the tenase complex, in which FVIII serves as a cofactor, thus this step is dependent on FVIII activity. The time for fibrin clot formation was recorded using Amelung KC4 Micro Clinical Coagulation Analyzer (Amelung, Trinity Biotech, Ireland). A standard curve was constructed using reference plasma and the results were expressed in a logarithmic-linear plot with log factor VIII activity vs linear clotting time. The factor VIII activities of samples in the presence or absence of galectin-1 and -3 were extrapolated from this curve.

Additionally, FVIII activity was also measured following the conversion of FX to FXa using a commercial Chromogenix™ assay (Instrumentation Laboratory, MA, USA) as per manufacturer's instructions. This assay uses purified bovine FIXa and FX rather than plasma. FX is activated to FXa by FIXa in the presence of calcium ions and phospholipids. This reaction is dependent on FVIIIa. The assay uses optimal amounts of calcium ions, phospholipids and FIXa together with an excess of FX, thus the rate of FX activation is linearly related to the FVIII activity in the sample. The amount of FXa generated is determined by the hydrolysis of a synthetic chromogenic substrate. The colour intensity is read spectrophotometrically at 405 nm. Increasing concentrations of

galectin-1 (1-10 μ M) were preincubated with 1nM rFVIII and FXa generation was measured as per kit protocol. A standard curve was constructed using known FVIII concentrations allowing extrapolation of FVIII activity in test samples.

2.20 *In vivo* clearance studies

2.20.1 Animal husbandry

Male and female VWF^{-/-} 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.

2.20.2 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.

2.20.3 Mouse Genotyping

Heterozygote VWF^{+/-} mice on a C57Bl/6J background (Jackson Labs) were bred and the offspring assessed for VWF^{-/-} homozygosity by PCR. Ear clippings were taken

from all offspring at wean age (three weeks of age). Ear samples were digested in 150µl DirectPCR Mouse Ear Lysis Reagent (Viagen Biotech, USA), and 0.2mg/mL proteinase K (Sigma-Aldrich, Ireland) at 55°C for 4-5 hours with shaking. Proteinase K was inactivated by heating samples to 85°C for 45 minutes. PCR was carried out on 1µl tail lysate with PrimeSTAR™ HS DNA Polymerase (Takara, Clontech, USA) according to manufacturer’s specifications (Table 2.7).

Reagent	Amount
5x PrimeSTAR Buffer	10µl
dNTPs	0.2mM
Forward primer	0.2µM
Reverse primer	0.2µM
Template	100ng
PrimeSTAR HS DNA Polymerase	0.5µl
Sterilised dH ₂ O up to	50µl

Table 2.7 PCR protocol for mouse genotyping using PrimeSTAR DNA polymerase

A multiplex reaction was set up using the primers outlined in table (2.8) and the PCR conditions detailed in table (2.9).

Primer name	5'-3' Sequence
VWF/GT-s	GAGGCTGGGTACTATAAGCTC
VWF/GT-as7	GCCATCTTGAAGTTGGATGG
VWF/GT-as10	ATGGAAGGATTGGAGCTACG

Table 2.8 Primers designed for genotyping VWF^{-/-} mice

Step	Temperature	Time
Hotstart	95°C	2 mins
Denature	95°C	30 seconds
Annealing	56°C	30 seconds
Extension	72°C	1 min/kb
30 cycles		

Table 2.9 PCR program used for genotyping VWF^{-/-} mice

1.8% agarose (w/v) was dissolved in Tris Borate EDTA (TBE) buffer (90mM Tris, 90mM Boric acid, 2mM EDTA, pH 8.3) by heating. The gels were poured into a horizontal gel electrophoresis tank (BRL 58 Horizontal Gel Electrophoresis Apparatus minitank; Gibco, UK). Ethidium bromide was used to visualise the DNA bands by exposure to ultraviolet light. DNA samples were prepared with 10x Loading Dye (4g sucrose, 25mg Orange G; Sigma Aldrich, Ireland, in 10 mL dH₂O). The samples were run in parallel with 50bp DNA ladder (New England BioLabs, UK). The gels were run at 70V for 45 minutes and a further 10 minutes at 90V and visualised under UV light in a UVP BioSpectrum GelDoc Imaging system. Expectant bands for heterozygous mice are 730bp and 570bp, while wild-type and homozygous mice have only a single band at 730bp and 570bp, respectively (Figure 2.12).

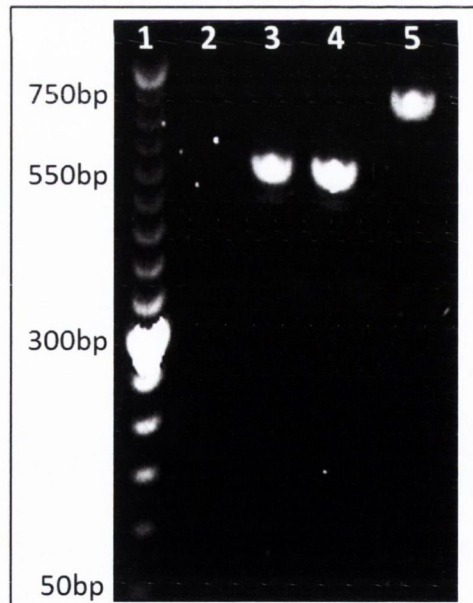


Figure 2.12 Mouse Genotyping

Lane 1: 50bp ladder (NEB, UK),

Lane 2: negative control

Lane 3 and 4; homozygous mice with a band size of 570bp,

Lane 5: wild-type mice display a band size of 730bp. Heterozygous mice express both bands.

2.20.4 VWF clearance studies

2.20.4.1 Administration of VWF intravenously

In keeping with previous biodistribution studies with human VWF in this mouse model a VWF dosage of 37.5U/kg was chosen (Lenting *et al*, 2004; Shi *et al*, 2012). This equates to approximately 0.75U of VWF for a typical 20g mouse. Normal pooled human reference plasma contains 1U or 10µg/mL VWF thus the dosage chosen for human VWF in study is physiologically relevant. However other pharmacokinetic studies with murine VWF in this mouse model have administered up to 200U/kg (Pruss *et al*, 2011) suggesting that markedly high plasma VWF levels are required before saturation is reached. VWF^{-/-} mice were immobilised in a restraint unit. Gentle heat was applied to the tail to encourage vasodilation. The tail veins, which run the length of both lateral aspects of the tail, were inspected for quality and a suitable site was chosen. The tail was stabilized between the thumb and forefinger. Digital pressure served as a tourniquet. 30nM of rVWF or variants thereof was administered in sterile PBS in a volume of 100µl via lateral tail vein injection. After removal of the needle, pressure was applied to the site for up to 1 minute.

2.20.4.2 Anaesthetic selection and preparation

For non-recovery blood collection procedures tribromoethanol (TBE) was selected as a suitable anaesthetic. This particular compound has several advantages over other common inhalation agents. TBE has a rapid induction (2-3 minutes) of short-term surgical anaesthesia lasting approximately 20 minutes without significant cardiac depression as seen with inhalation agents. Consequently this allows for sufficient blood volume collection in our studies. TBE has a good margin of safety with

low mortality rates of 1% (Papaioannou & Fox, 1993). TBE is administered by a single intraperitoneal injection and thus is simpler to use than inhalation agents which require equipment for induction and maintenance of anaesthesia and pose a higher occupation risk due to their volatile nature. The stock solution of anaesthetic was prepared by dissolving 2,2,2 tribromoethanol (Sigma-Aldrich, Ireland) in T-amyl alcohol (Sigma-Aldrich, Ireland) to a final concentration of 1.6g/mL. The solution is protected from light and kept at room temperature. A working stock, of 2.5% (v/v) in sterile saline (150mM NaCl) was prepared for injection. The dose used was 0.02mL/g of body weight.

2.20.4.3 Intraperitoneal anaesthetic delivery

At the appropriate time points post injection, mice were anaesthetised with an intraperitoneal injection of 2.5% tribromoethanol (0.2mL per 10g of body weight). Once the righting and pedal reflexes were lost, mice were placed the prone position, the forelimb stretched and a deep incision made in the axilla close to the thorax. The subclavian or axillary artery was severed and blood collected into heparin-coated microcontainers (BD Unitech, Ireland). Three to eight mice per time point were used.

2.20.4.4 Plasma collection and analysis

Blood samples were centrifuged at 2,500g for 15 minutes at room temperature and plasma was removed. Plasma VWF:Ag levels were subsequently measured by ELISA (section 2.3.1). Human reference plasma was used as a standard for quantifying full length VWF constructs analysed while a purified A1A2A3 VWF standard was used to determine the clearance rates for these truncated VWF constructs. Residual VWF value was expressed at a percentage of the amount injected which was corrected for

the dilution factor of total mouse blood volume. Clearance values were graphed as residual VWF (%) relative to the amount injected against time (minutes). The intra-assay coefficients of variation for each recombinant VWF variant injected was less than 20% at each time point measured.

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:

$$Y=(Y_0-\text{Plateau}) * e^{(-K * X)} + \text{Plateau}$$

Where Y refers to the amount of residual VWF antigen in plasma relative to the amount injected, X=time, K=rate constant, Plateau=0 and SEM=standard error of the mean and $\ln 2$ =natural log of 2.

The equation for monoexponential MRT was given as:

$$\text{MRT} = 1/K \pm \text{SEM}$$

Monoexponential half-life was given as:

$$t_{1/2} = \ln 2 / K \pm \text{SEM}$$

2.20.5 *In vivo* macrophage depletion

Clodronate is a bisphosphonate drug which is metabolised to a toxic analog of ATP. Consequently its accumulation intracellularly inhibits mitochondrial oxygen consumption resulting in cell death. Free clodronate does not pass easily across cell membranes and only has a half-life of approximately 15 minutes. Thus clodronate is

encapsulated in a lipid bilayer of liposomes, which specifically targets it to phagocytic cells such as macrophages (van Rooijen *et al*, 1996). Administration of clodronate liposomes intravenously primarily targets macrophages localised in the liver and spleen (van Rooijen & Hendrikx, 2010). Clodronate liposomes are significantly advantageous over other chemical agents used to deplete macrophages as clodronate has a very specific mode of action with limited unwanted side effects. Liver and splenic macrophage cells begin to repopulate approximately a week after administration (van Rooijen *et al*, 1989).

2.20.5.1 Clodronate liposomes preparation and administration

Clodronate liposomes were used to transiently deplete macrophage populations *in vivo*. Clodronate liposomes were administered via intravenous injection to selectively target splenic and hepatic macrophage populations including Kupffer cells and red pulp macrophages. These populations have been previously demonstrated to regulate VWF clearance. 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 5mg/mL solution (Van Rooijen and Sanders 1994). Mice were intravenously injected with clodronate liposomes (100 μ L/10g body weight) 24 hours prior to the injection of VWF.

2.20.5.2 Flow cytometry assessment of macrophages depletion

To confirm depletion of macrophage populations, spleens were harvested from mice who received clodronate liposomes and control PBS liposomes. Flow cytometry analysis of F4/80+/CD11B+ splenic cell populations was performed (carried out by Dr.

Hendrik Nel, Inflammation and Immunity Research Group, Trinity College Dublin). In the red pulp and marginal zone of splenic tissues, monocytes and macrophages express the macrophage markers CD11b and F4/80. Cells were co-labelled with APC anti-F4/80 (BM8) and PE anti-CD11b (M1/70) (eBiosciences). The presence of these cell surface markers was assessed by flow cytometry with data collection on a CyAn (Beckman Coulter). Appropriate isotype control antibodies were used and data were plotted on logarithmic scale dot-plots.

2.21 Data analysis and statistics

Experiments were performed in triplicate and data are presented as mean $\pm$ SEM. To assess statistical differences in the galectin binding studies the data was analysed with Student's unpaired 2-tailed *t* test. $P > 0.05$ = non-significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. For mouse clearance studies, a one way ANOVA with post-hoc Dunnett's test for multiple comparisons was used. All data analysis was performed using GraphPad Prism software (GraphPad Prism version 5.0 for Windows; GraphPad Software, CA, USA).

3 Defining the molecular basis underlying the physiological interaction between von Willebrand factor and galectin-1 in plasma

3.1 Galectins

Galectins, historically termed soluble-type or S-type lectins, are a family of highly conserved carbohydrate binding proteins characterised by their affinity for β -galactoside containing glycans, with significant sequence homologies in their carbohydrate recognition domains (CRD). Each CRD comprises approximately 135 amino acids folded into 5 or 6 β -sheet structures. Currently fifteen mammalian galectins have been identified. These have been sub-grouped into three main types on the basis of the spatial arrangement of their CRD. Group 1 are the proto-type galectins, and consist of a single CRD, (e.g. galectin-1). Group 2 are chimeric type galectins. These contain a C-terminal CRD, and an N-terminal non-lectin binding domain, (galectin-3 is the sole member of this family). Finally, Group 3 include the tandem-repeat galectins which possess two distinct CRDs, (e.g. galectin-8).

Galectins are expressed in many human tissues, including endothelial cells, platelets and macrophages (Lotan *et al*, 1994; Herrmann *et al*, 2002; Thijssen *et al*, 2008; Van Den Brûle *et al*, 2004; Romaniuk *et al*, 2010). Even though galectin genes do not encode a classical secretory signal many galectins have also been identified in the extracellular compartment. Galectins have been implicated in mediating diverse array of both physiological and pathological functions, including cell adhesion, immune modulation and inflammation (Stowell *et al*, 2010; Toscano *et al*, 2007; Hughes, 2001;

Rabinovich & Toscano, 2009; Romaniuk *et al*, 2010). Lectin arrays and frontal affinity chromatography have been used to elucidate the sugar binding specificities for several members of the galectin family. The CRD of galectins typically recognise the basic disaccharide ligand Gal β 1-4GlcNAc, or its linkage isomer Gal β 1-3GlcNAc (N-acetyllactosamine or LacNAc). Moreover, within this glycan structure, three specific hydroxyl groups have been shown to be of particular importance in regulating galectin interactions (4-OH and 6-OH of the galactose moiety and position 3-OH of the N-acetylglucosamine (GlcNAc) (Hirabayashi *et al*, 2002). Consequently substitution or modification of these critical sites in the LacNAc ligand disrupts galectin affinity. Interestingly however the positions 2-OH and 3-OH of the terminal galactose can be modified and thus galectin binding specificities can be further diversified, allowing recognition of a vast array of heterogeneous glycan structures. For example, addition of blood group antigens to the basic LacNAc ligand significantly enhances binding for several members of the galectin family, including galectin-1, galectin-3 and galectin-8 (Stowell *et al*, 2008; Hirabayashi *et al*, 2002). Furthermore galectin-1 affinity is enhanced with increased branching of complex N-glycan chains. In contrast galectin-3 affinity is unaffected by the level of branching but rather its affinity is enhanced by the presence of repeating LacNAc units (Hirabayashi *et al*, 2002).

Extensive literature exists proposing galectins as inflammatory mediators, regulating both adaptive and innate immune responses (Rabinovich *et al*, 2004). While some members act as negative regulators of immune responses and modulate T-cell homeostasis (e.g. galectin-1), others serve to propagate pro-inflammatory responses promoting T-cell proliferation (e.g. galectin-3) (Yang *et al*, 1996). Differential repertoires of glycans expressed on Th1 versus Th2 cells results in distinct galectin

binding. For example Th1 cells fail to express the protective α 2-6 sialic acid residues found in Th2 glycans. Thus galectin-1 adheres to Th1 cells inducing cell death (Toscano *et al*, 2007). Consequently galectin-1 promotes a polarised Th2 response and thus immune suppression. In keeping with these data, galectin-1 deficient mice display enhanced susceptibility to experimentally induced autoimmune disorders including encephalomyelitis and arthritis (Rabinovich *et al*, 1999; Offner *et al*, 1990). In addition to its role in T cell modulation, galectin-1 also modulates autoantibody-mediated inflammation. Administration of galectin-1 prevented the development of experimental myasthenia gravis in rabbits and reduced functionality of inhibitory antibodies (Levi *et al*, 1983).

Galectins are not limited to binding host glycan structures, but can also recognise β -galactoside containing glycans present on microorganisms. In this manner, galectins have been demonstrated to act as defence lectins, recognising pathogenic glycan motifs on bacteria and inducing cell death (Stowell *et al*, 2010).

3.1.1 Galectins in haemostasis

In recent years, novel roles for galectins have been highlighted in thrombosis and haemostasis (Romaniuk *et al*, 2010, 2012; Saint-Lu *et al*, 2012; Zappelli *et al*, 2012; Diaz *et al*, 2013). Galectin-1 and -8 have both been shown to modulate platelet physiology. Galectin-1 triggers platelet activation in a carbohydrate-dependent manner, mediated through α IIb β 3 platelet receptor (Romaniuk *et al*, 2011). In this context, galectin-1 acts as a non-classical platelet agonist, triggering platelet activation through outside-in signalling via direct binding to α IIb β 3 integrin. It has also been proposed that galectin-1 has the ability to form multivalent lattice structures which may induce α IIb β 3 integrin

clustering and thereby mediate platelet activation. Upon galectin-1 stimulation, platelets undergo morphological changes and display increased surface expression of P-selectin as well as GPIIb-IIIa activation (Pacienza *et al*, 2008). Consequently, galectin-1 deficient mice demonstrate impaired platelet GPIIb-IIIa platelet signalling, reduced platelet adhesion to immobilised fibrinogen, and exhibit a bleeding phenotype (Romaniuk *et al*, 2012). Interestingly, the addition of exogenous galectin-1 failed to restore normal platelet activation in these mice, leading the authors to hypothesise that endogenous platelet-derived galectin-1, may be critical in an autocrine signalling pathway for modulating normal platelet physiology. Galectin-8 also promotes platelet activation. However this effect is modulated through the GPIb α platelet glycoprotein (Romaniuk *et al*, 2010). Importantly, galectin-8 was found to trigger both dense and alpha granule release. Additionally, members of the galectin family have been reported to directly interact with cofactors in the coagulation cascade. For example, galectin-8 expressed on megakaryocytes mediates coagulation Factor V endocytosis (Zappelli *et al*, 2012).

Plasma galectin levels have been studied in patients with thrombotic complications. Plasma galectin-3 levels were significantly elevated, over 4-fold, in patients with venous thrombosis (DeRoo *et al*, 2014). Moreover, in contrast to finding by Saint-Lu *et al*, galectin-3 deficient mice exhibited a 34% reduction in thrombus weight compared to controls in an inferior vena cava stasis model (DeRoo *et al*, 2014). Additionally wild-type mice displayed a statistically significant increase in plasma galectin-3 within 48 hours of inferior cava ligation and histology revealed galectin-3 to colocalise at the leukocyte/endothelial interface. These findings highlight a role for galectin-3 in promoting venous thrombosis (Diaz *et al*, 2013; DeRoo *et al*, 2014).

3.1.2 Galectins and VWF

Interestingly, Saint-lu *et al* recently reported that galectin-1 and galectin-3, both colocalise with VWF within EC. In addition coprecipitation studies demonstrated that both galectins remained associated with VWF in normal plasma following secretion into the peripheral circulation. Moreover, significantly decreased plasma concentrations of galectin-1 and -3 were demonstrated in VWF-deficient mice, correctable upon VWF gene transfer. Finally, and most critically, these galectins were found to directly modulate VWF functional activity. VWF string formation on endothelial cell surface was enhanced in galectin-1/galectin-3 double deficient mice, resulting in enhanced thrombus formation following ferric chloride-induced injury compared to wild-type controls (Saint-Lu *et al*, 2012).

Given this novel description of galectin-1 and -3 as binding partners of human VWF, and regulators of VWF functional activity, we sought to define the molecular basis underlying the physiological interaction between VWF and galectin. On the basis that the galectin-1, but not galectin-3, knockout mouse model exhibits altered haemostasis upon tail clip challenge (Romaniuk *et al*, 2012) we focused our study on examining the interaction between galectin-1 and VWF. Moreover, galectin-1 binding affinity, in contrast to galectin-3, is dramatically enhanced with increased branching of glycan chains (Hirabayashi *et al*, 2002) making VWF a highly suited ligand for galectin-1.

3.2 VWF binds galectin-1 in a dose-dependent and glycan-dependent manner

Recent data suggest that VWF circulates in normal plasma bound to carbohydrate-binding proteins galectin-1 and galectin-3. In order to characterise this physiological interaction, plasma-derived VWF (pd-VWF) was purified from commercial concentrate Haemate P® and its glycosylation profile modified using specific exoglycosidases. VWF-galectin binding interactions were then studied using recombinant human galectin-1 expressed in *E. coli* cells and modified immunosorbent assays with human pd-VWF, platelet-derived (plt-VWF) and recombinant VWF (rVWF).

Purified pd-VWF bound to galectin-1 (figure 3.1A) in a dose-dependent and saturable manner. The affinity, (as measured by the K_d apparent), was calculated as 13.9 ± 2.5 nM. This affinity is unusually high for a lectin-glycoprotein interaction. Interestingly however, Saint-lu et al also reported a galectin-VWF affinity in the range of 20-80nM (Saint-Lu *et al*, 2012). The high affinity nature of this interaction may be attributable to the clustering of glycan chains on pd-VWF. This glycan clustering results in increased local density of galectin ligands which has previously been shown to significantly increase galectin affinity in a synergistically manner (Dam & Brewer, 2008).

To assess the role of specific VWF glycan populations in modulating interaction with galectin 1, pd-VWF was subjected to enzymatic digestion with specific exoglycosidases to modify its glycan profile. 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-1 ($13.1 \pm 1\%$, $p < 0.001$, figure 3.1B). To determine the contribution of VWF O-linked glycans, VWF was

incubated with both PNGase F and O-glycosidase to remove both the N- and O-linked glycans (PNG-Ogly VWF). Combined removal of both N- and O-linked did not serve to further reduce galectin-1 binding compared to PNG-VWF alone ($15.5 \pm 2\%$ versus $13.1 \pm 1\%$, respectively, $p > 0.05$). Cumulatively, these data establish that the N-linked glycans of VWF are the critical mediators of its interaction with galectin-1. This is not surprising given that the majority of VWF N-linked glycans have been reported as complex-type glycans expressing LacNAc sequences (Canis *et al*, 2012). Additionally, VWF N-linked glycans are highly branched, (ranging from bi-, tri- and tetra-antennary structures), and are thus particularly suited for galectin-1 binding. The T-antigen, sialylated Gal(β 1-3)GalNAc, represents the predominant O-glycan chain for VWF. Structural analysis of galectin-1 CRD has revealed that a pentad amino acid motif resides with its CRD which confers significant steric hindrance for galectin-1 binding to T-antigen. Consequently galectin-1 displays no affinity for T-antigen structures (Bian *et al*, 2011). Our data are therefore in keeping with these findings and explain why removal of the O-linked glycans on VWF does not serve to influence galectin-1 binding.

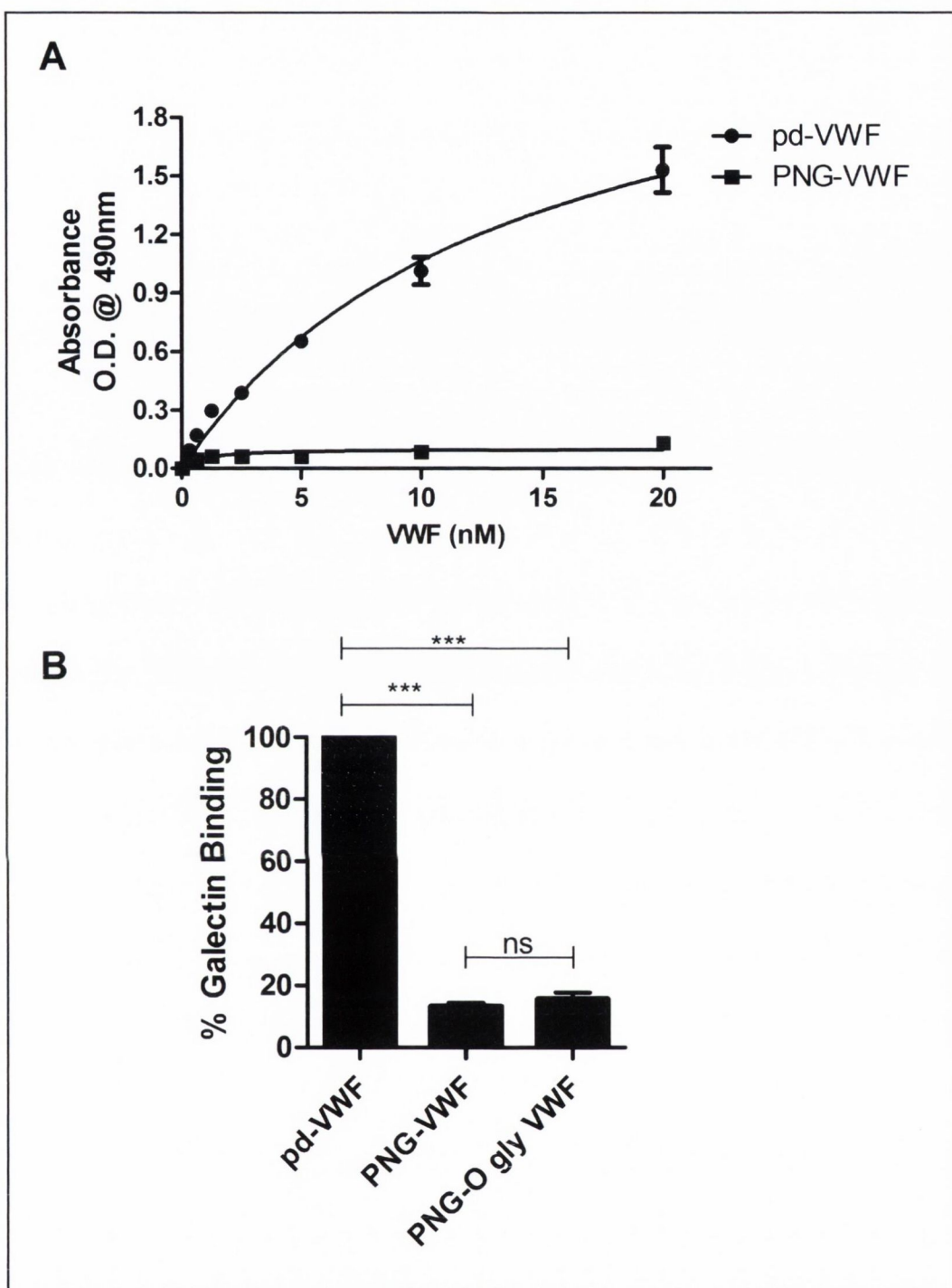


Figure 3.1 Galectin-1 binds to VWF in a dose dependent and N-linked glycan-specific manner (A) Galectin-1 displays dose-dependent and glycan-dependent binding for pd-VWF, (B) Modification of the glycan profile of pd-VWF demonstrated a key role for the N-linked glycans in mediating galectin-1 binding. Galectin binding is expressed as a percentage of pd-VWF at 2.5nM.

3.3 Terminal glycan modifications critically regulate the VWF/galectin interaction

3.3.1 Effect of VWF terminal sialic acid expression on galectin-1 binding

The N-linked glycans of pd-VWF have previously been demonstrated to carry a significant amount of terminal sialic acid (Canis *et al*, 2012). In fact, HPLC analysis has revealed that 80% of total sialic acid of VWF is present on its N-linked glycan chains, where it is predominantly α 2-6-linked to penultimate galactose residues. In contrast, O-linked sialic acid, constitutes only 20% of the total sialic acid expression on pd-VWF and may be α 2-3 or α 2-6 linked (McGrath *et al*, 2010). The sialylation status of VWF directly influences its functional activity and circulatory half-life. For example, terminal sialic acid on VWF significantly enhances its susceptibility to proteolysis by ADAMTS13 (McGrath *et al*, 2010). Additionally, sialic acid influences the interaction of VWF with the hepatic asialoglycoprotein clearance receptor (Grewal *et al*, 2008). To investigate the role of specific terminal glycan determinants, including sialic acid, in mediating the interaction of VWF with galectin-1, the VWF glycosylation profile was modified to generate a number of glycan variants. Complete removal of VWF terminal sialic acid via α 2-3,6,8,9 neuraminidase (Neu-VWF) digestion exposes the sub-terminal galectin ligand, (LacNAc) and resulted in markedly enhanced galectin-1 binding ($230.8 \pm 5\%$, $p < 0.0001$, figure. 3.2). Sialylation, (or indeed any modification, at position 6 of the galactose within LacNAc sequence), disrupts a critically conserved galectin binding residue (Hirabayashi *et al*, 2002). This is consistent with the observation that enzymatic removal of α 2-6 terminal sialic acid enhanced galectin binding. Conversely, specific

removal of VWF O-linked sialic acid via treatment with α 2-3 neuraminidase had no effect on galectin-1 binding. Galectins are more permissive to modification of position 3 on the sub-terminal galactose compared to position 6 and thus specific removal of VWF α 2-3 linked sialic does not serve to expose shielded galectin binding sites. Additionally this population of sialic acid is largely limited to the terminus of the O-linked glycans on VWF, which we have established as having a negligible contribution to the galectin-1/VWF interaction.

To assess the contribution of sub-terminal galactose in modulating galectin-1 binding, pd-VWF was exposed to sequential α 2-3,6,8,9 neuraminidase and galactosidase digestions to remove both terminal sialic acid and sub-terminal galactose residues (NeuGal-VWF). In contrast to the enhanced binding of galectin-1 with Neu-VWF, binding of galectin-1 with NeuGal-VWF was significantly reduced ($51.2 \pm 4\%$ compared to pdVWF; $p < 0.005$; figure 3.2).

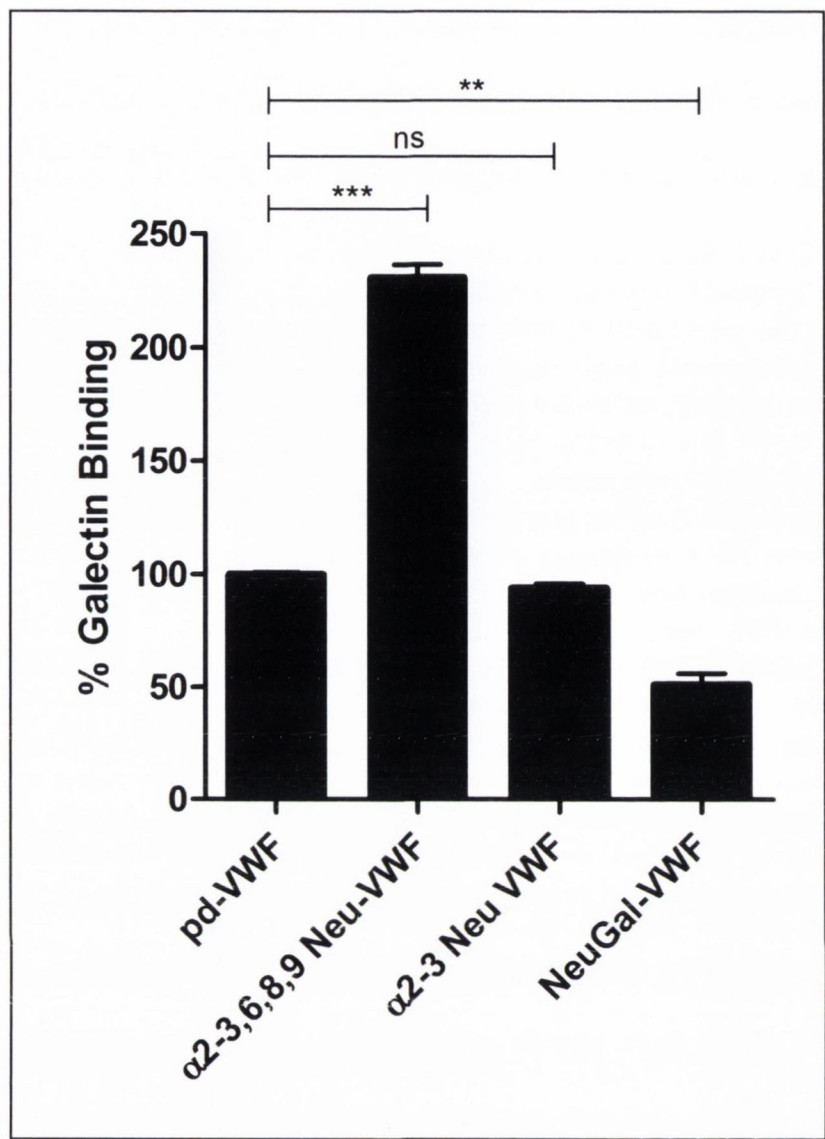


Figure 3.2 Terminal sialic acid and sub-terminal galactose on VWF N-linked glycans modulate galectin-1 binding. Galectin binding to glycan-modified pd-VWF is expressed as a percentage of untreated pd-VWF at 2.5nM, non-saturating conditions.

3.3.2 Effect of terminal ABO(H) determinants of galectin-1 binding

In addition to sialic acid residues, ABO(H) blood group antigens are terminally expressed on 10% of the N-linked glycans of pd-VWF (Matsui *et al*, 1992). Interestingly, they are also present on a small proportion of VWF O-linked glycans (Canis *et al*, 2010). Moreover, ABO(H) determinants influence pd-VWF susceptibility to ADAMTS13 proteolysis (Bowen, 2003). Furthermore, blood group antigens also significantly modulate the plasma half-life of VWF in humans (Gallinaro *et al*, 2008; O'Donnell *et al*, 2005). Importantly however, the exact molecular mechanisms through which these single sugar modifications serve to influence VWF biology remains poorly defined. To investigate whether ABO(H) antigen expression on VWF influences its interaction with galectin-1, VWF was purified from blood group specific plasma (Group AB, O and Bombay) using cryoprecipitation and gel filtration. Interestingly, a significant effect of ABO blood group on galectin-1 binding was observed (figure 3.3). VWF from group AB individuals bound to galectin-1 (figure, 3.3B) significantly better than group O VWF ($272.6 \pm 10\%$ vs $142.2 \pm 14\%$ $p < 0.05$). Conversely, no significant difference in binding was observed between group O and Bombay VWF ($142.2 \pm 14\%$ vs $100.0 \pm 8.8\%$, respectively, $p > 0.05$). Members of the galectin family have previously been demonstrated to display increased affinity for A and B blood group containing glycans on both micro-array and affinity chromatography analysis (Stowell *et al*, 2008; Hirabayashi *et al*, 2002). Moreover, galectin-4 and galectin-8 have been shown to adhere to and induce apoptosis in blood group antigen expression *E. coli* cells (Stowell *et al*, 2010).

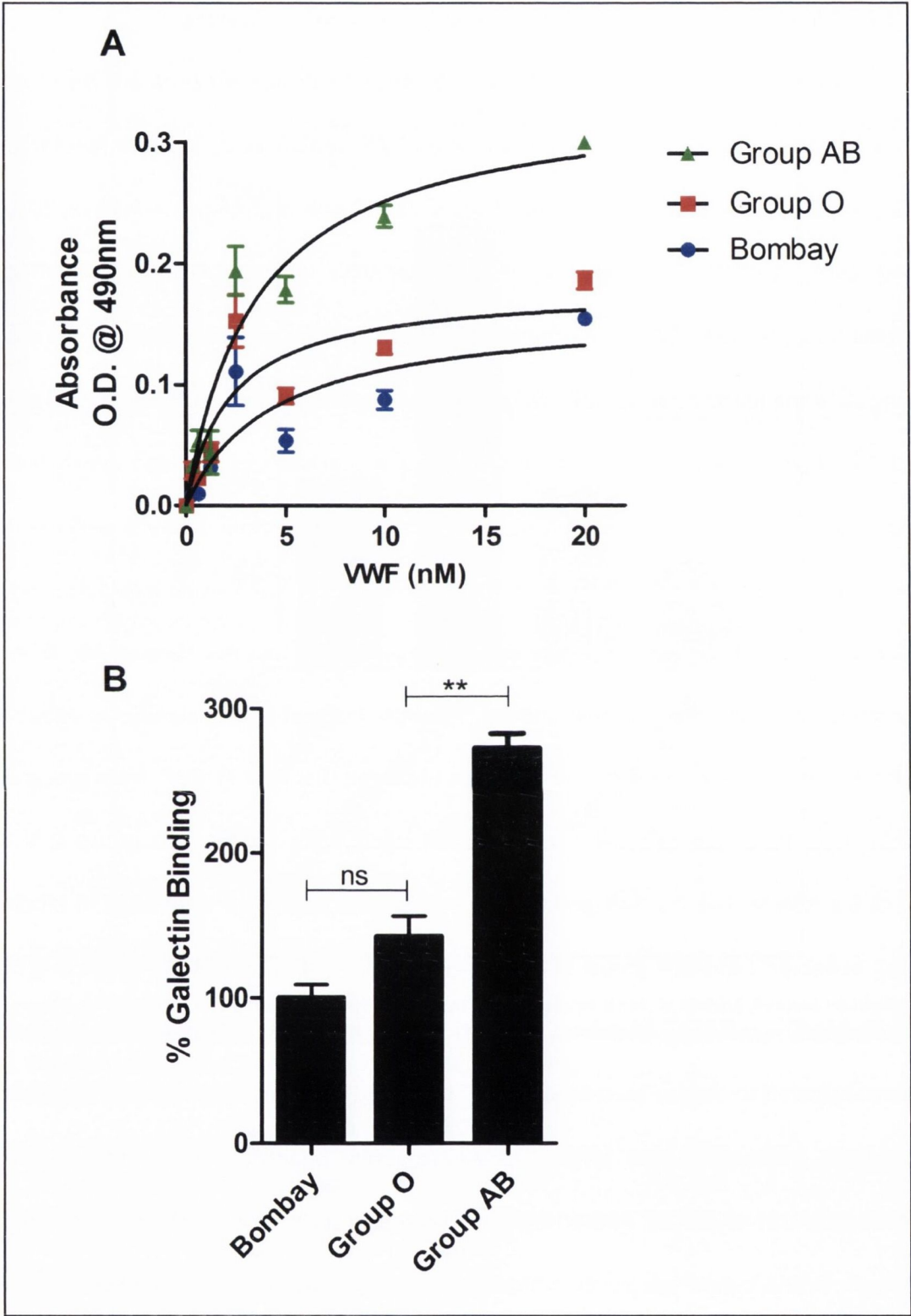


Figure 3.3 Terminal ABO(H) VWF glycan determinants regulate galectin-1 binding (A) Galectin-1 binding with blood group-specific pd-VWF, (B) Galectin-1 binding with 10nM blood group-specific pd-VWF.

3.4 Cellular origin of VWF modulates galectin-1 binding

Within normal plasma, the majority of circulating VWF is derived from endothelial cells. However significant proportion of circulating VWF is present within platelets (Zucker *et al*, 1979). Importantly, the post-translational modification of VWF synthesized within megakaryocytes differs significantly to that of VWF produced within endothelial cells (figure 3.4A). In particular, total sialic acid and galactose expression are reduced 2-fold on plt-VWF (Williams *et al*, 1994). In addition, plt-VWF fails to express terminal ABO(H) blood group antigens and is also enriched in ultra-large multimers (Brown *et al*, 2002). Interestingly plt-VWF has been characterised as functionally distinct from endothelial VWF. These differences are likely to be in part due to its differential glycosylation profile. To characterize galectin affinity for this distinct glycoform of VWF, plt-VWF was isolated and purified by platelet snap freeze-thawing followed by immuno-affinity chromatography. Plt-VWF bound significantly less to galectin-1 compared to pd-VWF ($72.1 \pm 5\%$ $p < 0.05$, figure 3.4B). The glycosylation profile of recombinant VWF (rVWF), produced in HEK293T cells, also differs significantly to that of pd-VWF. In addition, multimer composition is different and terminal ABO(H) blood group antigens are not expressed (figure 3.4A). In keeping with this, the binding of rVWF expressed in HEK293T cells to galectin-1 was significantly reduced ($45.0 \pm 3\%$ $p < 0.0001$) compared to either pd-VWF or plt-VWF respectively. Cumulatively these data indicate that VWF from different cellular origins, with differential glycosylation and multimeric profiles, exhibit distinct binding interactions with galectin-1.

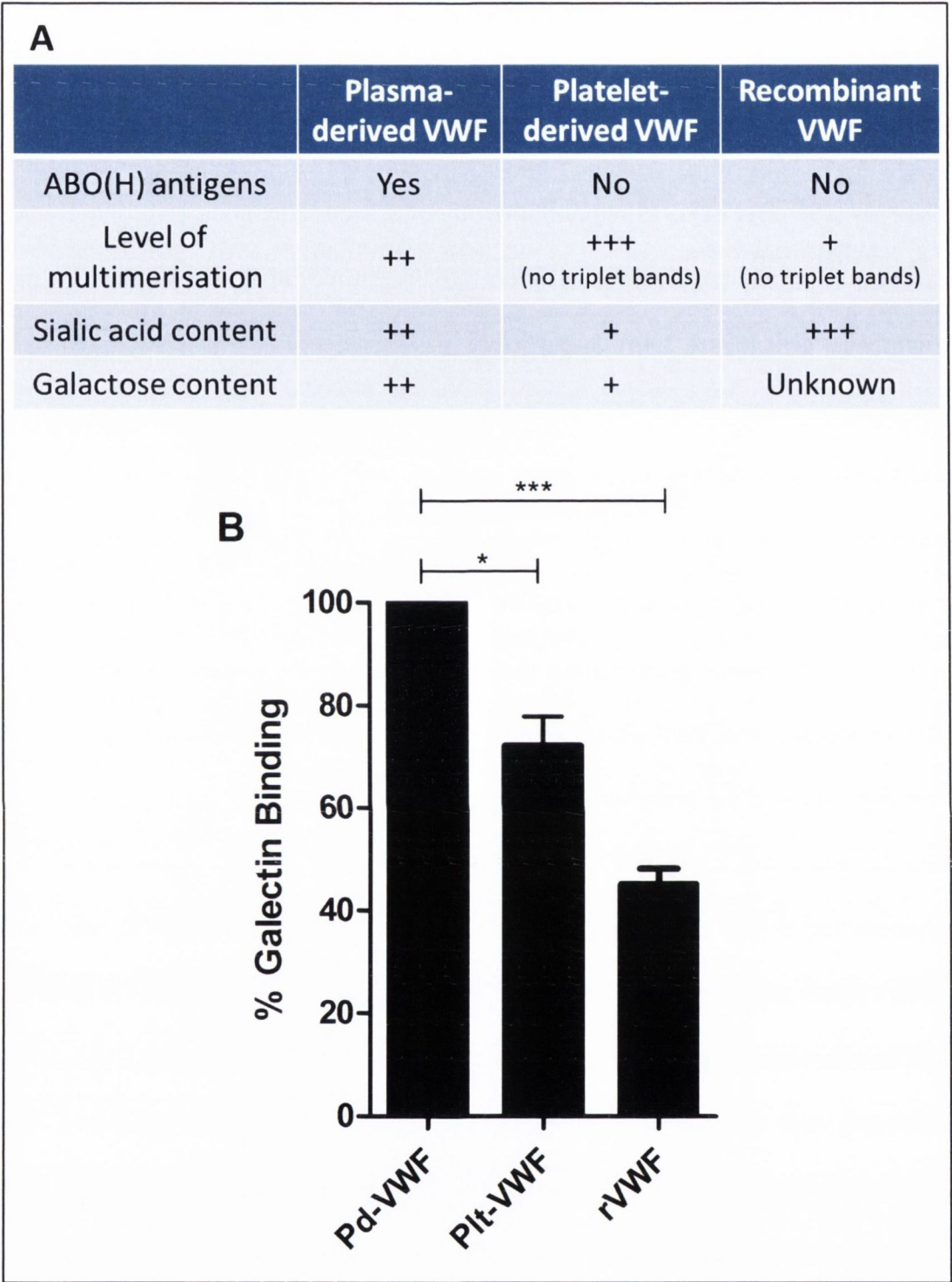


Figure 3.4 Cellular origins influence galectin-1 binding

(A) A table highlighting differential glycosylation and multimerisation patterns between VWF of different cellular origins, (B) Galectin-1 binding was expressed as a percentage of pdVWF at 2.5nM. Galectin-1 displays distinct binding preferences for pd-VWF, plt-VWF and rVWF.

3.5 VWF multimeric composition regulates galectin-1 binding

VWF multimerisation is a critical determinant of functional activity. Upon release from endothelial cells, pd-VWF exists as elongated strings of ultra-large VWF (UL-VWF) multimers. These haemostatically active forms of the protein are then cleaved by the zinc metalloprotease ADAMTS13. This proteolysis results in a heterogeneous population of high molecular weight multimers (HMWM) and low molecular weight multimers (LMWM) of VWF within circulation, (ranging from 500-20,000 kDa in size).

To investigate whether multimeric structure influences the ability of VWF to interact with galectin-1, HMW-VWF and LMW-VWF were isolated from group AB and group O plasmas by cryoprecipitation and gel filtration chromatography. The level of VWF multimerisation was assessed by determining the ratio of VWF antigen to collagen binding activity. Additionally VWF multimer analysis was visualised using agarose gel electrophoresis. Binding studies utilising size-fractionated, blood group specific VWF demonstrated that galectin-1 preferentially bound to HMWM compared to LMWM of the same blood group. Galectin-1 binding was reduced to $43.6 \pm 5\%$ for LMWM AB-VWF compared to HMWM AB-VWF, ($p < 0.0001$, figure 3.5). Similarly the binding of galectin-1 with O-VWF LMWM was decreased by $37.7 \pm 5\%$ compared to HMWM of the same blood group, ($p < 0.0001$). Cumulatively, these novel data demonstrate that VWF post-translational modifications, including glycosylation and multimerisation, are crucial regulators of VWF/galectin-1 interaction.

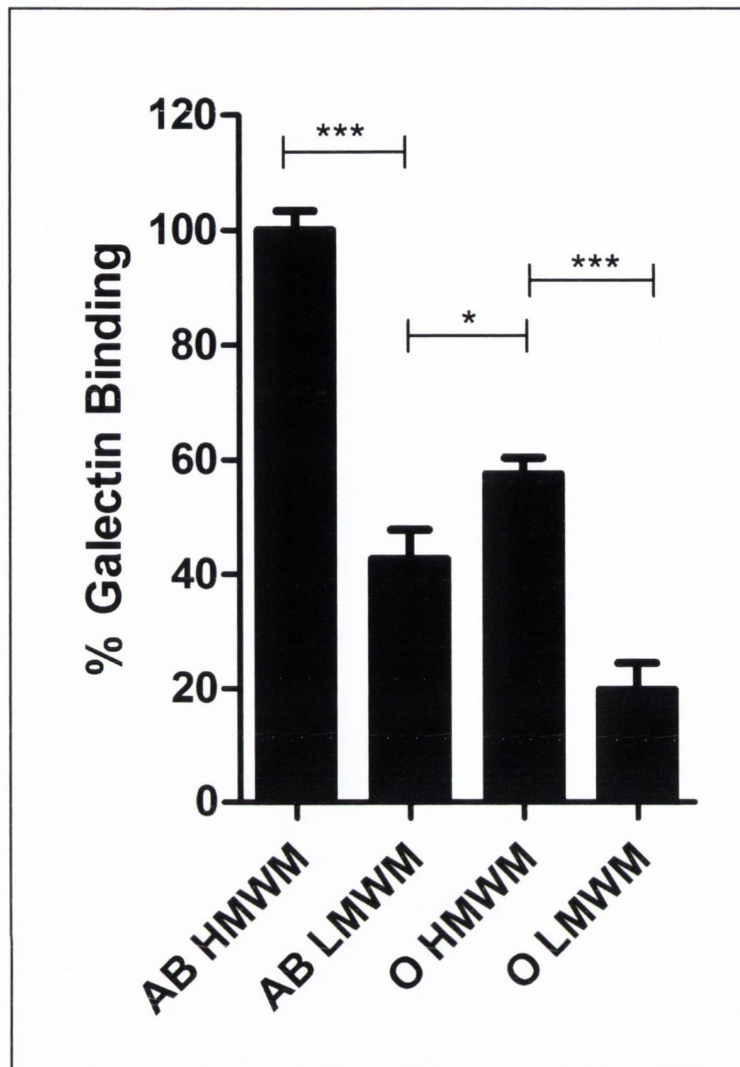


Figure 3.5 Post translational glycosylation and multimerisation of VWF are important regulators of its interaction with galectin-1
Galectin-1 binding was assessed for equi-molar concentrations of HMWM and LMWM of blood-group specific VWF and expressed as a percentage of maximal binding.

3.6 The VWF A domains play an important role in modulating the interaction with galectin-1

A series of truncated VWF domain fragments were constructed to assess the contribution of individual VWF regions to galectin binding. These VWF fragments were recombinantly expressed in HEK293T cells and included N and/or C-terminal truncations of VWF; A1-CK, D'-A3 and A1-A3 (figure 3.6). The fragments expressed included a poly-histidine tag to facilitate purification.

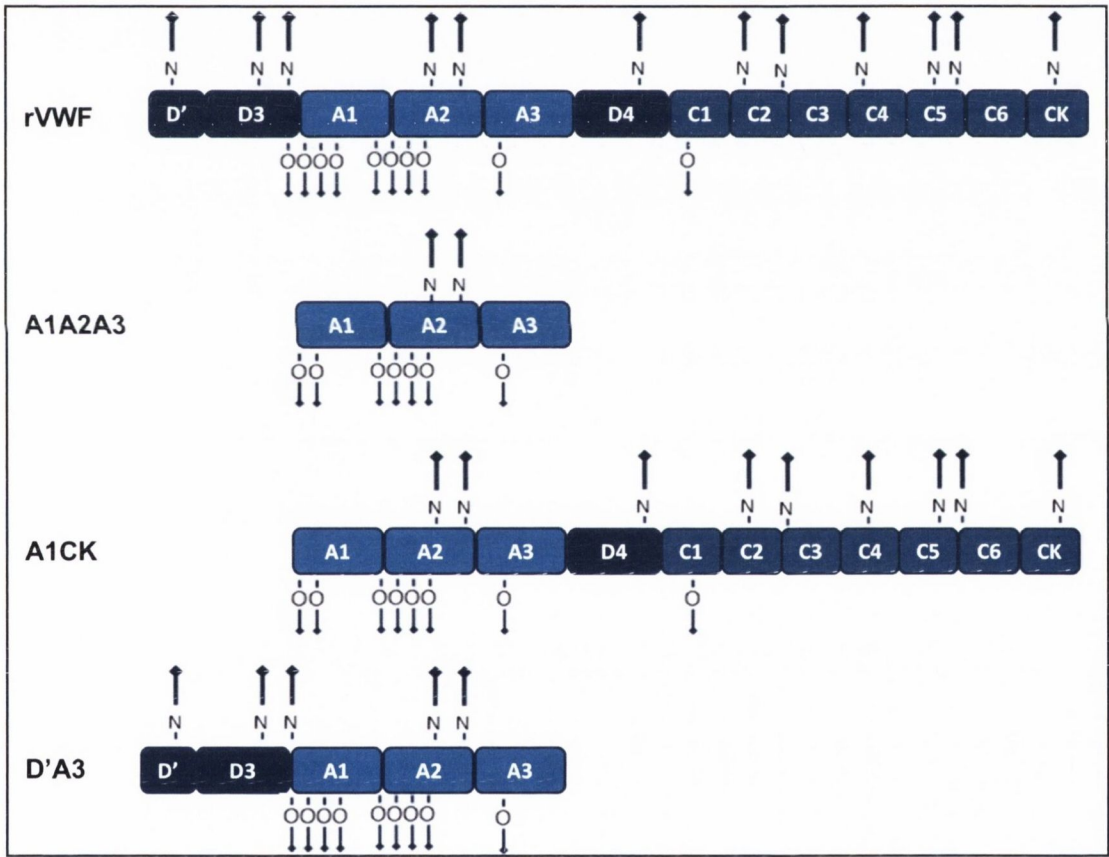


Figure 3.6 An illustration of the specific VWF domain fragments utilised and their associated glycan sites

The monomeric tri-domain A1A2A3 fragment, (which contains only two N-glycan ligands; N1515 and N1574) bound to galectin-1 ($28.1 \pm 2\%$ binding compared to full length multimer rVWF, figure 3.7). The additional of the C-terminal domains to the A domain fragment (A1-CK) served to further enhance galectin-1 binding. This is unsurprising considering that the C-terminal tail of VWF contains an addition 7 N-linked glycan sites. Paradoxically, the addition of the N-terminal D'D3 domains to the A1A2A3 fragment significantly decreased galectin-1 binding compared to A1A2A3 alone. These data suggest that the N-terminal D domains negatively regulate the interaction of VWF with galectin-1. Taken together these findings nevertheless suggest that the A domains of VWF are involved in regulating galectin-1 binding.

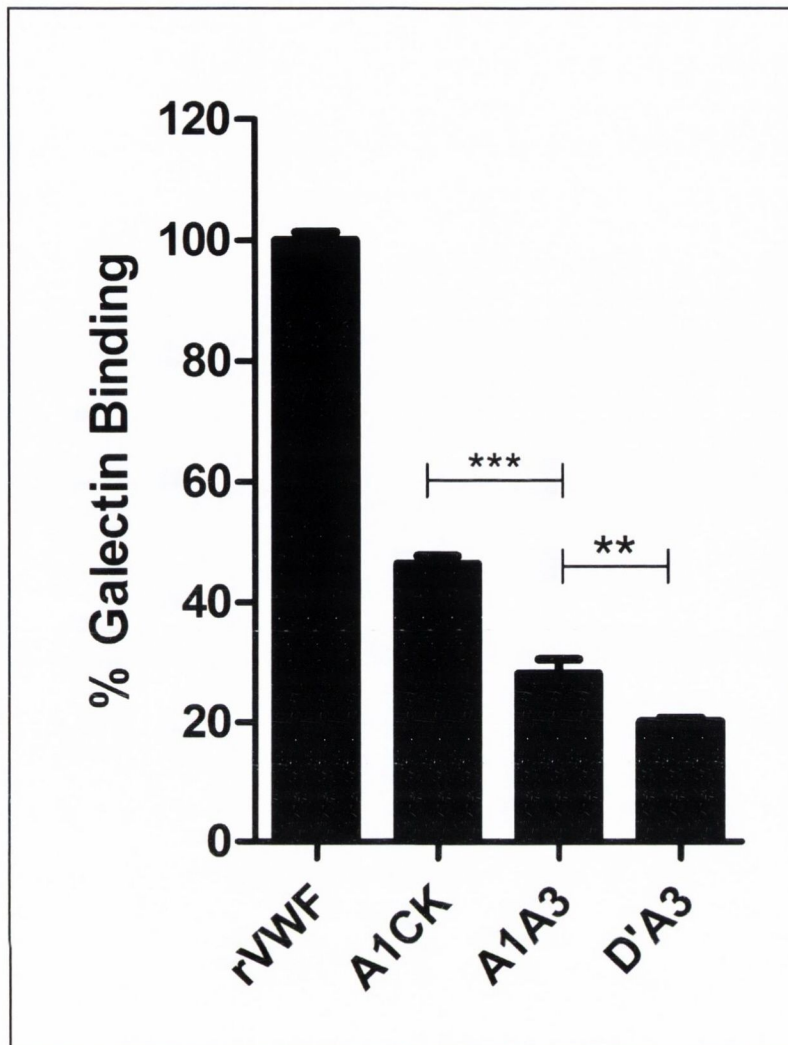


Figure 3.7 VWF domain specific binding of galectin-1
Galectin-1 binding was assessed for equi-molar concentrations various domain truncations of VWF and expressed as a percentage of full length rVWF at 5nM.

3.7 Structural conformation of VWF A domains is a key regulator of the VWF-galectin-1 interaction

Upon vessel damage, VWF within the circulation is subject to increased shear stress. Shear forces of approximately 5000s^{-1} promote a conformational transition in VWF (Schneider *et al*, 2007; Siedlecki *et al*, 1996). VWF multimers adopt an elongated structure, exposing the GPIIb α binding site within A1. This conformationally active VWF is perfectly suited to anchor flowing platelets and thereby enable primary haemostasis. Moreover, the tensile stress of attached platelets subsequently assists ADAMTS13 proteolysis of VWF multimers, likely associated with exposure of the cryptic ADAMTS13 cleavage site within A2 domain (Shim *et al*, 2008). This serves to limit the haemostatic activity of VWF and prevent excessive thrombi formation.

To further investigate the role of the VWF A domains conformation in modulating galectin-1 interaction, pd-VWF was incubated with ristocetin. Incubation with ristocetin induces allosteric changes in the VWF A domains promoting GPIIb α binding (Dong *et al*, 2001) and exposing the cryptic ADAMTS13 cleavage site mimicking the effects of shear *in vitro* (Chen *et al*, 2012). Interestingly, incubation with increasing concentrations of ristocetin dramatically enhanced binding of pd-VWF to galectin-1 ($914 \pm 50\%$, $p < 0.05$; figure 3.8A). Further evidence for the critical contribution of the VWF A domains conformation was observed in binding studies utilizing VWD type 2B mimic mutation, R1450E. This substitution has previously been shown to disrupt electrostatic interactions with the residue D1269 in the A1 domain reorienting this domain into a permissive alignment for GPIIb α binding (Yago *et al*, 2008). In keeping with the increased galectin-1 binding observed following VWF treatment with ristocetin, galectins-1 also exhibited enhanced affinity for VWF R1450E ($164.6 \pm 9\%$

$p < 0.05$, figure 3.8B). Collectively these findings suggest that the structural conformation of the VWF A domains modulates the VWF/galectin-1 interaction.

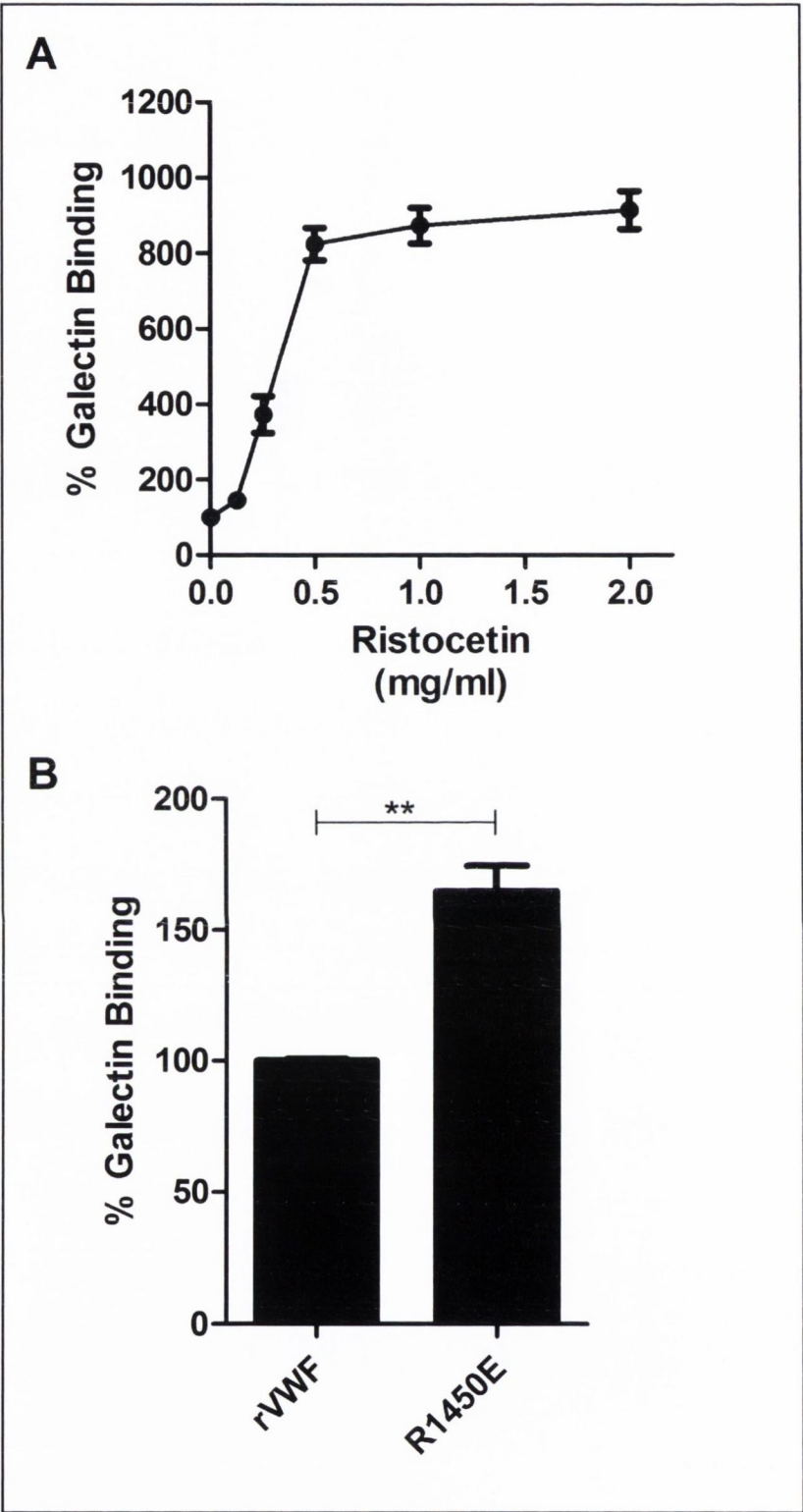


Figure 3.8 Structural conformation of VWF A domains critically influence galectin-1 binding (A) Ristocetin promotes galectin-1 binding to pd-VWF in a dose-dependent manner (B) VWF A1 mutation, R1450E, enhances galectin-1 binding. Galectin-1 binding was assessed at non-saturating 5nM rVWF.

3.8 VWF A2 domain N-linked glycans contribute to galectin-1 binding

Given that the VWF A domains and their conformation regulate galectin-1 binding, the specific contribution of glycans within the A domains was further assessed. In contrast to the multiple glycans featured in other VWF domains, there are only two N-linked glycan sites within VWF A domains (figure 3.9), residing at position N1515 and N1574 within A2 (Canis *et al*, 2012). Importantly, the glycan at position N1574 has previously been demonstrated to directly modulate VWF proteolysis. This glycan lies adjacent to the ADAMTS13 cleavage site in the VWF A2 domain and its removal, by site directed mutagenesis, results in enhanced ADAMTS13 proteolysis (McKinnon *et al*, 2008).

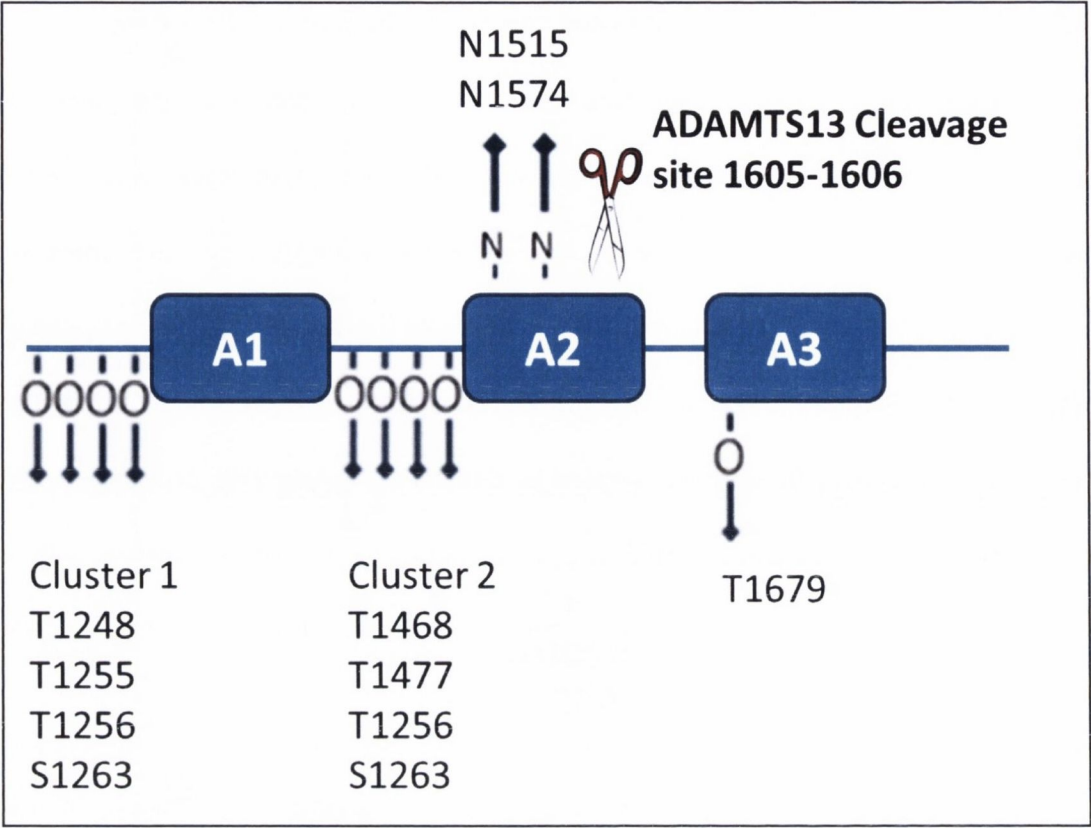


Figure 3.9 Diagrammatic representations of VWF A domain glycan structures

To investigate the contribution of A2 domain N-linked glycans in mediating galectin-1 binding, targeted removal of individual glycans located at N1515 and N1574 was carried out via site-directed mutagenesis. These two glycan variants of VWF, N1515Q and N1574Q, have been previously shown to display normal collagen binding and a full range of multimers, indicating that the overall global conformation is not significantly altered (McKinnon *et al*, 2008). Targeted removal of the glycans at position N1515 and N1574 significantly reduced binding to galectin-1, $67.6 \pm 3\%$ and $67.6 \pm 3\%$, respectively, compared to rVWF (figure 3.10; $p < 0.05$). Taken together, these data suggest that specific glycan sites within VWF A2 domain contribute significantly in mediating the VWF/galectin-1 interaction.

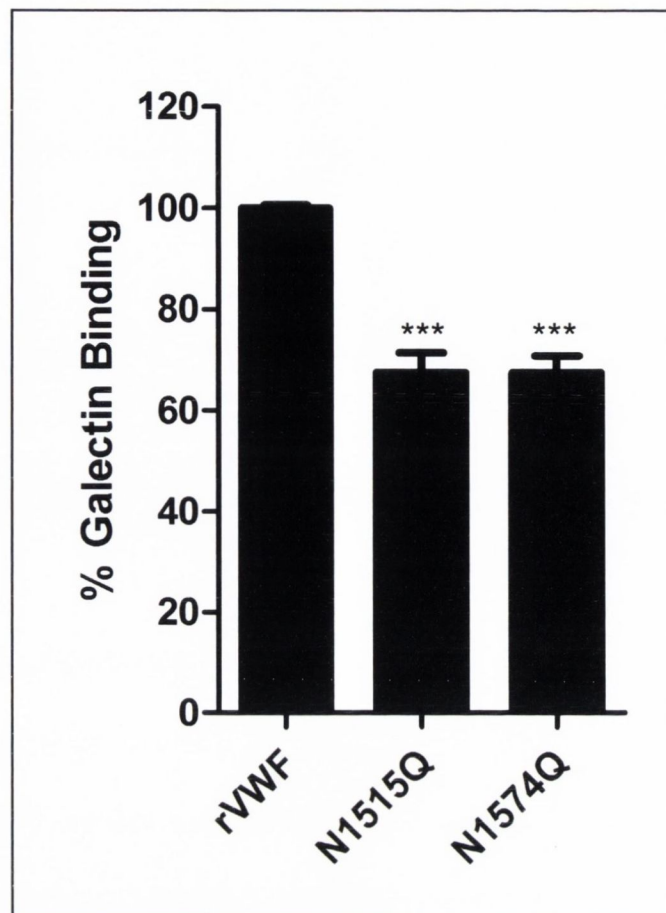


Figure 3.10 Specific N-glycan sites within VWF A2 domain mediate galectin-1 binding
Galectin-1 binding with glycan mutants, N1515Q and N1574Q, was assessed and expressed as a percentage of 5nM rVWF.

3.9 Galectin-1 constitutes a novel binding partner for ADAMTS13

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. The critical physiological role of ADAMTS13 is highlighted in patients with an inherited or acquired deficiency in ADAMTS13 which manifests as thrombocytopenia purpura (TTP). This life threatening condition is characterised by excess widespread deposition of UL-VWF and subsequent formation of platelet rich thrombi in the microvasculature (Levy *et al*, 2001).

ADAMTS13 is heavily glycosylated with 10 potential N-linked glycosylation sites identified using glycan mapping tools. These are clustered within the metalloprotease, spacer and CUB domains (Zhou & Tsai, 2009). Moreover, ADAMTS13 has been shown to express six O-linked fucosylated glycans, localised within the thrombospondin type 1 repeats (TSR) (Ricketts *et al*, 2007). Both the N-linked and O-linked glycans are of direct functional significance for ADAMTS13. The N-linked glycans have been shown to be essential for ADAMTS13 expression. In addition, specific N-linked sites are reported to enhance its ability to cleave VWF (Zhou & Tsai, 2009). Additionally, ADAMTS13 unique O-fucosylation oligosaccharides are critical for efficient secretion of the glycoprotein (Ricketts *et al*, 2007). Given the putative role for galectin-1 in modulating VWF string formation at the endothelial cell surface, we further investigated whether galectin-1 might also interact with ADAMTS13.

Firstly a modified ELISA assay was employed, coating the wells with various concentrations (0-20nM) of recombinant ADAMTS13 produced via stable transfection of HEK293T cells and purified by affinity chromatography. Interestingly, galectin-1

bound to ADAMTS13 in a dose-dependent manner and with a strong affinity, within the physiological concentration range of ADAMTS13, 3-5nM, (figure 3.11A). Moreover this novel dose-dependent interaction was confirmed using SPR analysis (figure 3.11B). ADAMTS13 requires conformational changes in VWF to permit cleavage in the A2 domain. However co-immunoprecipitation studies in normal human plasma have shown that 3% of plasma ADAMTS13 circulates in complex with VWF (Feys *et al*, 2009). Work by Zanardelli has shown that the D4 domain of VWF contains a constitutively exposed site mediating ADAMTS13 binding (Zanardelli *et al*, 2009).

To assess glycan-dependent binding of galectin-1, ADAMTS13 was subjected to PNGase F treatment to enzymatically remove the N-linked glycans. The digest was confirmed by examining the change in molecular weight as seen by the shift in migration on a SDS-PAGE gel (figure 3.12A). Surprisingly galectin-1 did not display reduced affinity for PNG-ADAMTS13 compared to unmodified ADAMTS13 (figure 3.12B), indicating that the N-linked glycans of ADAMTS13 do not mediate galectin-1 binding. Further studies will be required to define the regions of ADAMTS13 involved in regulating galectin-1 binding.

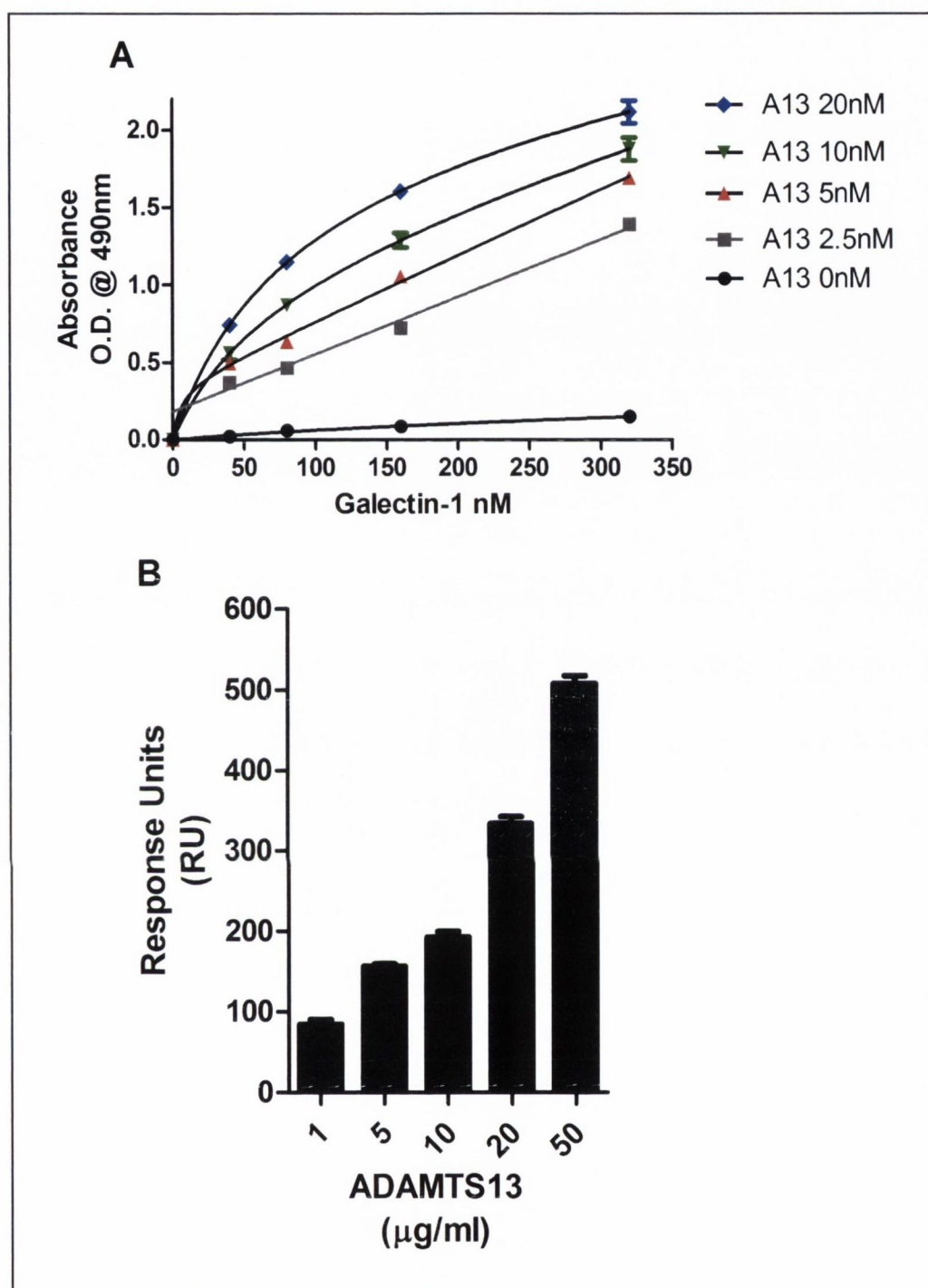


Figure 3.11 Galectin-1 represents a novel binding partner for ADAMTS13
 Galectin-1 binds to recombinant ADAMTS13 in a dose-dependent manner as assessed by immunosorbant assays (A) and SPR analysis (B).

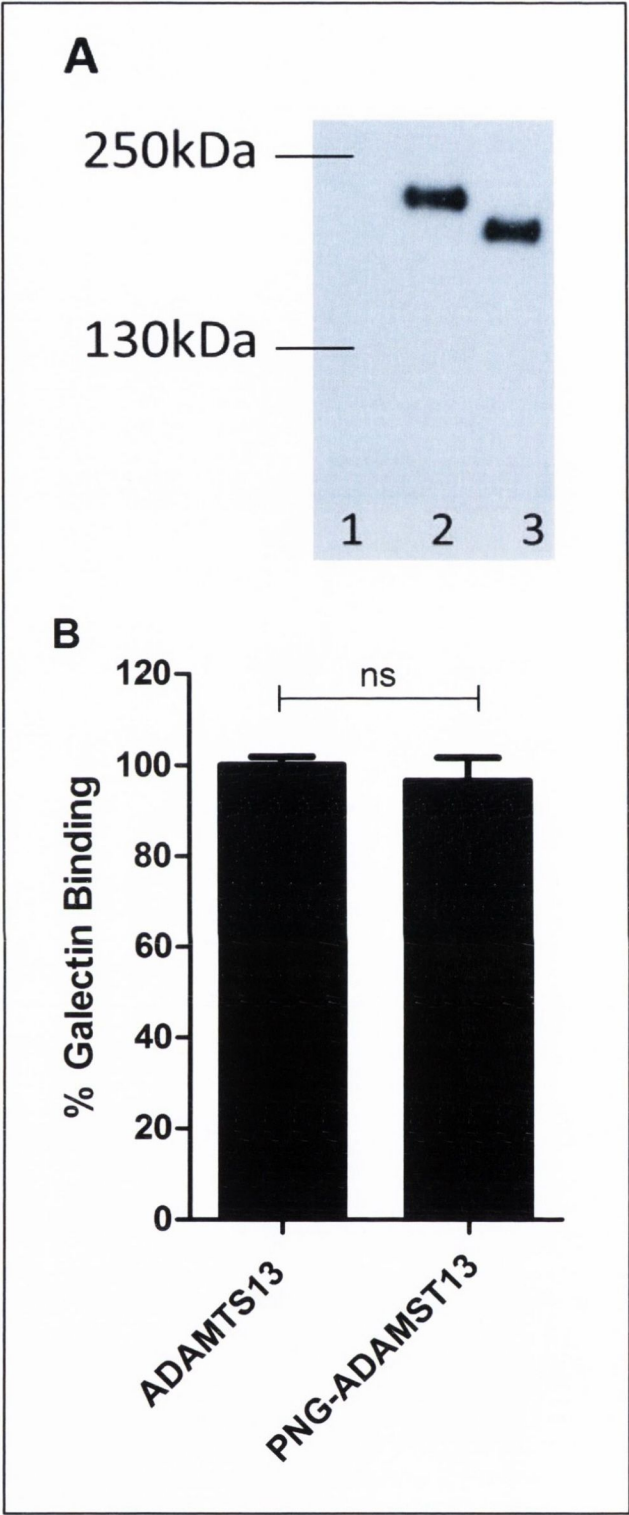


Figure 3.12 The N-linked glycans of ADAMTS13 do not mediate galectin-1 binding
(A) ADAMTS13 SDS-PAGE electrophoresis, Lane 1; Ladder, Lane 2; ADAMTS13 control undigested (~190kDa), Lane 3; PNG-ADAMTS13 (~170kDa)
(B) Galectin-1 binding to ADAMTS13 is unaffected by removal of its N-linked glycans. Galectin-1 binding was assessed at 4nM ADAMTS13.

3.10 Discussion

More than half of all known mammalian proteins express glycans structures. VWF is heavily glycosylated, with glycan structures contributing more than 20% of its total monomeric mass. Moreover, VWF carbohydrate structures critically influence a variety of its functional properties, including plasma clearance and susceptibility to ADAMTS13 proteolysis. However despite the prevalence of glycans on VWF, the molecular mechanisms underlying how the glycan structures modulate VWF biology remains elusive. Interestingly, recent evidence demonstrates that VWF circulates complexed with galectin-1 and galectin-3. Moreover, these novel binding partners also directly modulate the haemostatic activity of VWF.

Saint-Lu *et al* reported that in the absence of galectins, more VWF strings were released from stimulated HUVECs in an *in vitro* perfusion assay. This effect was verified *in vivo*, whereby enhanced VWF-platelet string formation was visible in ferric-chloride-treated mesenteric vessels in dual galectin-1 and -3 knock-out mice. Consequently these mice exhibited more rapid formation of initial thrombi at sites of ferric-chloride-induced injury. Cumulatively these studies suggest that galectin -1 and -3 negatively modulate VWF-dependant thrombus formation at the endothelial surface. Critically this represents the first report of human VWF interacting with soluble lectins in normal plasma.

Given the physiological significance of the VWF-galectin interaction, we sought to further characterise the specific determinants of VWF which mediate galectin-1 binding. Initially our results demonstrated that galectin-1 bound to pd-VWF in a dose dependant, saturable and glycan-dependant manner. The N-glycans of VWF are the principal glycan population mediating the VWF-galectin-1 interaction. Interestingly the

markedly high affinity interaction reported by Saint-Lu was confirmed in our assays. Typically, galectin-1 affinity for immobilized glycans expressing LacNAc is in the 2-4 μ M range (Leppänen *et al*, 2005). Notably however, galectin-1 displays multivalent properties. Secreted as a non-covalently linked homodimer, galectin-1 possesses two CRD allowing it to form galectin-glycan clusters or lattices promoting high affinity binding (Rabinovich *et al*, 2007). Therefore, the multimeric nature of both VWF and galectin-1 likely facilitate interactions between multiple galectin-1 CRDs and multiple VWF glycans to promote high-affinity binding.

VWF haemostatic activity is critically modulated via regulatory proteolysis by ADAMTS13. Limiting the availability of UL-VWF is of direct physiological significance, preventing the accumulation of VWF-platelet thrombi with the vasculature. Specific VWF glycan terminal determinants are known to critically influence susceptibility to proteolysis. While α 2-6 linked sialic acid protects VWF from non-specific degradation by circulating serine proteases, in contrast this terminal sialic acid specifically targets it for degradation by ADAMTS13 (McGrath *et al*, 2010). Herein we demonstrate that the galectin-1/VWF interaction is also influenced by VWF sialylation. Removal of the entire capping sialic residues, using α 2-3,6,8,9 Neuraminidase, resulted in a markedly enhanced affinity for galectin-1. This likely reflects the fact that the sub-terminal galectin ligand, LacNAc (Gal β 1-3(4)GlcNAc) on the N-linked glycans becomes accessible for binding. In keeping with the crucial role of penultimate galactose, sequential digestion with neuraminidase and β -galactosidase resulted in a significant loss in galectin-1 binding. Consequently these data highlight a critical role for terminal sialic acid in regulating galectin-1 binding to VWF. Importantly, quantitative changes in N-linked VWF sialic acid expression have been reported in some disease states (Grewal *et*

al, 2008) and in some patients with VWD (Gralnick *et al*, 1975; Ellies *et al*, 2002; van Schooten *et al*, 2007).

VWF is one of only three circulating plasma glycoproteins known to express ABO(H) antigen determinants. These terminal sugar moieties are covalently attached to 13% of its N-linked glycans and a further 1% of the O-linked glycans. Expression of ABO determinants on VWF are of direct clinical significance critically regulating VWF plasma levels, such that the circulatory half-life of VWF is significantly shorter in group O compared to non-group O individuals (Gallinaro *et al*, 2008). Additionally, ADAMTS13-mediated proteolysis of VWF has been shown to be ABO dependent with preferential cleavage in the order O>B>A>AB, with an additional susceptibility of Bombay VWF compared to group O (Bowen, 2003; O'Donnell *et al*, 2005). The significant influence of ABO(H) antigens on VWF biology is striking considering only a single sugar difference exists between group O versus group A and B. However, the molecular mechanisms through which these glycan effects are mediated remain unknown. Interestingly we have now demonstrated that ABO blood group phenotype influences the interaction of VWF with galectin-1 with enhanced affinity for group AB VWF compared to group O. These data demonstrate a role for terminal A (GalNAc) and B (Gal) sugar moieties in promoting galectin binding to VWF. In contrast, expression of α 1-2 fucose residues appears to be of lesser importance, since galectin-1 binding for Bombay VWF was not significantly different to group O VWF. These data indicate that within normal plasma galectin-1 may interact differentially with VWF from individuals of different blood group. Since ABO(H) expression levels on VWF also differ in different vascular beds, and following DDAVP administration, galectin-1 affinity will also vary accordingly (Brown *et al*, 2002).

In addition to endothelial cells, alpha granules of platelets also store VWF and can contribute to circulatory VWF levels via regulated secretion. Plt-VWF is known to differ functionally from VWF of endothelial origin. It has a reduced affinity for the platelet glycoprotein GPIb α and conversely an enhanced affinity for GPIIb-IIIa (Williams *et al*, 1994). Additionally, plt-VWF is not found complexed with procoagulant FVIII. The different functional properties of plt-VWF are thought to be in part mediated by its distinctive glycosylation profile. Indeed work by McGrath *et al*, highlighted that a reduction in sialic acid content on plt-VWF N-linked glycans contributes directly to its decreased susceptibility to ADAMTS13-mediated proteolysis (McGrath *et al*, 2013). Galectin-1 displayed significantly reduced affinity for plt-VWF compared to pd-VWF. This decreased affinity may be attributed to 50% less sub-terminal galactose expression which acts as the preferential ligand for galectin binding.

Interestingly galectin-1 also bound significantly less well to rVWF compared to pd-VWF. Recombinant proteins manufactured in heterologous cell systems have been characterised as having distinct post-translational modifications, including glycosylation. In particular, rVWF fails to express ABO(H) blood group antigen determinants on its glycan structures. Additionally, work by Turecek *et al*. report that rVWF, manufactured in CHO cell line, is characterised by a significant increase in sialic acid content (Turecek *et al*, 2009). Another distinctive characteristic of rVWF is its reduced capacity to multimerise compared to pd-VWF. By isolating specific fractions of HMWM and LMWM of pd-VWF, it was shown that galectin-1 displays a significantly enhanced affinity for haemostatically-active HMWM over LMWM of the same blood group. Local aspects of glycan presentation, such as multivalency, are known to regulate the avidity of lectin binding. Clustering of glycan chains into spatial proximity,

as would occur in highly multimerised pd-VWF, results in increased local density of ligands suited for galectin binding. This “cluster glycoside” effect is of particular significance for galectin-1, which exists as a homodimer. Given that the variation in VWF glycosylation and multimerisation profile regulates its ability to interact differentially with this lectin this may represent a mechanism by which glycan structures modulate VWF functional activity *in vivo*.

Using a series of specific truncated VWF domains, an important role for VWF A domains has been identified in regulating galectin-1 binding. In keeping with the relative abundance of glycans, A1-CK retained $46.3 \pm 1\%$ of binding for galectin-1, compared to full length VWF. Almost 30%, of this binding appears to be supplied by the A1A2A3 domains. This region of VWF possesses only two N-glycan sites, N1515 and N1574 as potential galectin ligands. The incorporation of the N-terminal region, D'-D3 significantly reduced galectin-1 binding compared to A1A2A3 alone. This finding is surprising unusual given that it contains an additional three N-glycan sites. Cumulatively these results indicate an important role for VWF glycan sites within the A domains in mediating the interaction with galectin-1. Furthermore galectin-1 affinity can be significantly influenced by the addition of the C-terminal domains and their associated glycans. Recently, accumulating evidence points to a modulatory role for D'-D3 in limiting A domain function, in particular reducing GPIb α binding (Ulrichs *et al*, 2006) and extended circulatory clearance (Lenting *et al*, 2004). Furthermore, Tischer and colleagues have recently elucidated that in fact it is the amino acid linker between D3 and A1, (Q1238-E1260) which contributes to the conformational balance that maintains quaternary A domain structure in the inactive state of VWF negatively regulating platelet GPIb α interactions (Tischer *et al*, 2013). The D'-D3 domains and

linker region may act to restrict conformational freedom of the polypeptide backbone of the tri-domain fragment and in turn negatively regulate access for galectin-1 to critical binding sites with the A domains. VWF A domains regulate a number of the multifunctional aspects of VWF including platelet GPIb α binding, ADAMTS13 cleavage and collagen binding. Given the localisation of crucial functional motifs within the A domains, further studies will be required to define the relevance of galectin-1 binding within these critical domains.

Galectin-1 appears to be particularly susceptible to ristocetin-induced conformational changes within VWF, enhancing binding nearly 10-fold, which is likely to be attributed to improved access to neighbouring N-glycans sites within A2. Similarly, a gain of function mutation of VWF, R1450E, which constitutively disrupts A domain conformation significantly enhanced galectin-1 binding. Collectively, these data indicate that galectin-1 may preferentially bind “active” or unwound VWF.

Given that the A domains of VWF can contribute to galectin-1 binding, the role of specific A domain glycans was assessed. Only two N-linked glycan sites are located within A1A2A3 at position N1515 and N1574. Site-directed mutagenesis was used to mutate the respective asparagine residues at position 1515 or 1574 to glutamine within the VWF A2 domain to create the two glycan variants N1515Q and N1574Q within full length rVWF. Mass spectrometry of pd-VWF, has revealed these two N-linked sites to have greater density of complex type bi- to tetra-antennary glycan chains with multiple fucosylated species (Canis *et al*, 2012). These features make them ideally suited as galectin ligands *in vivo*.

Specific mutation of either of these single individual N-linked glycans, within a full length recombinant VWF construct, reduced galectin-1 affinity for VWF by ~30%.

This effect is striking considering that only a single glycan site out of a possible 12 has been removed. Interestingly, these specific glycans have previously been demonstrated to influence VWF biology. Removal of the glycan structure at N1574 enhances ADAMTS13 affinity for VWF 2-fold and consequently increases VWF susceptibility to ADAMTS13 mediated proteolysis (McKinnon *et al*, 2008). Given that galectin-1 and -3 are negative modulators of VWF string formation at the endothelial cell surface, these data may suggest a putative role for these lectins in regulating ADAMTS13 proteolysis via the N-glycans adjacent to the cleavage site. However further studies will be warranted to investigate this hypothesis.

Further to this, we sought to examine if in fact galectin-1 could interact with ADAMTS13 which is also heavily glycosylated. Galectin-1 displayed high affinity for ADAMTS13, within physiological concentration ranges. However, somewhat surprisingly, this interaction occurs independent of the N-linked glycans on ADAMTS13. Thus further studies are warranted to determine the possible contribution of the O-fucosylation sites in regulating galectin-1 binding. Moreover, recently published data suggest that the TSR domains of ADAMTS13 contain 2 consensus sequences for another unique glycosylation feature, C-mannosylation (Sorvillo *et al*, 2014). This occurs whereby a mannose sugar is covalently attached to the carbon backbone of a tryptophan residue. These unique features could represent another possible determinant for galectin binding. Given that galectin-1 may bind to ADAMTS13 in normal plasma, in addition to circulating complexed with VWF, these findings have implications for fully understanding how galectins may influence VWF multimer assembly along the endothelial cell surface.

These novel data define the molecular determinants responsible for mediating the interaction between VWF and galectin-1. The findings demonstrate that the N-linked oligosaccharide structures of VWF are involved in mediating galectin-1 binding. In particular, expression of terminal AB blood group antigens, and expression of sub-terminal galactose moieties, following loss of capping sialic acid, both markedly enhance galectin-1 binding affinity. The extent of VWF multimerisation and conformational freedom of the A domains further regulate galectin-1 binding. Cumulatively the VWF/galectin interaction may provide insight into the mechanisms through which glycan expression may modulate VWF function *in vivo*.

4 Identification of galectin-1 and galectin-3 as novel binding partners for human FVIII

Plasma glycoprotein, factor VIII (FVIII) plays a crucial role in the coagulation pathway acting as a cofactor for Factor Xa (FXa) generation by Factor IXa (FIXa). The physiological importance of FVIII is illustrated in patients with quantitative or qualitative defects in FVIII which manifests as the bleeding disorder haemophilia A. Conversely, elevated plasma FVIII is an established independent risk factor for venous and arterial thrombosis, and stroke (Koster *et al*, 1995; O'Donnell & Laffan, 2001; Folsom *et al*, 1999).

Recent studies suggest that plasma FVIII is primarily derived from liver sinusoidal endothelial cells (Shahani *et al*, 2014; Fahs *et al*, 2014). FVIII forms a high affinity non-covalent complex with VWF. VWF serves as a molecular chaperone for procoagulant FVIII, binding approximately 95% of total plasma FVIII via the FVIII light chain. This interaction with VWF significantly prolongs the half-life of plasma FVIII and protects it from premature proteolytic degradation. The dissociation constant (K_d) for the VWF-FVIII interaction is approximately 0.4nM (Saenko & Scandella, 1995). However, following vascular damage and initiation of coagulation, plasma FVIII is cleaved at positions Arg 372, Arg 740 and Arg 1689 by thrombin. This cleavage significantly attenuates the affinity of FVIII for VWF. Consequently free activated FVIII (FVIIIa) is released. Activation of FVIII results in exposure of critical interactive sites for anionic phospholipids, FIXa and substrate FX thereby promoting FVIII activity as a non-enzymatic cofactor in the tenase complex.

During biosynthesis, FVIII undergoes extensive post-translational modifications including significant glycosylation (figure 4.1). The native heterodimer protein has 21 N-glycosylation sites, including 17 sites in its B domain. FVIII glycans are heterogeneous in nature including complex-type bi- to tetra-antennary core fucosylated oligosaccharides. Additionally, high mannose structures have also been reported in the A1 and C1 domains (Hironaka *et al*, 1992). Interestingly, FVIII has been reported to express ABO(H) blood group antigen structures on up to 10% of its N-linked glycans (Hironaka *et al*, 1992). Seven O-linked glycan sites have been identified, located within the B domain. These consist primarily of a sialylated T-antigen structure (Mazsaroff *et al*, 1997).

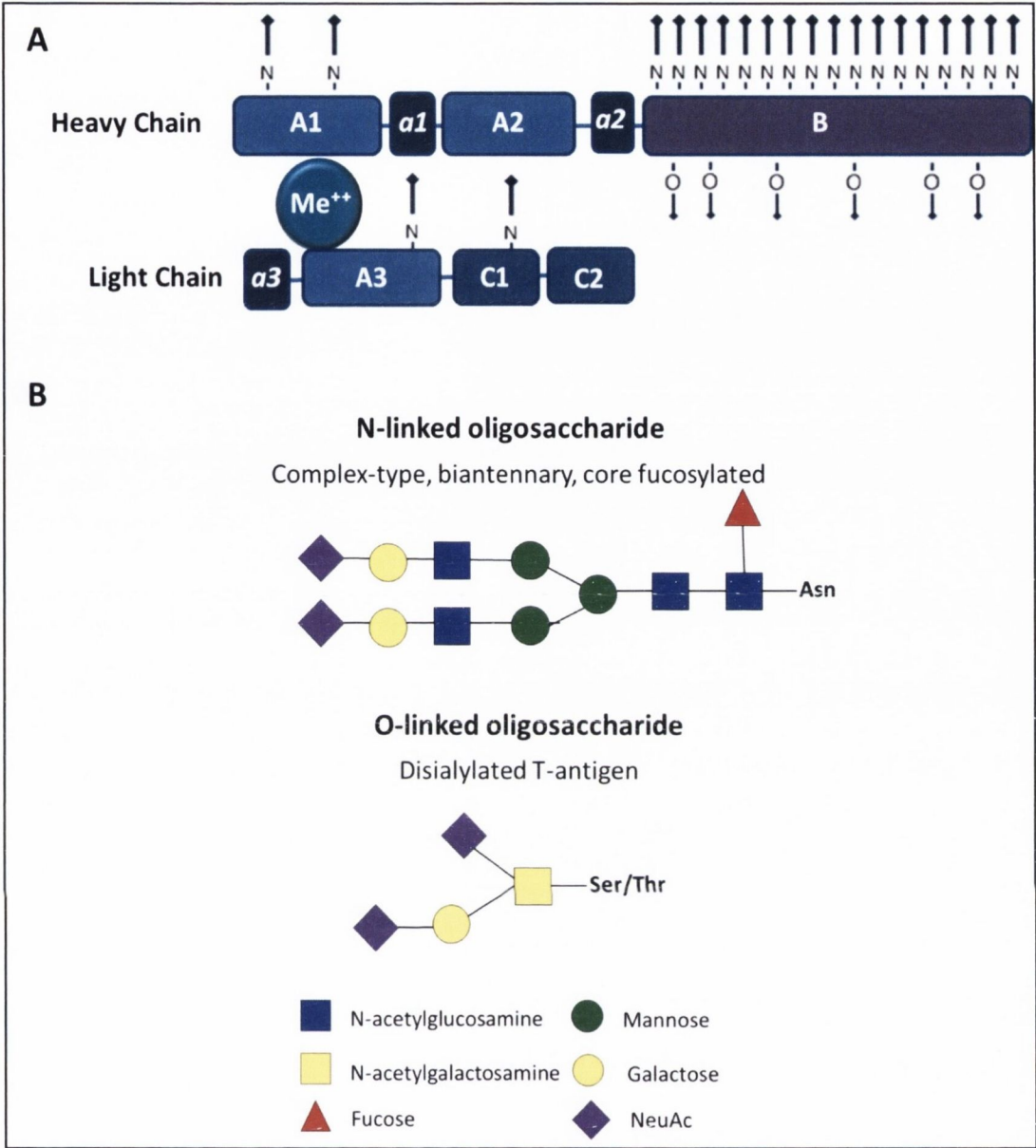


Figure 4.1 Diagrammatic FVIII structure

(A) Multi-domain structure of heterodimeric FVIII and associated glycan sites (B) Structure of the most common N- and O-linked oligosaccharides found on FVIII

FVIII glycans are of physiological significance as they modulate key aspects of its biology. For example, the resident ER chaperone lectins, calnexin and calreticulin both interact with FVIII B domain glycans to promote optimal protein folding. Consequently inhibition of N-glycosylation with tunicamycin ablates FVIII secretion. These data serve to emphasize the importance of FVIII glycan structures in regulating its intracellular trafficking (Pipe *et al*, 1998).

Within the circulation, FVIII can interact with a number of lectins including the asialoglycoprotein receptor (ASGPR) and the mannose receptor CD206. Bovenschen *et al* have demonstrated that the glycan-rich B domain of FVIII binds to ASGPR. Moreover, *in vivo* studies showed that FVIII plasma half-life was significantly prolonged in the presence of an ASGPR antagonist (Bovenschen *et al*, 2005). It is well established that commercial FVIII concentrates devoid of the heavily glycosylated B domain display similar circulatory half-lives as full length FVIII products. Consequently it has been proposed that the role of ASGPR may be primarily in regulating quality control of incompletely glycosylated FVIII.

ABO(H) blood groups sugars have been shown to directly influence the plasma levels of both VWF and FVIII *in vivo* (Orstavik *et al*, 1985). Consequently blood group O individuals have approximately 25% less circulating VWF and FVIII compared to non-O individuals. While the blood group effect on FVIII levels is mediated primarily through alterations in plasma VWF levels, a number of studies report a small but significant effect for FVIII independent of VWF (Morange *et al*, 2005; Tirado *et al*, 2005; Souto *et al*, 2000). The significant effect of blood group in regulating circulating FVIII levels is surprising, given that the ABO(H) determinants only differ with respect to a single

terminal sugar residue. However the manner in which ABO(H) sugars contribute independently in regulating FVIII plasma levels remains undefined.

Cumulatively, current data highlights the critical importance of FVIII carbohydrates in normal physiology. However the molecular mechanisms through which FVIII glycan determinants modulate their effects remain poorly understood. In Chapter 3 we outlined the molecular determinants modulating the galectin-VWF interaction. On the basis of the fact that FVIII and VWF share similar glycan profiles we further sought to investigate if galectins might also constitute novel binding partners for FVIII, and moreover modulate FVIII function.

4.1 Galectin-1 and -3 bind rFVIII with high affinity in a glycan-dependant manner

Glycans constitute a significant proportion of the mass of FVIII (figure 4.1A) and influence a various aspects of its biology. Given the high affinity of galectins for VWF, we assessed their ability to interact with FVIII. Firstly immunosorbant assays were employed, coating the wells with purified rFVIII (Advate). Of importance, the major glycan structures of this rFVIII product expressed in CHO cells has previously been reported to be comparable to that of human plasma-derived FVIII (pd-FVIII) (Hironaka *et al*, 1992; Medzihradszky *et al*, 1997). In addition, this rFVIII product does not contain any VWF. Both galectin-1 and galectin-3 bound to immobilised rFVIII in a dose-dependent and specific manner (figure 4.2 A and B, respectively). In contrast, neither galectin demonstrated any binding to the control glycoprotein ovalbumin. Ovalbumin was the first protein to provide definitive evidence for the existence of carbohydrates structures covalently coupled to a protein. It is now one of the best characterised glycoproteins, expressing sialylated hybrid-type glycans and high mannose structures (Harvey *et al*, 2000). However as the glycans fail to express a terminal galactose required for galectin binding ovalbumin served as a negative control glycoprotein in our studies. Equilibrium binding constants ($K_{d_{app}}$) were calculated using a non-linear regression fit model (0.18 ± 0.06 nM and 0.19 ± 0.1 nM for galectin-1 and galectin-3, respectively).

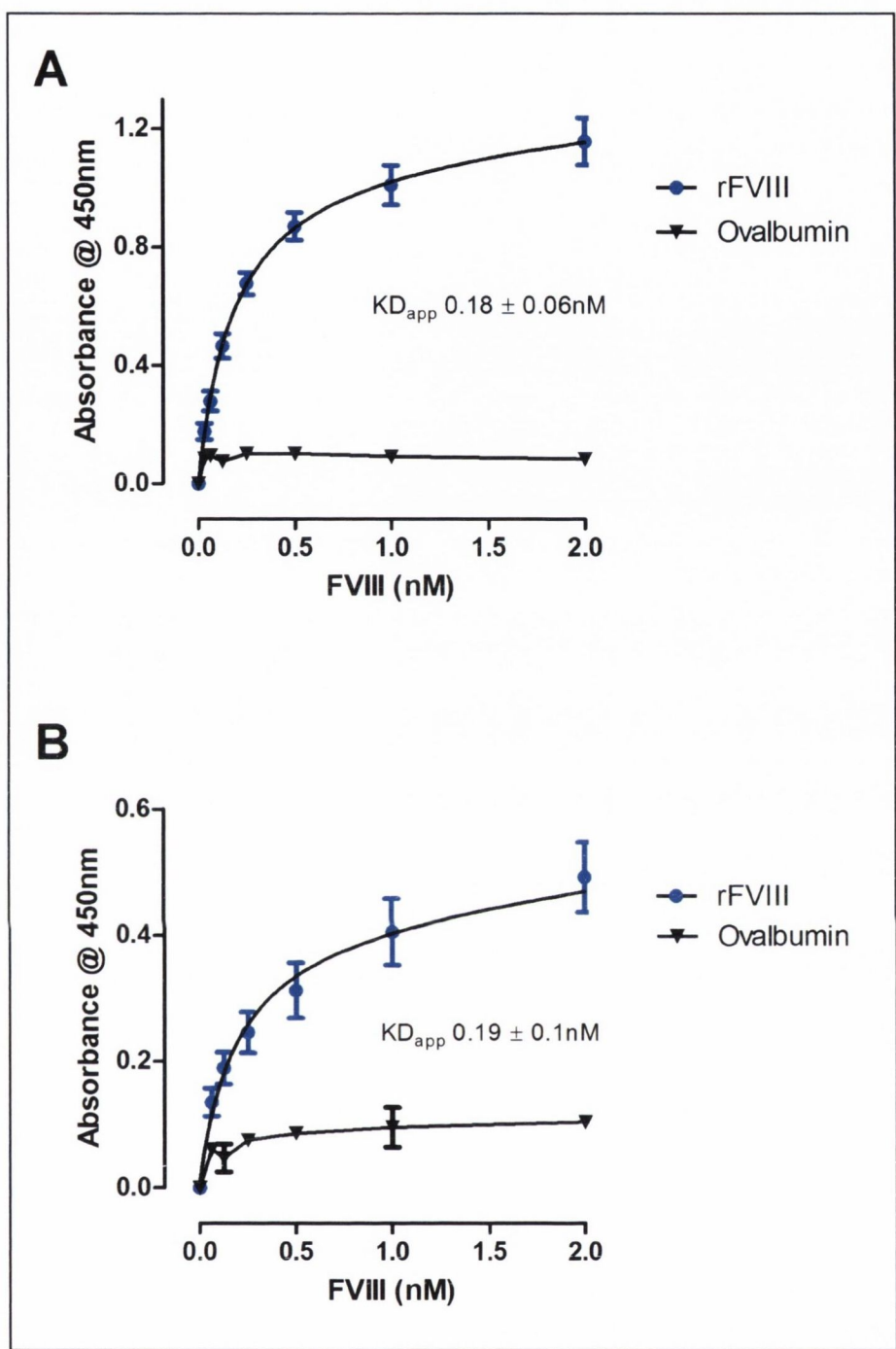


Figure 4.2 Galectins constitute novel binding partners for rFVIII
(A) Galectin-1 and (B) galectin-3 bind specifically to rFVIII in a dose-dependent and saturable manner.

To further quantify the galectin-FVIII interaction, surface plasmon resonance (SPR) studies were performed. rFVIII was coupled to a CM5 sensor chip using amine coupling chemistry as defined by manufacturer's instructions. rFVIII was surface attached at a limiting density of 1000RU, optimal for kinetic analysis avoiding mass transfer and steric hindrance effects. Regeneration optimisation revealed 10mM NaOH with a 30 second contact time to be suitable, maintaining a constant baseline value and reproducibility upon analyte injection. SPR studies confirmed dose-dependent binding of rFVIII to both galectin-1 and galectin-3 (figure 4.3 and 4.4, respectively). The calculated K_{on} and K_{off} data obtained using a heterogeneous ligand model demonstrated half maximal binding at 0.47 ± 0.11 nM and 0.12 ± 0.10 nM for galectin-1 and -3 respectively (figure 4.3 and 4.4). These high affinities are unusual compared to the previously described lectin-glycoprotein interactions. Interestingly, the only other galectin ligand reporting such high affinity is actually VWF. These data demonstrate for the first time that galectin-1 and -3 can both bind to free human rFVIII. Moreover the high affinity values suggest that under physiological conditions free FVIII should be capable of interacting with galectin-1 and galectin-3.

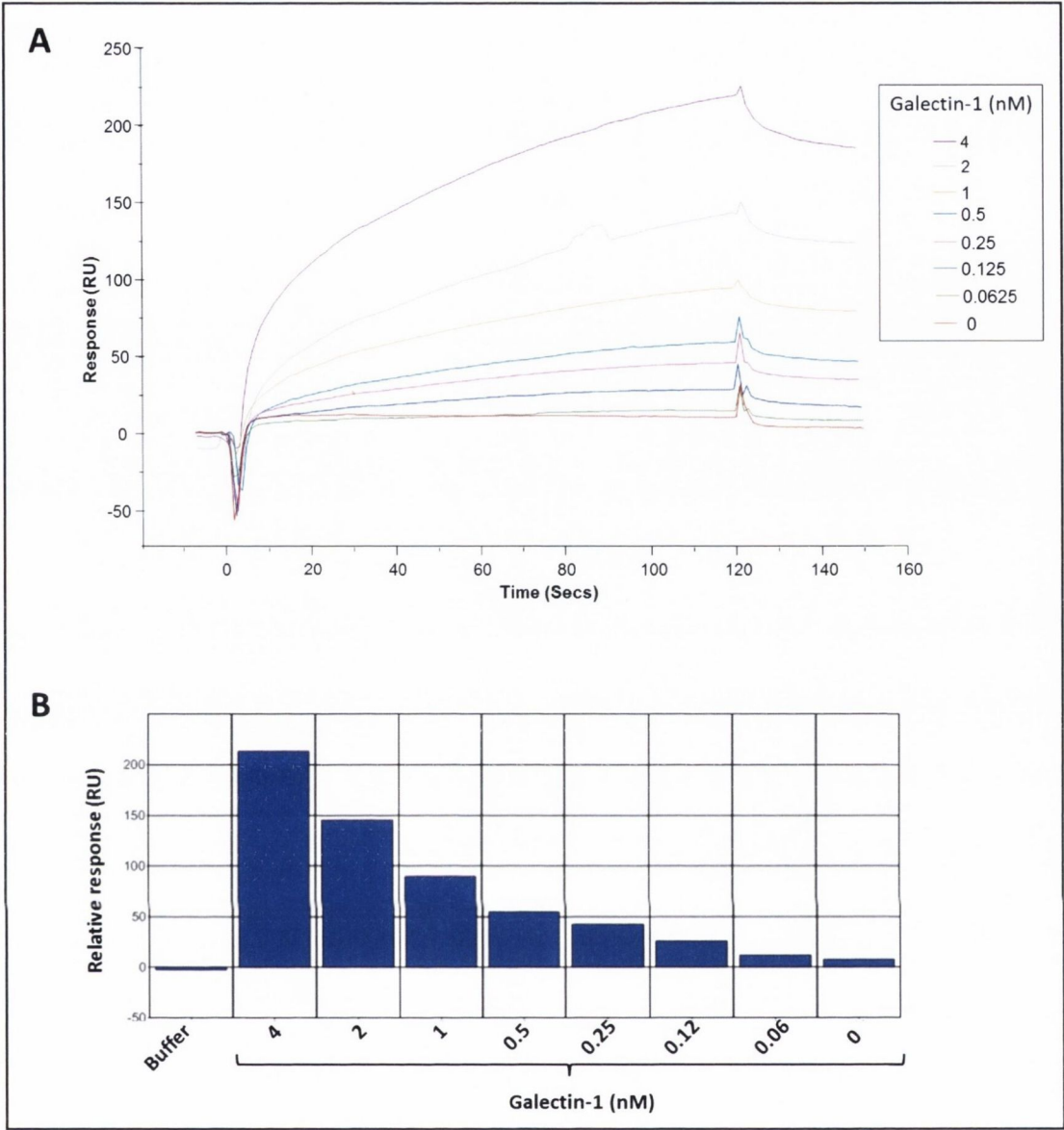


Figure 4.3 Surface plasmon resonance analysis of the galectin-1 interaction with rFVIII
(A) Galectin-1 sensorgram reveals dose-dependent and reversible binding to rFVIII by SPR analysis, (B) Galectin-1 binding analysis.

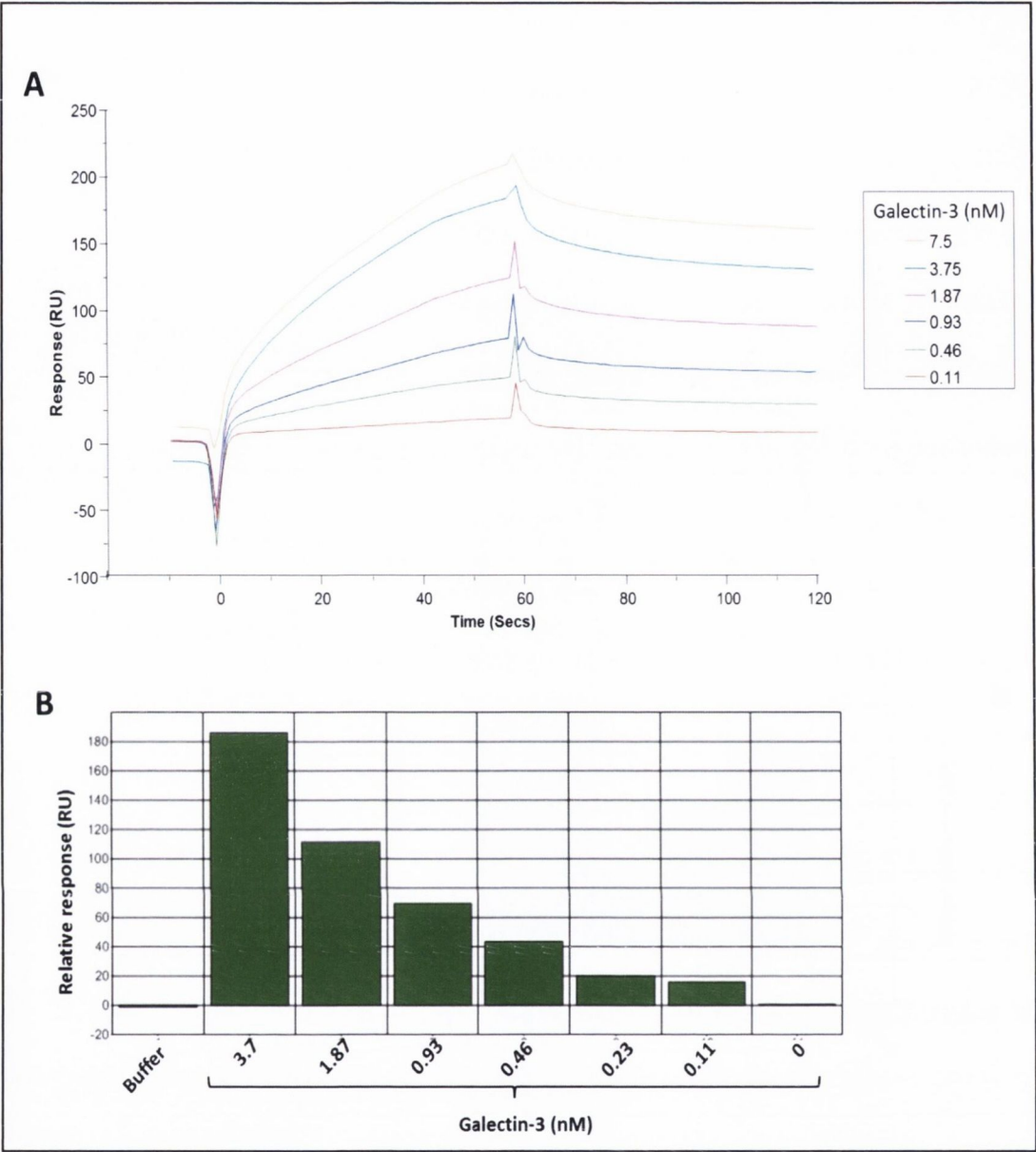


Figure 4.4 Surface plasmon resonance analysis of the galectin-3 interaction with rFVIII
(A) Galectin-3 sensorgram reveals dose-dependent and reversible binding to rFVIII by SPR analysis, (B) Galectin-3 binding analysis.

4.2 FVIII N and O-linked glycans regulate galectin binding

To determine the role of specific FVIII glycan determinants in regulating interaction with the galectins, rFVIII glycan profile was modified with a series of exoglycosidases. The efficacy of these digestions was confirmed with specific lectin ELISAs (section 2.5.1). The N-linked glycans of rFVIII were observed to be the critical mediators of the FVIII-galectin interaction since their removal using PNGase F (PNG-FVIII) served to significantly reduce galectin-1 and galectin-3 binding (to $8.6 \pm 1\%$ and $30.2 \pm 2\%$, respectively, figure 4.5A and B, respectively, $p < 0.0001$). To further assess the possible contribution of FVIII O-linked glycans in modulating galectin binding, rFVIII was sequentially digested with PNGase F and O-glycosidase (PNG-Ogly-FVIII) to remove both the N- and O-linked glycans. This digest significantly further attenuated galectin-3 binding ($16.5 \pm 2\%$; $p < 0.05$). In contrast, O-glycan removal had no significant effect on galectin-1 binding compared to PNG-FVIII ($15.0 \pm 0.9\%$ versus $8.6 \pm 1.5\%$, respectively; $p > 0.05$, figure 4.5A). Cumulatively, these data establish the N-linked glycans of rFVIII as the principal ligands involved in binding both galectin-1 and galectin-3. However, our data also suggest that FVIII O-linked glycans may also have a modest additional role in contributing to galectin-3/FVIII interaction.

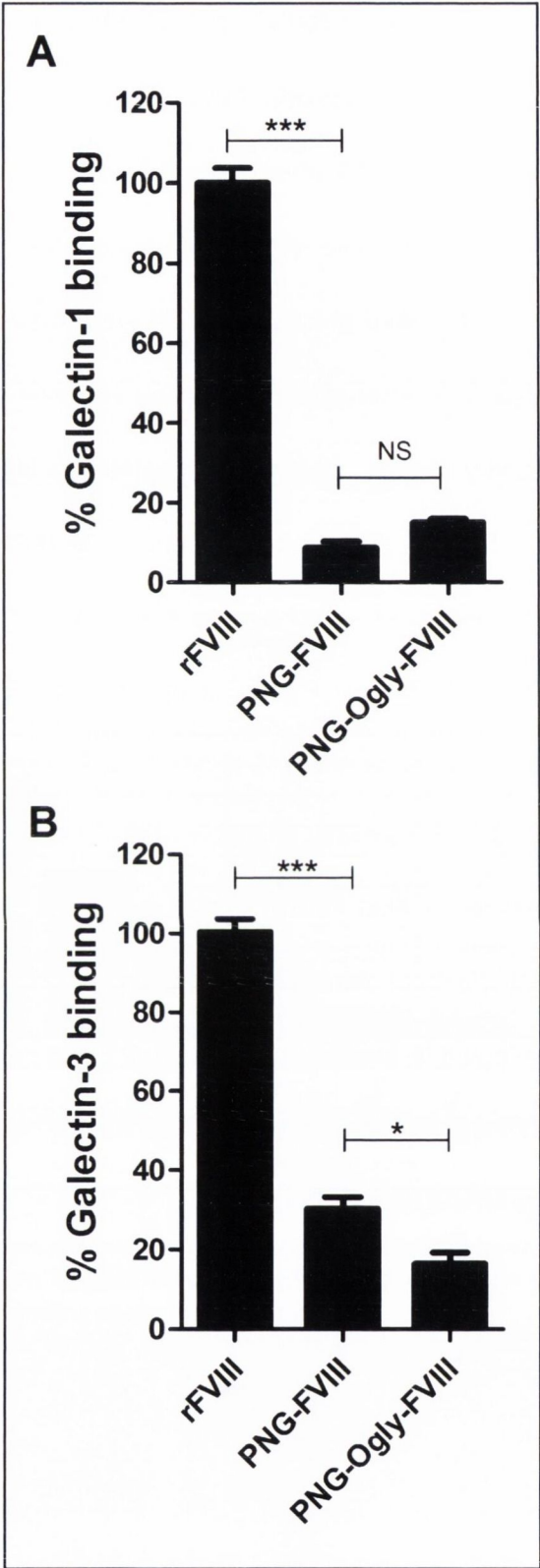


Figure 4.5 The N- and O-linked glycans of FVIII contribute to galectin binding
Modifying the glycan profile of rFVIII revealed the N-linked glycans support the majority of (A) galectin-1 and (B) galectin-3 binding. Galectin binding to glycan-modified rFVIII was expressed as a percentage of untreated rFVIII at non-saturating 0.5nM.

4.3 Differential FVIII glycosylation critically regulates its interaction with galectin-1 and -3

In light of the critical importance of FVIII glycans in regulating its interaction with galectins, galectin binding to various commercial recombinant FVIII (rFVIII) concentrates was examined. It is well known that non-human cell lines do not replicate human post-translational modifications, including glycosylation. Nevertheless heterologous cell systems are widely used for biopharmaceutical protein production. rFVIII concentrates manufactured in various cell lines are characterised by the expression of differential glycosylation profile compared to that of pd-FVIII. In particular, a number of FVIII concentrates have been characterised by the presence of non-human glyco-epitopes, including the presence of galactose α 1-3 galactose (Gal- α 1-3Gal) on the N-glycans of BHK derived FVIII (Hironaka *et al*, 1992), and the expression N-glycolylneuraminic acid (NeuGc) on the glycan termini of CHO derived FVIII (Varki, 2009). Importantly, high levels of antibodies against both NeuGc and Gal- α 1-3Gal are found in normal human plasma (Macher & Galili, 2008; Diaz *et al*, 2009). In immunosorbant assays both galectin-1 and -3 were found to display modestly enhanced binding for BHK-rFVIII, ($107.3 \pm 2\%$ and $128.5 \pm 3\%$, respectively), compared to CHO-rFVIII (figure 4.6A and B, respectively, $p < 0.05$). These data are consistent with the finding that α 1-3 galactosylation constitutes a preferential galectin-binding ligand. The addition of this extra galactose residue is only permitted onto the 3-OH position of the nonreducing terminal galactose as the 4-OH and 6-OH of the galactose moiety are essential galectin binding regions (Hirabayashi *et al*, 2002). This ligand has been described to enhance galectin-1, -2, -3 and -8 binding in frontal affinity chromatography analysis. Collectively, these findings suggest even subtle changes in

FVIII glycosylation profiles may significantly influence its ability to interact with galectins. Consequently commercial full length rFVIII therapeutics derived, from different cellular origins display differential galectin binding affinities.

Early analysis of the structure-function relationship of FVIII revealed that the B domain, comprising over 30% of the total molecular mass of FVIII, was completely dispensable for its cofactor activity (Toole *et al*, 1986). Thus Pittman *et al* constructed a BDD-variant of rFVIII, (deleting residues 760-1639), and observed the secreted protein yield improved by over 30% (Pittman *et al*, 1993).

Currently, BDD-rFVIII protein is manufactured for therapeutic use by Wyeth Pharmaceuticals in CHO cell lines (Refacto). The ability of galectin-1 and -3 to interact with this commonly used rFVIII concentrate was examined by immunosorbant assay. In keeping with the loss of the majority of its glycans, both galectin-1 and -3 binding were significantly attenuated for BDD-rFVIII compared to full length rFVIII manufactured in the same cell line ($42.2 \pm 1\%$ and $19.6 \pm 1\%$, respectively), figure 4.6A and B, $p < 0.0001$). Interestingly, galectin-1 still retained a significant proportion of binding for BDD-FVIII even with the loss of approximately 80% of its suitable ligands. Cumulatively, these data also show that the majority of galectin-1 and galectin-3 binding is supported by the glycans within the B domain of FVIII. However it appears that glycans residing outside the B domain also significantly contribute to regulate galectin-1 binding. Given that activated FVIII exists as a B-domainless heterotrimer, these novel findings suggest that in addition to binding free full length FVIII, galectin-1, in contrast to galectin-3, may be capable of interacting with FVIIIa. This suggests that galectin-1 binding may occur at or near regions important for FVIII function.

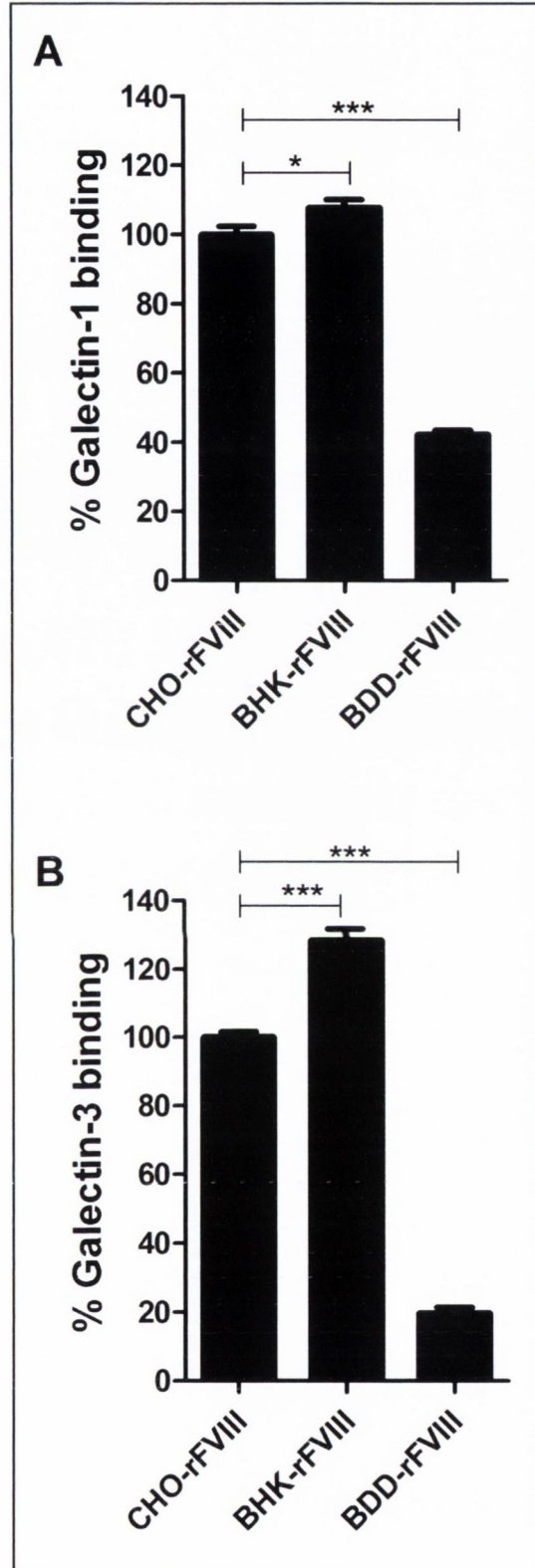


Figure 4.6 Galectin binding is distinct with various therapeutic rFVIII concentrates (A) Galectin-1 and (B) galectin-3 binding was assessed for rFVIII derived from CHO and BHK cells and a recombinant B domain deleted (BDD) variant. Galectin binding expressed as a percentage of binding to 0.5nM CHO-rFVIII.

4.4 The high-mannose oligosaccharides of rFVIII contribute significantly to galectin-1 binding

To further investigate the role of the non-B domain glycans in modulating FVIII-galectin-1 interaction additional studies were performed. FVIII has 4 non-B domain glycans; N41 and N239 in the A1 domain, N1810 in the A3 domain and N2118 in the C1 domain. Of these, N239 and N2118 have been identified as high mannose-type oligosaccharides (Medzihradzsky *et al*, 1997). To further determine the potential role for these glycan sites in influencing galectin-1/FVIII interaction, BDD-FVIII was subjected to enzymatic digestion with either endoglycosidase H (Endo H BDD-FVIII), which selectively removes FVIII high mannose structures, or with PNG F to cleave all remaining glycan structures (PNG BDD-FVIII). Importantly, galectin-1 binding was significantly reduced for Endo H BDD-FVIII compared to unmodified BDD-FVIII (figure 4.7A). Moreover there was further modest reduction in galectin-1 binding with PNG BDD-FVIII compared to Endo H BDD-FVIII ($24.1 \pm 0.6\%$ versus $35.8 \pm 3\%$, respectively, $p < 0.05$ figure 4.7B). These findings suggest that the majority of galectin-1 binding with BDD is supported by the high mannose glycans expressed on FVIII.

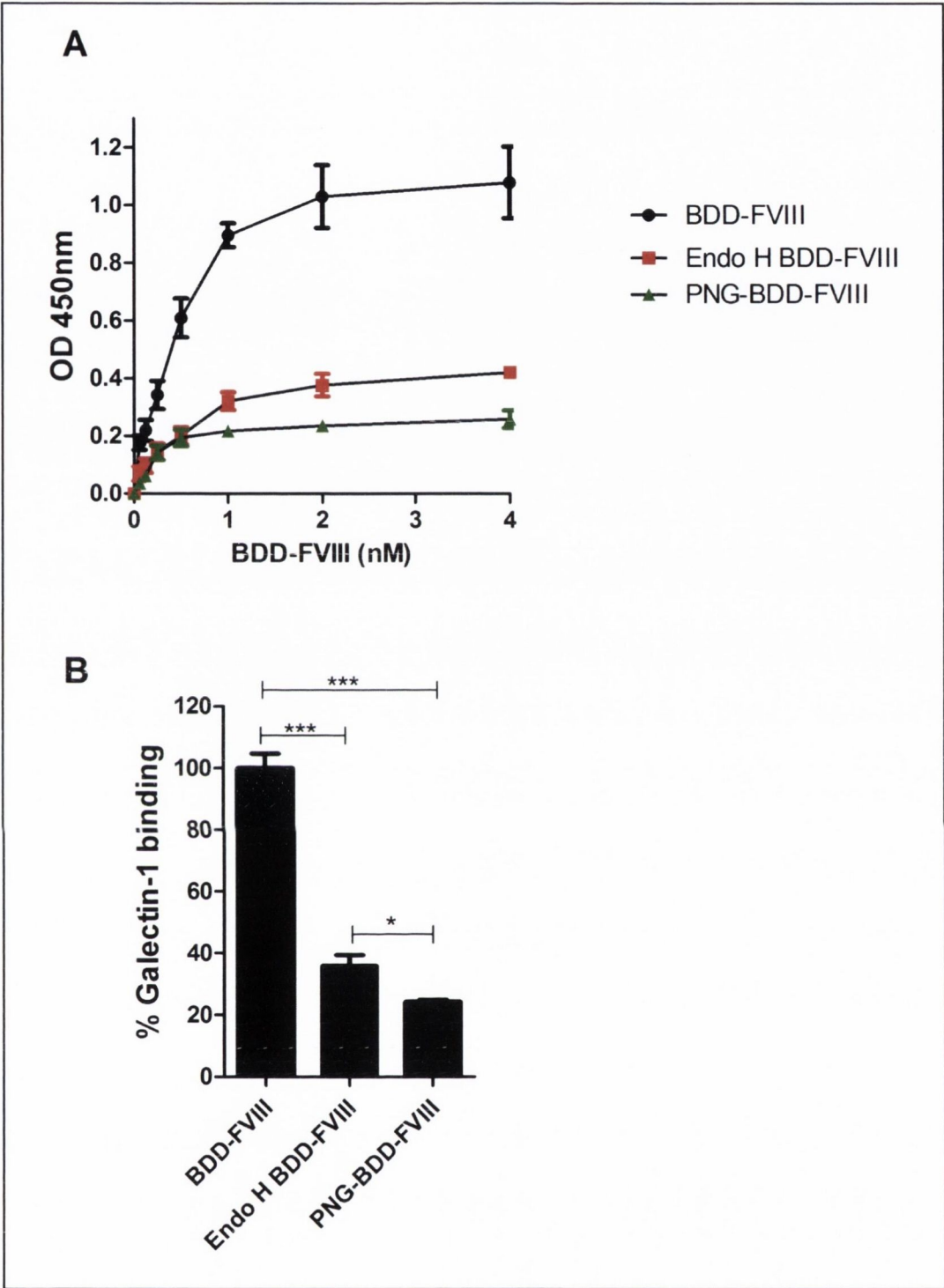


Figure 4.7 Removal of the high mannose oligosaccharides outside the B domain of FVIII reduced galectin-1 binding
(A) Galectin-1 binding with BDD-FVIII modified by exoglycosidase enzymes to remove high mannose chains (Endo-H BDD-FVIII) or all N-linked glycans (PNG-BDD-FVIII), (B) galectin-1 binding with modified BDD-FVIII at 1nM.

4.5 Galectin-1 binds to plasma-derived FVIII

On the basis of the high affinity interaction of galectin-1/-3 with rFVIII it was of interest to investigate whether these galectins also interact with plasma derived FVIII. However, given the previous studies identifying that galectins and VWF circulate as a high affinity complex it was difficult to directly study the galectin-FVIII interaction *in vivo*. Consequently, a pull-down experiment was performed utilising VWF-deficient plasma obtained from VWF knockout mice. These mice are homozygous null for the VWF gene and thus have no plasma VWF. Cobalt chelate agarose beads coated with recombinant galectin-1 or -3 or control untreated beads were incubated with VWF deficient plasma. After sufficient washing, loading dye with reducing agent was added, samples were boiled and the supernatants run on a SDS-PAGE gel. The lanes were then blotted for FVIII using a specific heavy chain antibody. For control purposes the lanes were also blotted for galectin-1 and -3 using specific antibodies. Galectin-1 proteins appears as a monomer and dimer at 15 and 30kDa, respectively (figure 4.8, lane 2). In contrast galectin-3 protein was present as monomeric and dimeric fragments at 30 and 60kDa, respectively (lane 3). Galectin-1 coated beads precipitated FVIII from VWF deficient plasma samples (figure 4.8 lane 2). Conversely, no FVIII was observed with control untreated beads or galectin-3 coated beads. Collectively, these studies confirm that galectin-1 can bind free FVIII within the plasma milieu.

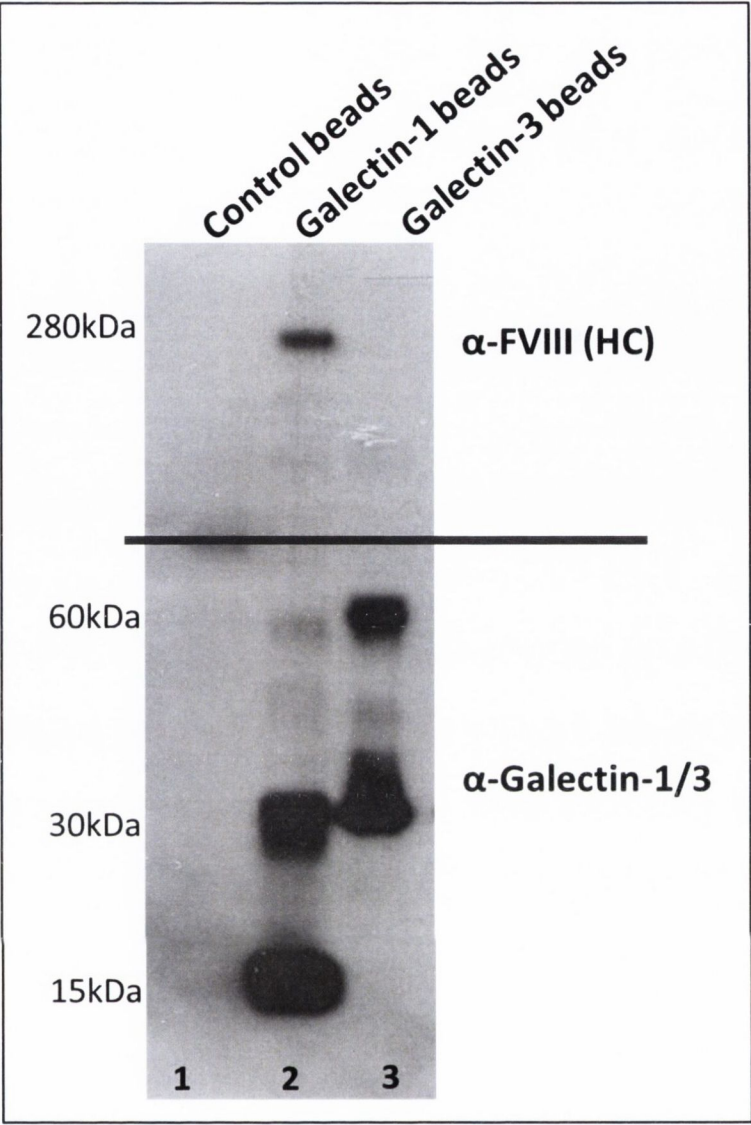


Figure 4.8 Galectin-1 binds to free FVIII in plasma
Galectin-1 coated beads precipitate FVIII, at approximately 280kDa, from plasma in the absence of VWF (lane 2). No FVIII is observed from untreated beads (lane 1) or galectin-3 coated beads (lane 3).

4.6 Galectin-1 negatively modulates FVIII cofactor activity *in vitro*

To investigate whether galectin-1 and/or galectin-3 binding may influence FVIII function, FVIII activity was assessed using a one stage FVIII clotting assay. However, on the basis that galectins may bind to numerous different plasma glycoproteins, a FVIII-galectin complex was firstly allowed to form before incubating this complex into FVIII-deficient plasma. In the presence of increasing concentrations of galectin-1 (0-17 μM) pre-complexed with full length rFVIII, a significant and dose-dependent prolongation in the clotting time was observed (58.5 ± 0.2 seconds in the presence of 17 μM of galectin-1 compared to 26.6 ± 3 seconds for control; $p < 0.001$, figure 4.9A). In contrast, no effect on FVIII clotting activity was observed following galectin-3 binding (up to 50 μM). The inhibitory effect of galectin-1 was observed as a dose-dependent reduction in FVIII cofactor activity (figure 4.9B). As the B domain constitutes the major galectin-3 binding site within FVIII it is perhaps not surprising that this galectin does not influence FVIII function. Interestingly, the increase in APTT observed with galectin-1 preincubation occurred in a FVIII dependant manner, since addition of galectin-1 to normal plasma did not influence clotting activity as measured by APTT (data not shown). Whether FVIII in complex with its carrier VWF is still a suitable galectin-1 ligand remains to be determined. Cumulatively these findings demonstrate that binding of galectin-1 can inhibit the cofactor activity of free FVIII *in vitro*. In contrast, galectin-3 fails to influence FVIIIa activity suggesting that galectin-3 binding to the B domain of FVIII does not modulate FVIII function.

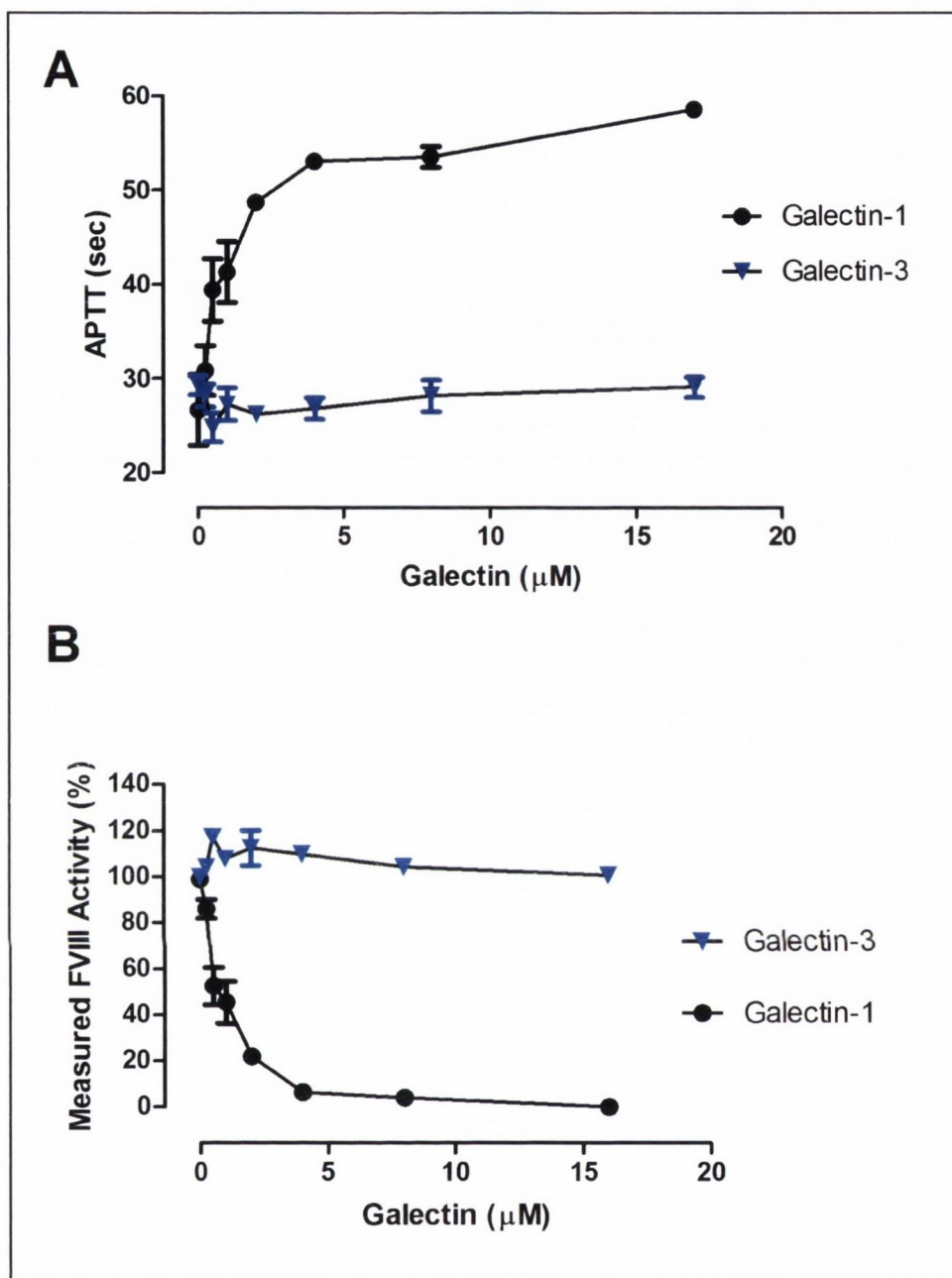


Figure 4.9 Galectin-1 attenuates FVIII activity *in vitro*

(A) Galectin-1 prolongs APTT clotting time in a FVIII-dependent manner, via inhibition of FVIII cofactor activity (B) No effect is observed for galectin-3.

To further characterise the inhibitory effect of galectin-1 on FVIII activity, additional studies were performed in a purified system to eliminate any confounding effects caused by galectin binding to other plasma glycoproteins. In this system FVIII activity was measured by the rate of conversion of FX to FXa using a commercial FXa generation assay. Increasing concentrations of galectin-1 and galectin-3 were pre-incubated with rFVIII before the addition of reagent and substrate as per kit protocol. Galectin-1 again reduced FVIII cofactor activity in a dose-dependent manner with an IC_{50} of $0.40\mu M$ (figure 4.10). In keeping with prior data, galectin-3 has no effect on FXa generation (data not shown). The IC_{50} value calculated for galectin-1 in this assay currently exceeds what has been reported for plasma galectin levels *in vivo* under physiological conditions. Importantly, galectins are known to circulate in complex with VWF and are also expressed in platelets and endothelial cells (Romaniuk *et al*, 2012; Thijssen *et al*, 2008). Thus it is possible that galectin concentrations at sites of vascular injury may be significant higher than in normal plasma. Moreover recent studies have highlighted that liver sinusoidal endothelial cells (LESECs) are the primary FVIII-producing cell type in the human liver (Shahani *et al*, 2014; Fahs *et al*, 2014). Additionally, galectin-1 is the only galectin found to be expressed in LESECs (Lotan *et al*, 1994). This raises the question of whether galectin-1, which lacks a secretory signal peptide, may be co-secreted with FVIII from these cells. Further studies will be required to fully understand the molecular mechanism by which galectin-1 inhibits FVIII activity.

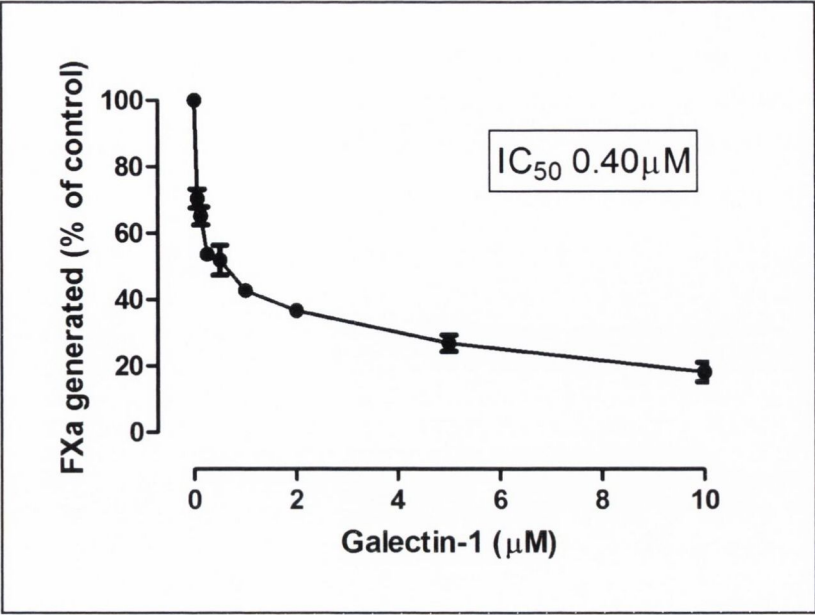


Figure 4.10 Galectin-1 reduces FVIII cofactor activity
Increasing concentrations of galectin-1 reduce the FVIII-dependent rate of conversion of FX to FXa, producing an IC_{50} value of 0.4μM.

4.7 Discussion

Since the glycosylation profiles of both VWF and FVIII share similar structures, we hypothesized that galectins might represent a novel family of lectins capable of interacting with human FVIII. This was assessed using purified proteins in immunosorbant assays and SPR analysis. Both revealed dose-dependent and high affinity binding of rFVIII with galectin-1 and -3. The high affinity was of particular interest. Since local aspects of glycan presentation are known to critically regulate the avidity of lectin binding, we speculate that this galectin-FVIII affinity is mediated primarily by the dense clustering of heavily branched N-linked glycans chains within B domain of FVIII. The increasing level of branching significantly promotes galectin-1 avidity. Moreover, the close proximity of multiple ligands is of relevance for galectin-3 interaction, which oligomerises upon ligand binding and can form pentamers.

To assess the glycan determinants mediating galectin binding, the glycosylation profile of rFVIII was modified with specific exoglycosidases. This revealed that the N-linked glycans of FVIII are critical in regulating galectin-1 and -3 binding. This is not unexpected as the principal N-linked glycans on FVIII are remarkably similar to those expressed on VWF. We also observed a role for the O-linked glycans of FVIII in mediating galectin-3 binding. However this finding is in keeping with Bian *et al* who reported that galectin-3 had a 2-fold higher affinity for the T-antigen which constitutes the primary O-linked glycan on FVIII (Bian *et al*, 2011).

A number of different recombinant FVIII concentrates are used in clinical practise. However glycosylation is species-specific and tissue-specific Hence rFVIII products manufactured in different non-human cell lines display distinct differences in glycosylation. These glycosylation differences result in altered galectin affinities.

Interestingly both galectin-1 and -3 displayed binding preferences for BHK-derived rFVIII compared to CHO-derived rFVIII. We speculate that the documented expression of α 1-3 galactose on the sub-terminal galactose of the N-linked glycan of BHK-rFVIII is important in contributing to this enhanced galectin binding.

The development of neutralising antibodies against rFVIII concentrates represents a significant issue in the treatment of haemophilia A patients. Some studies have suggested that patients receiving recombinant concentrates may have an increased rate of inhibitor development compared to patients receiving plasma derived FVIII products (Wight & Paisley, 2003). The molecular determinants that drive these immune responses remain elusive. Currently the presence of non-human glyco-epitopes is not thought to be causative for the high rate of inhibitor development in haemophilia A patients treated with rFVIII concentrates. Nevertheless the ability of circulating galectins, to recognise and adhere to non-human glyco-epitopes may have implications for understanding how altered glycosylation profiles may contribute to FVIII immunogenicity.

BDD-FVIII is another recombinant concentrate used in clinical practise. The B domain is heavily glycosylated containing all of FVIII O-linked glycans and approximately 80% of its N-linked glycans. Consequently, we observed a significantly reduced galectin-1 and -3 binding to BDD-FVIII. Of interest, galectin-1 binding remained just less than 50% for BDD-FVIII compared to full length rFVIII, even with the loss of the majority of its binding ligands. This novel finding led us to speculate that galectin-1 may be able to bind to FVIIIa and potentially modulate FVIII function. Consequently it is probable that patients treated with BDD commercial concentrate may display significantly altered galectin binding profiles compared to patients treated

with full length rFVIII products. Whether galectin binding may have physiological implications remains to be determined.

To further examine the role of glycans residing outside the B domain in contributing to galectin-1 interaction, BDD-FVIII was subjected to specific exoglycosidase digestion. We demonstrated a novel role for FVIII high mannose oligosaccharides in mediating galectin-1 binding. This was unanticipated since there is little evidence supporting a role for high mannose structures as galectin-1 ligands. Notwithstanding this, Krishnamoorthy *et al* presented compelling data demonstrating that galectin-1 specifically binds to high mannose carbohydrates expressed on the glycoprotein surface of HIV-1 virus to aid its adsorption to CD4⁺ T cells promoting virus infectivity (Krishnamoorthy *et al*, 2009). Interestingly the mannosylation of FVIII has previously been demonstrated to be responsible for its uptake into human dendritic cells (DC) via interaction with the mannose receptor, CD206 (Repešé *et al*, 2012). Moreover, Dasgupta *et al*. reported that site-directed mutagenesis of the mannose oligosaccharide in the C1 domain of FVIII completely abrogated the uptake of FVIII by DC and subsequent activation of human T cells *in vitro* (Dasgupta *et al*, 2007). Based upon these findings the authors hypothesised that novel FVIII products with reduced ability to interact with DCs could be developed by targeted removal of critical mannose sites. Further studies will be required to determine if specific affinity of galectin-1 for these key functional oligosaccharides may influence FVIII DC uptake and potential immunogenicity. Importantly galectins are found not only within circulation but also expressed on haematopoietic and immune-modulatory cells mediating endocytosis and immune surveillance.

We observed a strong interaction between rFVIII and galectin-1 and galectin-3 *in vitro* using SPR and immunosorbant assays. Thus it was of interest to examine if this interaction also could occur *in vivo*. Previous studies have demonstrated that galectin-1 and -3 circulate bound to VWF. Based on the relationship between VWF and FVIII in plasma, VWF-deficient plasma (obtained from VWF^{-/-} mice) was used in our assay. Using a pull-down experiment, we observed that only galectin-1 was capable of precipitating free plasma FVIII. Galectin-3 coated beads were unable to bind FVIII in plasma. Work by Cederfur *et al* has demonstrated that galectin-3 binds a significantly broader range of glycoproteins in serum than galectin-1, including $\alpha 2$ macroglobulin and haptoglobin. This suggests that galectin-3 may have a larger number of competing glycoproteins for binding in our VWF-deficient plasma (Cederfur *et al*, 2008).

Finally the question of whether the galectin/FVIII interaction could modulate FVIII function was assessed. Importantly galectin-1 and -3, when simply added to the normal plasma, failed to effect clotting time. However unexpectedly, galectin-1 pre-complexed with rFVIII resulted in a significant reduction in FVIII procoagulant activity. Conversely, galectin-3 failed to influence FVIII activity in these assays. This is perhaps unsurprising given that the B domain, which represents the major galectin-3 binding, is dispensable for FVIII activity. The ability of galectin-1 to attenuate FVIII function was confirmed in a purified FXa generation assay. Once again, galectin-1 reduced FVIII cofactor function dose-dependently. Although galectin-1 displayed an affinity for rFVIII in a nano-molar range, significantly higher concentrations of galectin were required to inhibit FVIII function *in vitro*. We speculate that these high concentrations are likely to be in part mediated by the loss of the heavily glycosylated B domain upon FVIII activation. Consequently the affinity of galectin-1 for FVIIIa, in these functional assays,

is expected to be significantly less than full length FVIII. Cumulatively, these findings point to a possible novel role for FVIII glycans, complexed with galectin-1, in modulating its procoagulant activity. The concentration of galectin-1 required to inhibit FVIII activity is in excess of that present in normal plasma. However several papers have demonstrated that galectin-1 and galectin-8 are expressed at significant levels in platelets (Romaniuk *et al*, 2010; Pacienza *et al*, 2008; Romaniuk *et al*, 2012). At sites of vascular injury with platelet activation it is therefore possible that local galectin concentration may be significantly increased. Importantly the effects of galectin-1 on platelet aggregation, granule release and adherence to fibrinogen also require high local concentrations of galectin-1 (ranging 0.1-1 μ M) (Romaniuk *et al*, 2012).

In conclusion, these data describe a novel interaction between FVIII and the galectin family. In light of the major role played by glycan determinants in modulating aspects of FVIII biology, understanding the biochemical basis underlying FVIII-lectin interaction *in vivo* may have significant implications for understanding how FVIII glycans mediate these effects.

5 Elucidating the molecular mechanisms responsible for VWF

circulatory clearance *in vivo*

The biosynthesis and structure-functional properties of VWF have been widely studied. However, the molecular mechanisms defining VWF circulatory clearance are poorly understood. Moreover, emerging evidence indicates that accelerated plasma clearance represents a pathophysiological mechanism in both VWD type 1 and type 2. Cohorts of patients with specific mutations resulting in reduced survival of VWF *in vivo* have been identified. In light of this, understanding VWF clearance is not only of scientific interest but also of direct clinical relevance. Consequently, we have undertaken a series of studies to elucidate the mechanisms regulating VWF clearance.

Haberichter *et al* have elegantly demonstrated that patients with accelerated VWF clearance (whom they termed VWD type 1C) can be identified by measuring steady-state plasma VWF propeptide to VWF antigen ratio (VWFpp/VWF:Ag) (Haberichter *et al*, 2006). Individuals with increased VWF clearance have reduced plasma levels of VWF, but normal plasma levels of VWFpp. Consequently, these patients have an increased ratio of VWFpp/VWF:Ag. A ratio greater than 2.3 is considered indicative of enhanced VWF clearance (Eikenboom *et al*, 2013). Determining plasma VWFpp/VWF:Ag ratio as a surrogate marker for VWF clearance is important since DDAVP treatment in these patients may be less clinically useful due to the shortened half-life of endogenous VWF. These patients with rapid clearance have enabled identification a number of key regions within the VWF glycoprotein where these mutations appear to cluster. Importantly, a significant inverse correlation exists

between the increase in VWFpp/VWF:Ag ratio and the reduction in VWF half-life. For example, patients with the R1205H (Vicenza) mutation, the archetypal fast clearance mutation, demonstrate the most elevated VWFpp/VWF:Ag ratios (15.8 ± 11.2) and the shortest VWF half-life (Casari *et al*, 2013b). This correlation is also evident in healthy subjects. Individuals with blood group O have lower plasma VWF:Ag levels, and also have elevated VWFpp/VWF:Ag ratios compared to non-group O (1.6 ± 0.1 versus 1.2 ± 0.5). These data suggest a reduction in group O VWF circulatory half-life (Gallinaro *et al*, 2008).

As part of the European Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand Disease (MCMDM-1VWD) study, VWFpp/VWF:Ag ratios were measured. The most markedly elevated VWFpp/VWF:Ag ratios were associated with mutations in the D3-A1 domains (Goodeve *et al*, 2007; Eikenboom *et al*, 2013). Collectively, these data suggest an important role for these VWF domains in regulating VWF clearance. Further evidence for an important role of A domains in regulating VWF survival *in vivo* has also been reported by Lenting *et al*. They observed that a truncated recombinant fragment of VWF comprising of A1A2A3 was cleared at a similar rate to that of full-length recombinant VWF (rVWF) (figure 5.1). Interestingly, addition of D'D3 to the N-terminal end of the A domain fragment served to significantly delay its clearance. In contrast, addition of D4-CK to the C-terminal end of the A domains resulted in significantly enhanced clearance. In light of the similar clearance rates for full-length VWF and the truncated A domain fragment, it seems likely that a receptor-recognition sites for clearance exists within the A1A2A3 domains of VWF. Moreover, the C-terminal end of VWF (D4-CK) may contain additional receptor-interactive sites for regulating A domain mediated clearance (Lenting *et al*,

2004). To date this work remains the only domain-specific investigation of VWF clearance. However the molecular mechanism and the cell type and receptors involved in the clearance of domain-specific regions of VWF remains undetermined.

In terms of the cellular basis underlying VWF circulatory clearance, hepatic and splenic macrophages have been documented to support binding and uptake. Firstly, Van Schooten *et al* demonstrated that VWF bound to both THP-1 macrophages and primary monocyte-derived macrophages *in vitro* (van Schooten *et al*, 2008). This binding was shown to result in rapid uptake, internalization and degradation of GFP-tagged VWF. A role for macrophages in regulating VWF clearance was further investigated using *in vivo* studies. These demonstrated that infused radiolabelled VWF was targeted to macrophage-rich regions of both the liver and spleen in VWF^{-/-} mice. Immunohistochemical analysis revealed VWF colocalized with CD68+ Kupffer cells in the liver, and with F4/80 macrophages in the red pulp of the spleen. Furthermore, chemical depletion of macrophages by gadolinium chloride resulted in a 2-fold prolongation in survival of infused VWF, in VWF^{-/-} mice. Despite this apparent role for macrophages in regulating VWF survival *in vivo*, specific macrophage receptors and VWF binding domains have not been fully defined. However, more recent data demonstrate that macrophage LRP1 can modulate VWF plasma levels (Rastegarlarlari *et al*, 2012).

The endocytic uptake of VWF to primary monocytes *in vitro* requires the presence of ristocetin, suggesting that the VWF-macrophage interaction may occur in a conformation-dependent manner requiring unfolding of VWF A domains. In addition, VWF binding to macrophages *in vitro* is enhanced if VWF is subject to shear stress (Castro-Núñez *et al*, 2012). Accelerated removal of VWF in its active conformation,

may be of physiological relevance, in order to prevent unnecessary formation of platelet rich thrombi within the circulation.

Shear-dependent activation is known to critically regulate a number of VWF interactions. Platelet tethering at sites of vascular injury requires shear-dependent exposure of the GPIIb α binding site within VWF A1 domain (Li *et al*, 2004). 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). Previous studies by Auton *et al* reported that the A2 domain of VWF is the least stable domain, and thus the most susceptible to unfolding. They concluded that the A2 domain is likely to be an important regulator of shear-dependent interactions (Auton *et al*, 2010). Given that VWF interacts with macrophage LRP1 clearance receptor in a conformation-dependent manner, this interaction may be mediated via cryptic sites located within VWF A domains that require shear-induced exposure *in vivo*, or ristocetin *in vitro* to promote binding.

These findings collectively support the hypothesis that the A domains of VWF may be important in regulating macrophage clearance. In this context, we sought to investigate the role of VWF A domains in regulating survival *in vivo*. To investigate the clearance of VWF *in vivo*, full-length VWF and truncated VWF fragments were recombinantly expressed in HEK293T cells and clearance was studied in VWF^{-/-} mice.

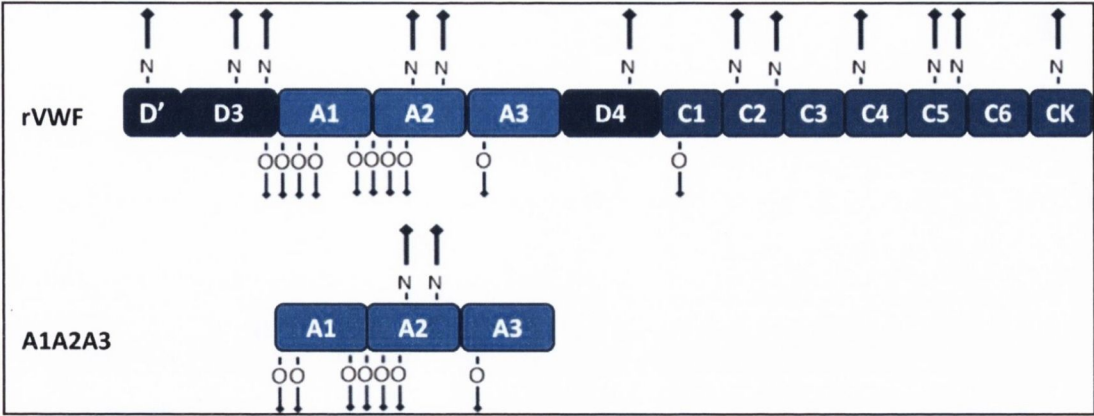


Figure 5.1 A schematic representation of a monomer of rVWF alongside A1A2A3 VWF fragment

5.1 VWF A domains regulate its clearance *in vivo*

For *in vivo* VWF clearance studies, VWF^{-/-} mice were intravenously injected via lateral tail vein with 30nM/100μL of rVWF or A1A2A3 VWF in sterile PBS. At appropriate time points, mice were anaesthetised with an intraperitoneal injection of 2.5% tribromoethanol and exsanguinated by subclavicle incision. Blood samples were collected into lithium heparin, centrifuged and plasma was removed and VWF:Ag level determination by ELISA. Clearance values were expressed as percentage residual VWF relative to the amount injected. Three to eight mice per time point were used.

Both rVWF and A1A2A3 VWF were cleared in a monophasic manner. The calculated clearance parameters, including percentage recovery at the earliest time point of 3 minutes, plasma half-life ($t_{1/2}$) and mean residence time (MRT) for rVWF and A1A2A3 VWF are shown below (table 5.1) and are illustrated in figure 5.3.

	% Recovery (mins)	$T^1/2$ (mins)	MRT (mins)
rVWF	72.0 ± 5.9	7.8 ± 0.4	11.2 ± 0.6
A1A2A3 VWF	82.7 ± 7.0	6.4 ± 0.3	9.2 ± 0.5

Table 5.1 Clearance parameters for rVWF and A1A2A3 VWF in VWF^{-/-} mice
Values expressed as mean ± SEM.

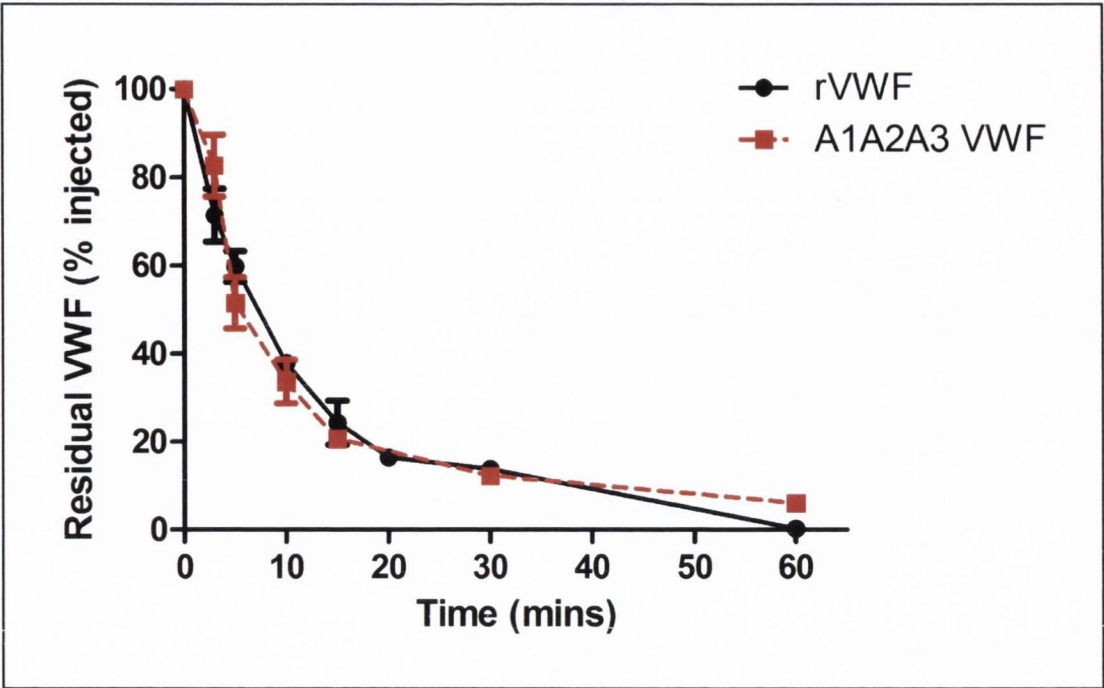


Figure 5.2 Monomeric A1A2A3 VWF fragment is cleared at a similar rate as full length rVWF in VWF^{-/-} mice
Data graphed as the mean $\pm$ SEM.

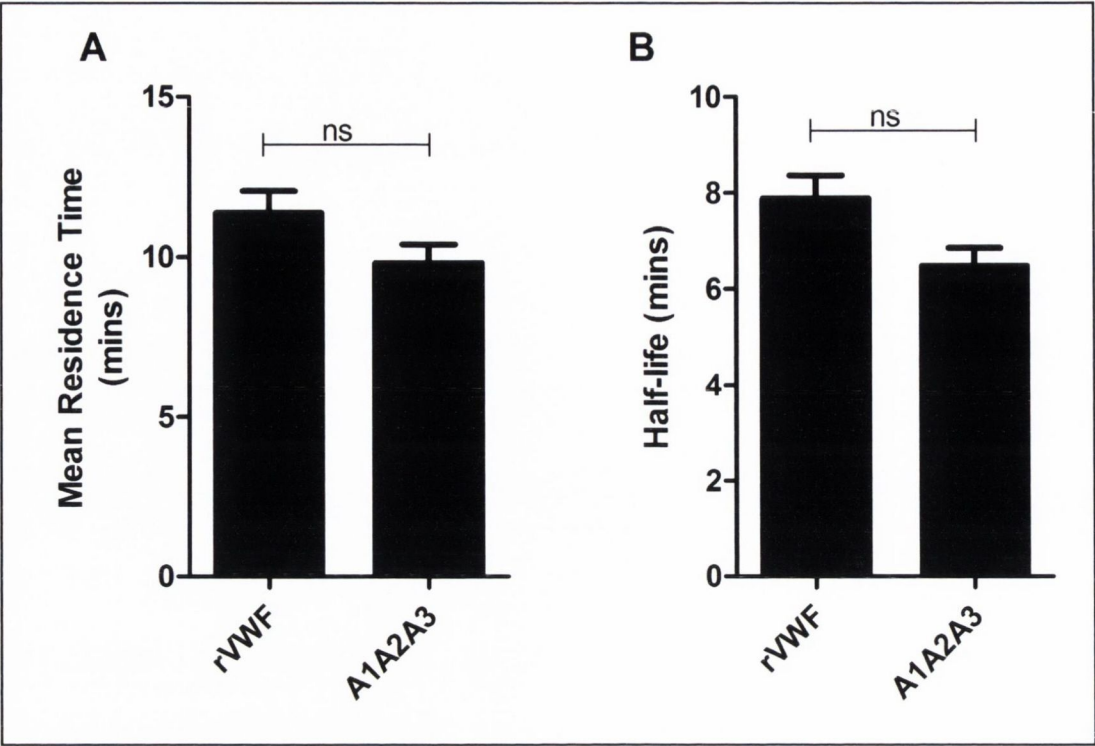


Figure 5.3 Clearance parameters for A1A2A3 VWF and rVWF
(A) Mean residence time values for rVWF vs A1A2A3 VWF
(B) Plasma half-life values for rVWF vs A1A2A3 VWF
Data are graphed as the mean $\pm$ SEM.

In keeping with a previous report, we observed that full length rVWF was cleared in a monophasic fashion (Pruss *et al*, 2011), with a MRT of 11.2 ± 0.6 minutes and a $t_{1/2}$ of 7.8 ± 0.4 minutes (table 5.1). Interestingly these values are not significantly different to those observed for the clearance of A1A2A3 VWF (MRT of 9.2 ± 0.5 minutes and $t_{1/2}$ of 6.4 ± 0.3 minutes, figures 5.2 and 5.3). Indeed, the rates of clearance for multimeric full length rVWF and monomeric A1A2A3 VWF were indistinguishable across the time points measured. These observations are in agreement with findings published by Lenting *et al*, who also described similar clearance kinetics for full length rVWF and the A1A2A3 VWF fragment (Lenting *et al*, 2004). Interestingly, significant differences have been reported for the rate of different recombinant VWF preparations in clearance studies. The differences in clearance rates likely reflect different VWF glycosylation profiles, which are dependent on the cell line used for VWF expression. All the recombinant variants described in this study, and that of Pruss *et al* (Pruss *et al*, 2011) were expressed in HEK293T cells. In contrast, Lenting *et al* employed BHK cells to express rVWF. Interestingly they reported a significantly longer $t_{1/2}$ different half-life for their recombinant variants. Nevertheless, despite the different cell line used, our data confirm the findings of Lenting *et al*. in suggesting that the A domains of VWF appear to be important in modulating VWF circulatory survival.

To investigate whether the infused full length rVWF or A1A2A3 VWF fragment may be proteolysed rather than cleared from the circulation, murine plasma samples from selected time points were analysed. As illustrated in figure 5.4A, there was no evidence of significant *in vivo* proteolysis of full length rVWF. Similarly, analysis of the murine plasma samples following A1A2A3 VWF administration again demonstrated no evidence of proteolysis (figure 5.4B).

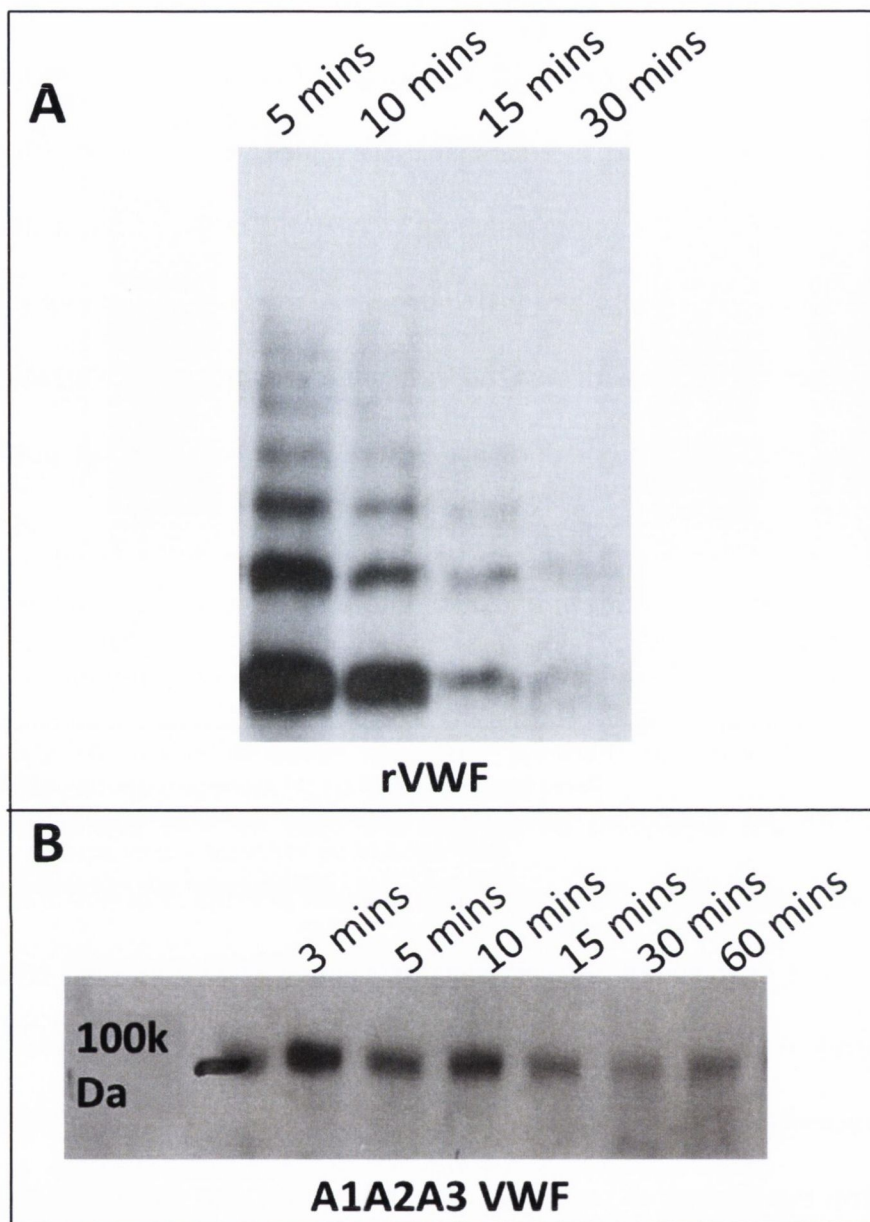


Figure 5.4 Clearance analysis of rVWF and A1A2A3 VWF

(A) VWF multimer gel analysis of plasma clearance samples at 5, 10, 15 and 30 minutes confirm the absence of proteolytic degradation of the protein

(B) Reducing SDS-PAGE gel analysis of murine plasma samples from A1A2A3 clearance, intact protein is observed at 100kDa.

5.2 Macrophages mediate the clearance of VWF via the A domains

Previous work has highlighted a particular role for hepatic and splenic macrophages in mediating VWF clearance. Depletion of macrophages *in vivo* increase VWF plasma levels 2.5-fold in wild-type mice (van Schooten *et al*, 2008). Given the apparent importance of macrophages in regulating VWF survival, clodronate liposomes were employed to assess their contribution in this study.

Mice were administered clodronate liposomes at a dose of 100 μ l/10g intravenously via lateral tail vein injection. This was injected 24 hours prior to VWF to allow sufficient time for macrophage depletion. To confirm depletion of macrophage populations, mouse spleens were harvested and flow cytometry analysis was performed (section 2.20.5). Splenic macrophage markers CD11b and F4/80 were used to co-label isolated mouse spleen cells. Subsequent flow cytometry analysis demonstrated that clodronate liposome treatment resulted in a reduction of F4/80+/CD11b+ macrophages of over 70% (figure 5.5).

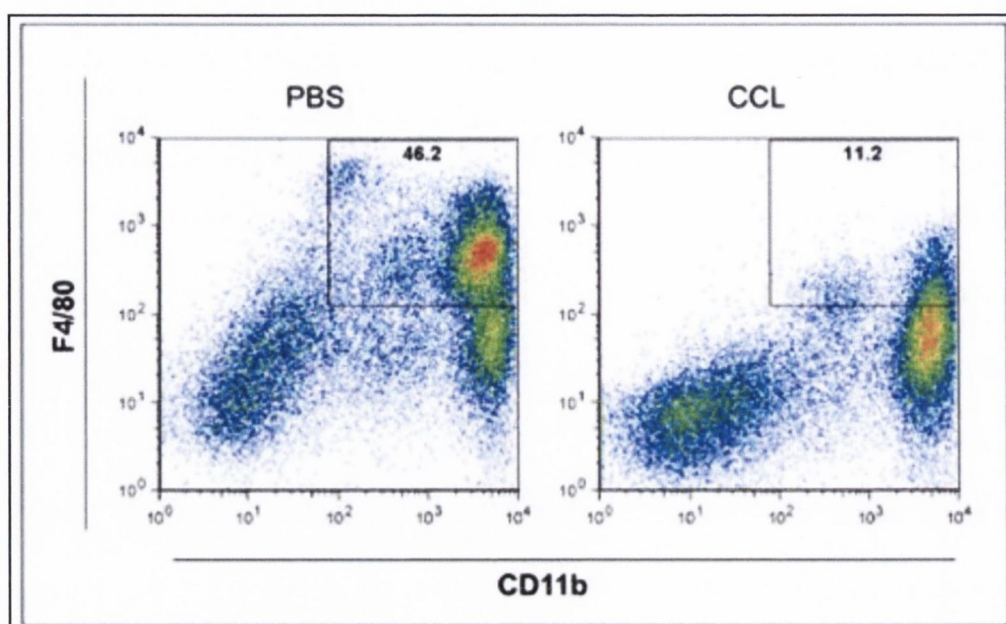


Figure 5.5 Flow cytometry analysis of C57Bl mouse spleen macrophage
Macrophages were labelled as F4/80+ and CD11b+ and measured after treatment with PBS or clodronate liposomes (CCL). Image provided by Dr. Hendrik Nel, Inflammation and Immunity Research Group, Trinity College Dublin.

In keeping with previous findings, depletion of macrophages *in vivo* resulted in a significantly prolonged survival of rVWF of approximately 1.5-fold. For example, at 3 minutes, macrophage depletion enhanced rVWF recovery from $71.4 \pm 5.9\%$, to $102.4 \pm 12\%$. Similarly, after 10 minutes rVWF survival was improved from $37.9 \pm 1.8\%$ in the absence of clodronate, to $70.0 \pm 5.0\%$ upon macrophage depletion (figure 5.6, $p < 0.01$).

Interestingly, clodronate treatment also significantly inhibited A1A2A3 VWF clearance ($106.8 \pm 3.4\%$ and $56.4 \pm 1.2\%$, at 3 and 10 minutes, respectively, figure 5.6, $p < 0.05$). Thus macrophage depletion led to improved survival of both rVWF and A1A2A3 VWF to a similar extent. These novel findings suggesting that macrophages not only contribute to the clearance of full length VWF but also to the clearance of an A domain fragment of VWF.

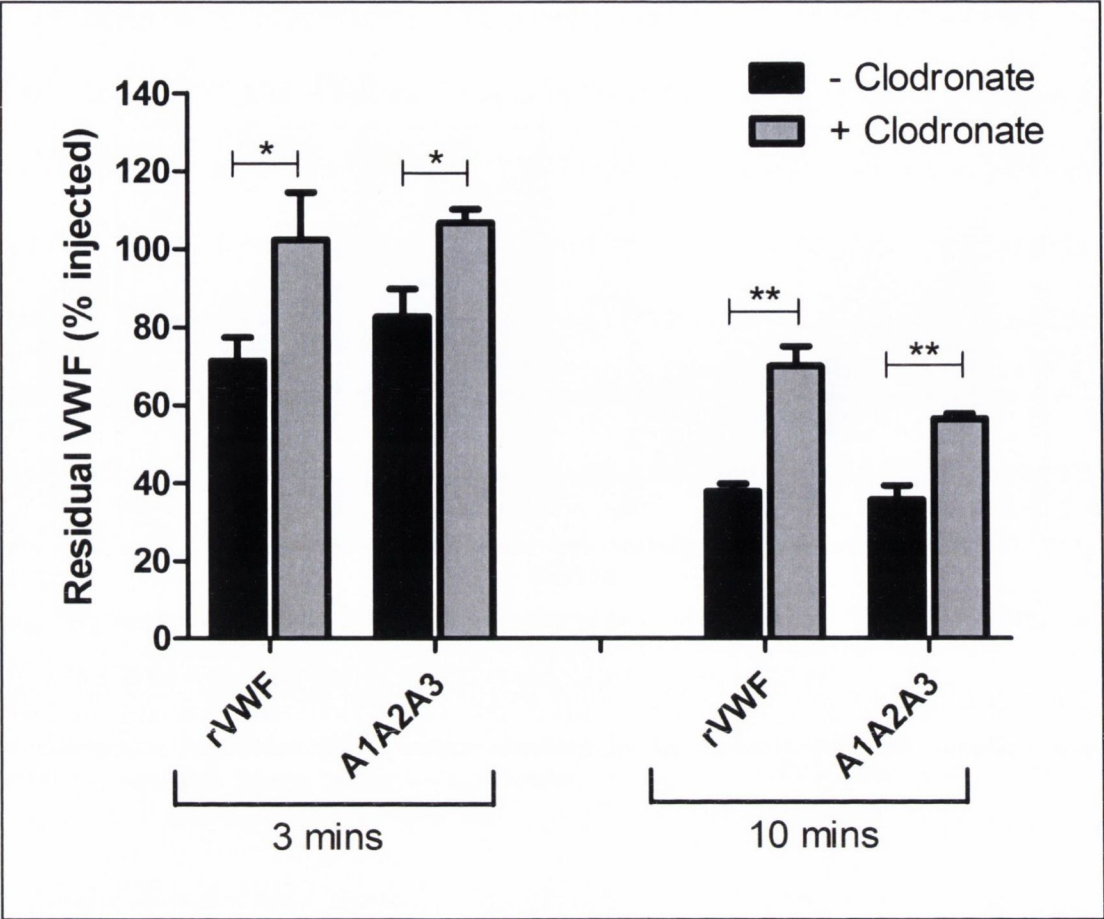


Figure 5.6 Macrophage depletion prolongs the survival of both rVWF and A1A2A3 VWF *in vivo*
Data graphed as mean $\pm$ SEM.

5.3 Binding of rVWF and A1A2A3 VWF to THP-1 macrophages *in vitro*

5.3.1 The VWF-macrophage interaction *in vitro* requires manganese cations

To further investigate the role of specific VWF domains in modulating the macrophage interaction, a THP-1 *in vitro* binding assay was established. Previous studies examining VWF-leucocyte interactions *in vitro* reported that VWF binding required the presence of divalent cations (Koivunen *et al*, 2001; Pendu *et al*, 2006). Thus we assessed the requirement of divalent cations for the VWF-THP-1 macrophage interaction. Plasma-derived VWF (pd-VWF) containing increasing concentrations of various divalent cations including Mn^{2+} , Ca^{2+} and Mg^{2+} was incubated with THP-1 macrophages, seeded on a 96 well plate, on ice (to prevent endocytosis and degradation) for 1 hour. After washing and fixing the cells, bound VWF was detected using a fluorescently tagged antibody (section 2.14.2). Fluorescence associated with the cell membrane was then quantified using the IN Cell Analyzer 1000 (GE Healthcare, UK). Surprisingly, no binding of VWF to THP-1 macrophages was observed in the presence of either Ca^{2+} or Mg^{2+} . However dose dependent VWF binding was detected in the presence of Mn^{2+} (figure 5.7). Taken together these findings highlight a critical role for Mn^{2+} in promoting VWF-THP-1 binding. On this basis of this manganese was included in subsequent THP-1 binding assays at a concentration of 1mM.

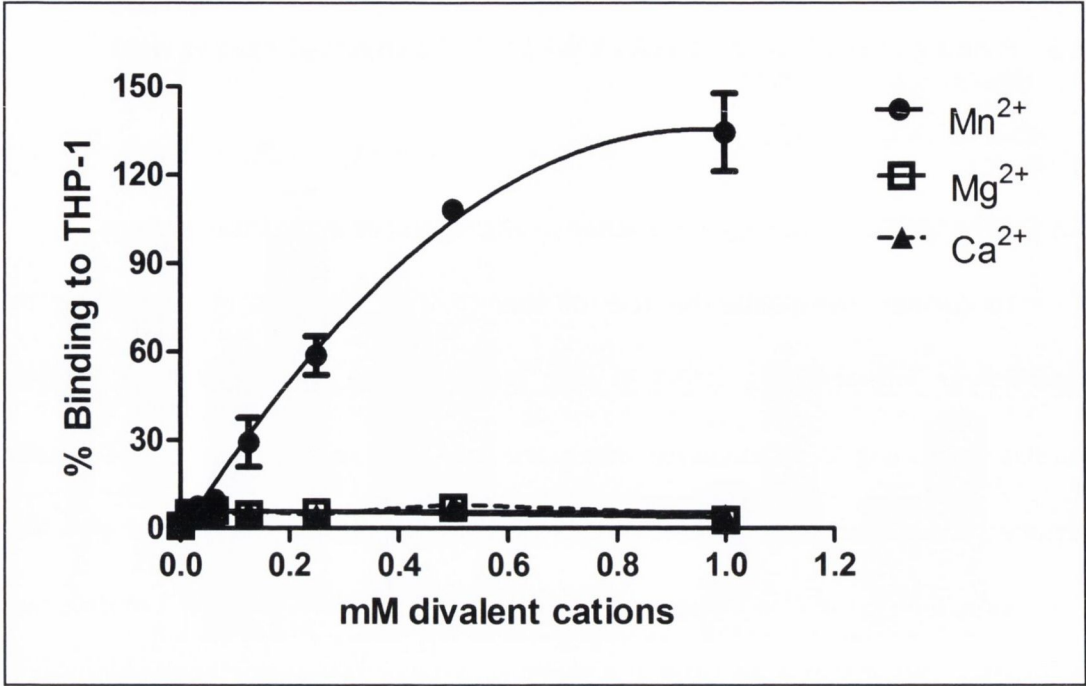


Figure 5.7 VWF binds to THP-1 macrophages in a manganese-dependent manner
Data are expressed as a percentage of maximum binding (mean $\pm$ SEM).

5.3.2 Ristocetin enhances VWF binding to THP-1 macrophages *in vitro*

Castro-Nunez *et al* reported that primary macrophages bound and endocytosed VWF in the presence of ristocetin (Castro-Núñez *et al*, 2012). Thus we examined the VWF binding to THP-1 macrophages in the presence and absence of ristocetin. Pd-VWF and rVWF were incubated with the cells in the presence of 1mg/mL of ristocetin. Bound VWF was detected and quantified. The level of VWF binding was expressed as percentage of the maximum binding observed. The presence of ristocetin resulted in both pd-VWF and r-VWF achieving near maximal binding to THP-1 macrophages ($90.6 \pm 5.0\%$ and $99.0 \pm 4.2\%$, respectively, figure 5.8; $p < 0.001$). Cumulatively these findings suggest that conformational changes induced in VWF by ristocetin promote VWF binding to THP-1 macrophages.

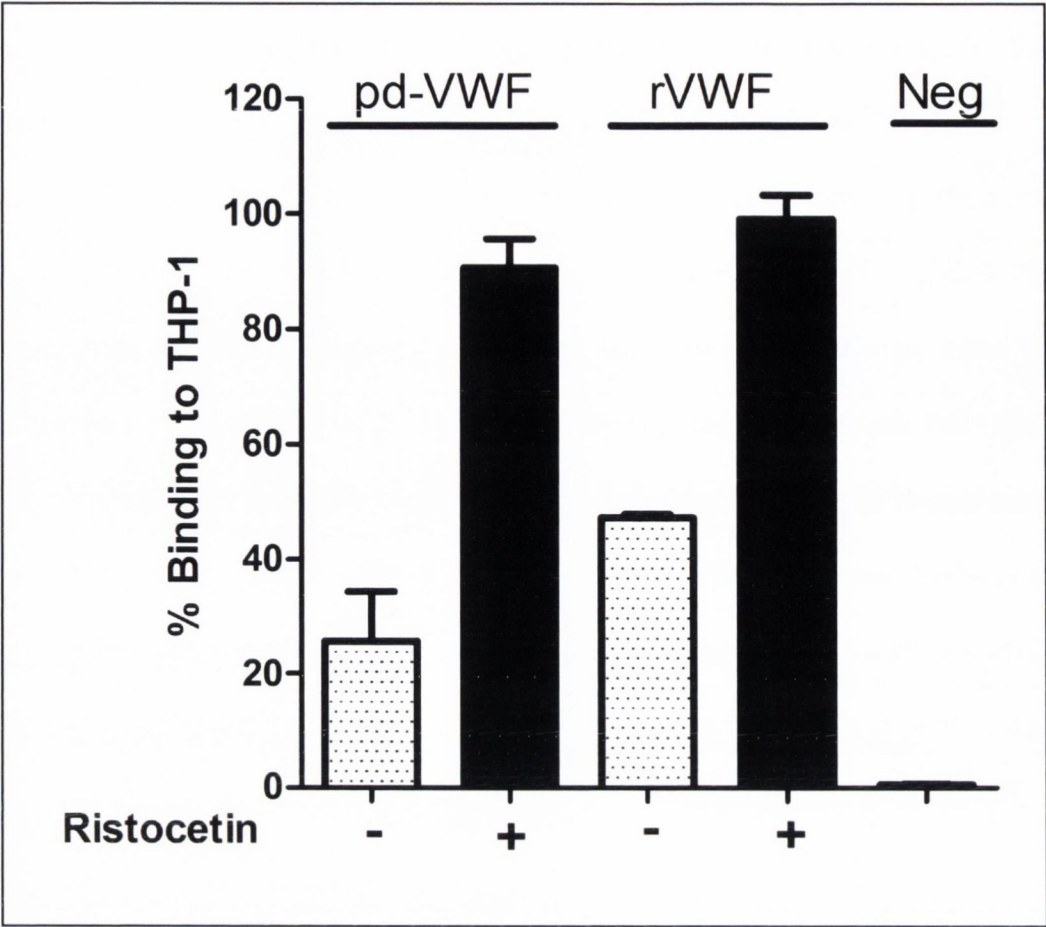


Figure 5.8 VWF binding to THP-1 macrophages is enhanced in the presence of ristocetin
Data are expressed as a percentage of maximum binding (mean $\pm$ SEM).

5.3.3 The A domains of VWF binds to THP-1 macrophages *in vitro*

In view of the *in vivo* experiments suggesting that VWF A domains may be important in regulating macrophage-mediated clearance, we assessed the ability of the A domains to bind to THP-1 macrophages *in vitro*. Both 1mM manganese divalent cations and 1mg/mL ristocetin were included as part of the binding conditions. A1A2A3 VWF was incubated with the THP-1 cells on ice. After washing and fixing the cells were probed with Penta-His antibody labelled with FTIC to detect binding of his-tagged A1A2A3. We observed A1A2A3 VWF bound to THP-1 macrophages in a dose-dependent manner (figure 5.9). These data imply that the A domains of VWF may contain macrophage interactive sites capable of mediating VWF binding *in vitro*. This is consistent with our findings that macrophages influence the clearance of A1A2A3 VWF *in vivo* also.

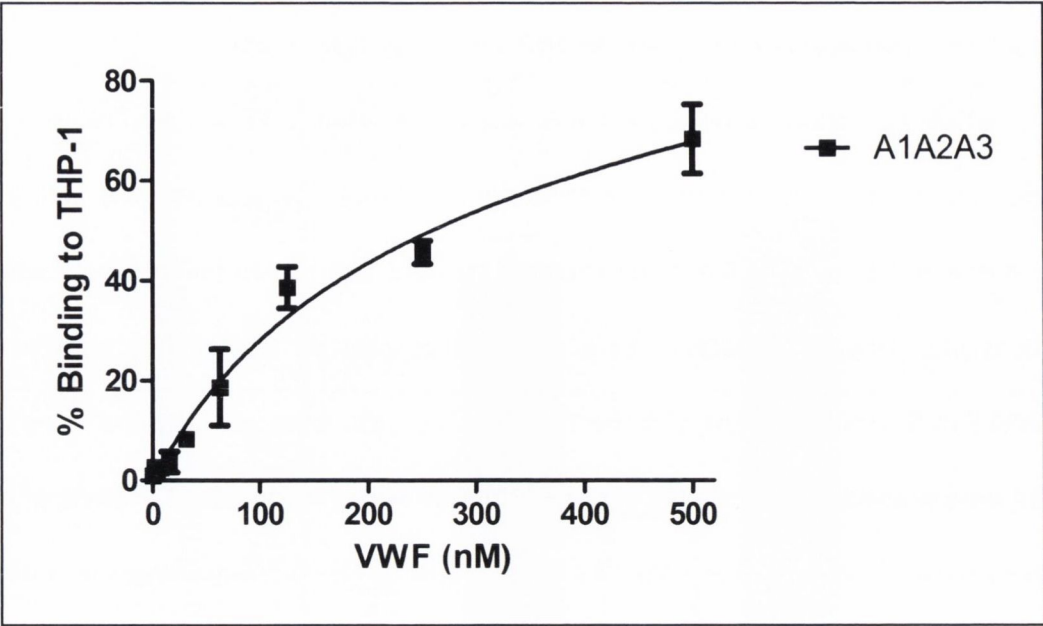


Figure 5.9 A1A2A3 VWF binds to THP-1 macrophages in a dose-dependent manner
Data are expressed as a percentage of the mean maximum binding $\pm$ SEM.

5.4 N-linked glycans within A2 regulate macrophage binding *in vitro*

The tri-domain A1A2A3 region of VWF is central in regulating a number of VWF functions including GPIIb α binding, ADAMTS13-mediated proteolysis and adherence to collagen. Both N and O-linked glycans expressed within the A domains of VWF have been shown to influence these functions. The O-linked glycans are found as clusters at the N- and C-terminal of the A1 domain and have been shown to negatively regulate GPIIb α binding under static and shear flow conditions (Nowak *et al*, 2012). Two complex-type N-linked glycans reside within the A2 domain. These have been demonstrated to stabilise the globular conformation of VWF and thereby regulate the interaction of VWF with ADAMTS13 (McKinnon *et al*, 2008). Moreover, a specific role has been demonstrated for the glycan at position N1574 in negatively modulating susceptibility to ADAMTS13 proteolysis (McKinnon *et al*, 2008). In this context we sought to examine a potential role for glycans in mediating macrophage binding. To this end, A1A2A3 VWF was subjected to PNGase treatment (PNG-A1A2A3) to remove the N-linked glycans in the A2 domain. The efficacy of the digest was confirmed by shift in electrophoretic mobility on SDS-PAGE electrophoresis. N-linked glycan removal served to markedly enhance A1A2A3 VWF binding to THP-1 macrophages *in vitro* by over 80% compared to untreated A1A2A3 (figure 5.10 $p<0.0001$). These novel data highlight a putative role for N-linked glycans in the A2 domain in modulating binding to THP-1 macrophages.

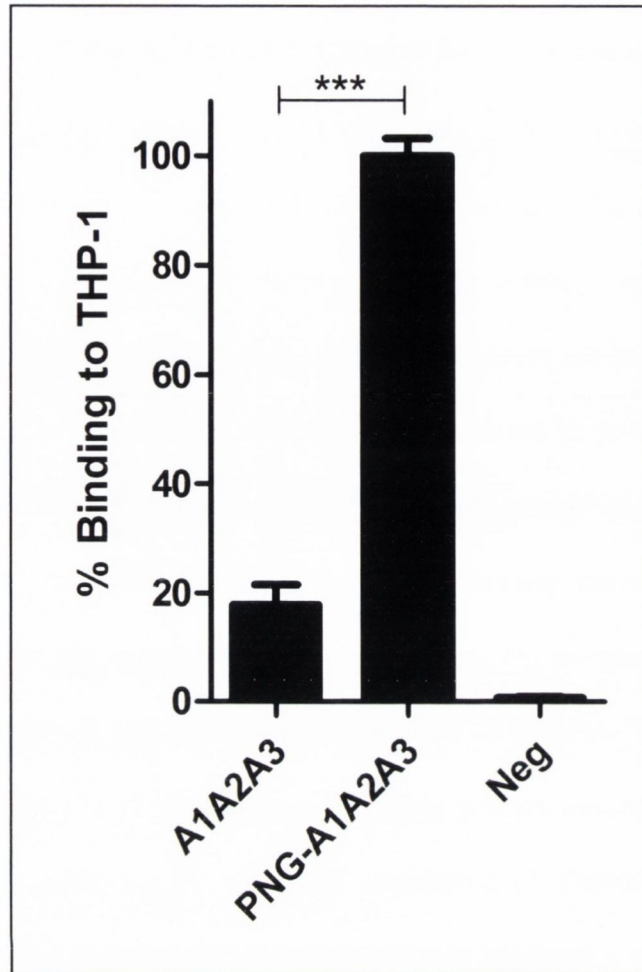


Figure 5.10 Removal of the N-linked glycans in A2 enhances binding to THP-1 macrophages
Data are expressed as a percentage of the mean maximum binding mean $\pm$ SEM.

On the basis of these observations, we hypothesised that the N-linked glycans in A2 may act to sterically shield critical VWF interactive residues required for macrophage binding. A detailed analysis of the crystal structure of the A2 domain was first published in 2009 by Zhang *et al.* Further to this, mass spectrometry analysis of human pd-VWF has provided extensive information regarding the N-glycome of VWF. In particular distinct N-glycan features at specific sites in VWF have been characterised (Canis *et al*, 2012). Utilising this information, a model of the VWF A2 domain with its associated glycans (figure 5.11) was constructed using Glycam Glycoprotein Builder software. N1515 and N1574 glycans structures were mapped onto the A2 domain crystal structure using this glycan modelling tool. This *in silico* analysis revealed that the complex glycans at N1515 and N1574 were both of significant size. They span approximately 33Å and 36Å in length, respectively (figure 5.11). Considering that the average diameter of the A2 domain is approximately 35Å, the potential importance of these N-linked glycans in shielding important interactive ligand sites and consequently modulating function can be appreciated.

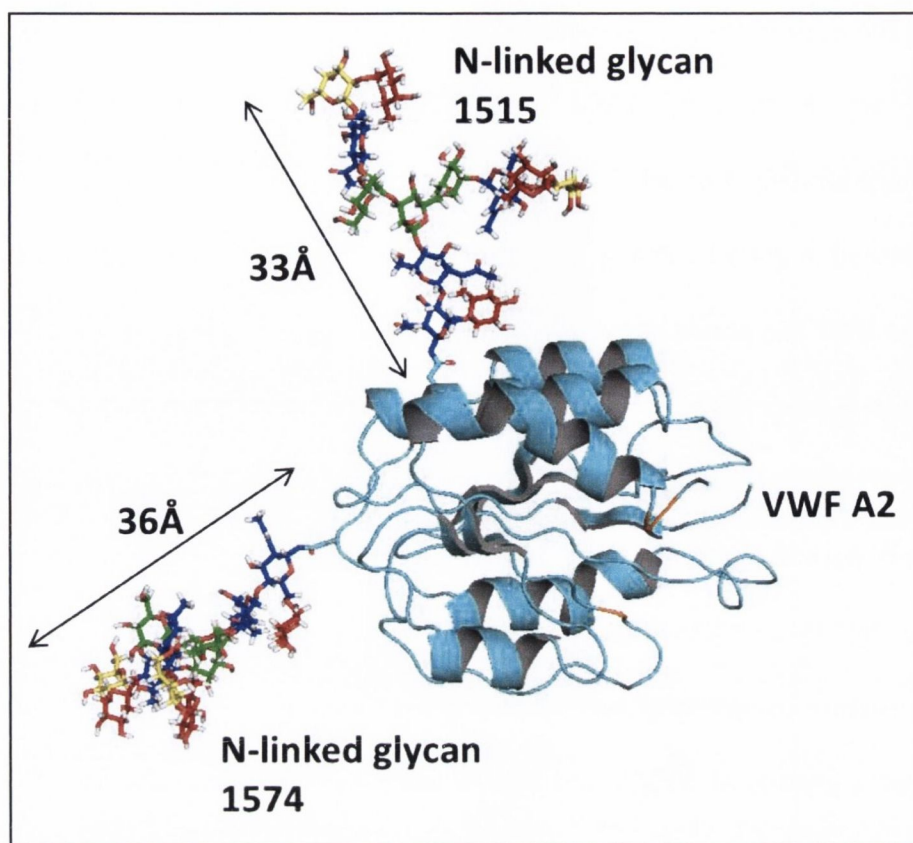


Figure 5.11 A glycan model to predicting the size contribution of the N-linked glycans to the A domains

The crystal structure of the A2 domain of VWF and relative positions of the N-linked glycans at 1515 and 1574 and their approximate length in angstroms (Å).

5.5 VWF N-linked glycans modulate *in vivo* survival

The N-linked glycans of VWF inhibit binding to THP-1 macrophages *in vitro*. Given that macrophages mediate VWF survival *in vivo* we speculated that this enhanced affinity of PNG-A1A2A3 for THP-1 macrophages may also influence *in vivo* clearance. To investigate this, rVWF was treated with exoglycosidase PNGase F (PNG-rVWF) resulting in a 90% reduction in the N-linked glycan population, as assessed by specific lectin, concavalin A. The clearance rate of PNG-rVWF was assessed in VWF^{-/-} mice. PNG-rVWF was cleared in a monophasic fashion. The calculated clearance parameters, including percentage recovery at the earliest time point of 3 minutes, *t*_{1/2} and MRT for rVWF and PNG-rVWF are shown below (table 5.2);

	% Recovery (mins)	<i>T</i> ¹ / ₂ (mins)	MRT (mins)
rVWF	72.0 ± 5.9	7.8 ± 0.4	11.2 ± 0.6
PNG-rVWF	57.8 ± 1.0	3.2 ± 0.09	4.7 ± 0.1

Table 5.2 Clearance parameters for rVWF and PNG-rVWF in VWF^{-/-} mice
Values expressed as the mean ± SEM.

Importantly, PNG-rVWF was cleared at such an accelerated rate than it was no longer detectable in the plasma beyond 20 minutes (figure 5.12). Both the *t*_{1/2} and MRT for PNG-rVWF were significantly reduced compared to untreated rVWF (figure 5.13; *p*<0.001). The asialoglycoprotein receptor (ASGPR) was the first VWF clearance receptor identified (Grewal *et al*, 2008). ASGPR is a lectin receptor binding VWF glycan structures containing β-linked galactose or N-acetylgalactosamine. Both of these sugars are expressed heavily by the N-linked glycans glycan structures on VWF. The

mechanism for the rapid clearance of PNG-rVWF is not yet clear but it is most likely mediated through ASGPR independent mechanisms. It is possible that removal of the N-linked glycans across the length of VWF may result in enhanced exposure of amino acid consensus sequences for non-lectin clearance receptors. Importantly, previous studies have shown that PNG-rVWF still displays a range of multimers comparable to undigested rVWF. PNG-rVWF also displays normal ability to bind to collagen (McGrath *et al*, 2010). This indicates that the overall global structure of VWF is unlikely to be significantly altered. Nevertheless, modest local conformation changes in PNG-rVWF are possible. Notably, PNG-rVWF has been characterised by increased ADAMTS13-mediated proteolysis *in vitro* indicating potential local conformational changes in the A2 domain of VWF enhancing access to the cryptic cleavage site (McKinnon *et al*, 2008).

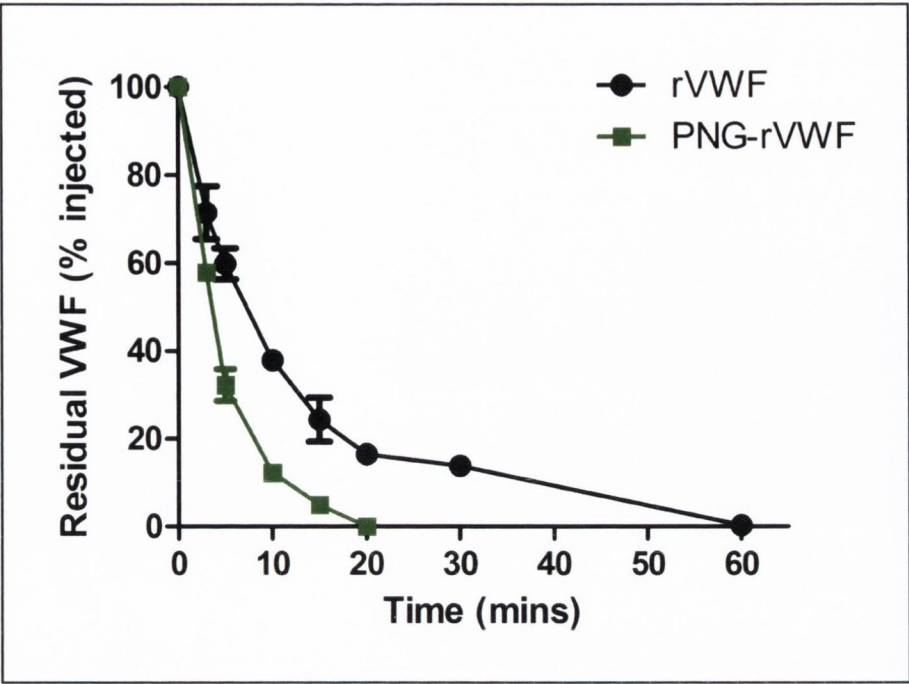


Figure 5.12 PNG-rVWF is cleared at an accelerated rate compared to untreated rVWF
Data graphed as the mean $\pm$ SEM.

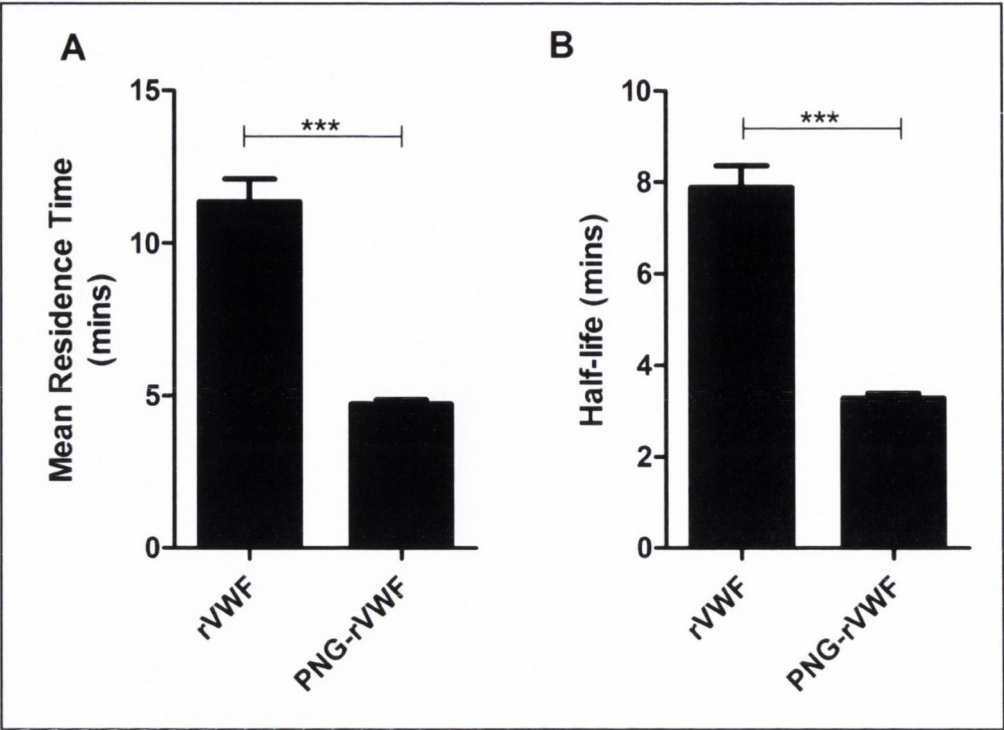


Figure 5.13 Clearance parameters for rVWF and PNG-rVWF
Data graphed as the mean $\pm$ SEM.

5.6 VWF A2 domain glycans regulate its clearance *in vivo*

Our *in vitro* findings suggest that the N-linked glycan within A2 may have a specific role in modulating VWF affinity for macrophages. Hence we sought to examine if the N-linked glycans within A2 may be important in regulating VWF survival *in vivo*. To this end, a fragment of VWF with the A2 domain deleted was constructed, (figure 5.14). Consequently this VWF variant (Δ A2-VWF) fails to express the N-linked glycans N1515 and N1574. These are the only N-linked glycans that reside within the A domains of VWF. Previous studies have assessed some of the functional characteristics of a Δ A2-VWF variant including FVIII, heparin and collagen binding (Koppelman *et al*, 1996; Lankhof *et al*, 1997). Collectively these studies have not reported any significant differences compared to wild-type VWF. This suggests that despite the deletion of the A2 domain, the global structural integrity and functional activity of Δ A2-VWF is maintained. Thus this variant was used to further examine the role of the A2 and its glycans in influencing VWF clearance in VWF^{-/-} mice. We observed that Δ A2-VWF was cleared in a monophasic manner. The calculated clearance parameters for Δ A2-VWF compared with rVWF are shown below (table 5.3).

	% Recovery (mins)	T ¹ / ₂ (mins)	MRT (mins)
rVWF	72.0 ± 5.9	7.8 ± 0.4	11.2 ± 0.6
Δ A2-VWF	83.6 ± 4.9	6.6 ± 0.4	9.6 ± 0.5

Table 5.3 Clearance parameters for rVWF and Δ A2-VWF in VWF^{-/-} mice
Values expressed as mean ± SEM.

Δ A2-VWF and rVWF were cleared at similar rates in VWF^{-/-} mice (figure 5.15). Δ A2-VWF had a plasma t_{1/2} of 6.6 ± 0.4 minutes compared to 7.8 ± 0.4 minutes for

rVWF (figure 5.16). These novel findings suggest that the VWF A2 domain is not the sole regulator of VWF circulatory clearance in this mouse model. Importantly, Lenting *et al* have previously demonstrated the addition of the N-terminal D'D3 domains to the A domains of VWF served to reduce its rate of clearance, while the addition of C-terminal D4-CK domains accelerate the clearance of A1A2A3 (Lenting *et al*, 2004). Consequently these findings establish that VWF domains outside the A domains can contribute to VWF clearance. Given that Δ A2-VWF resists ADAMTS13 cleavage (Lankhof *et al*, 1997), these data confirm that ADAMTS13-mediated proteolysis of multimeric VWF is not necessary for clearance. This is also in agreement with our earlier data (figure 5.4) depicting the clearance of rVWF without the appearance of proteolytic fragments in murine plasma samples.

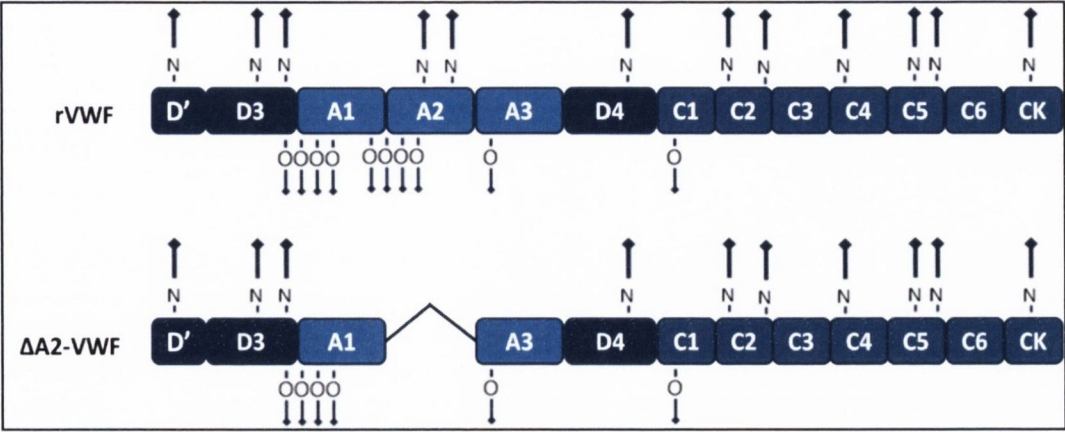


Figure 5.14 A schematic domain diagram of $\Delta A2$ -VWF with its associated glycans

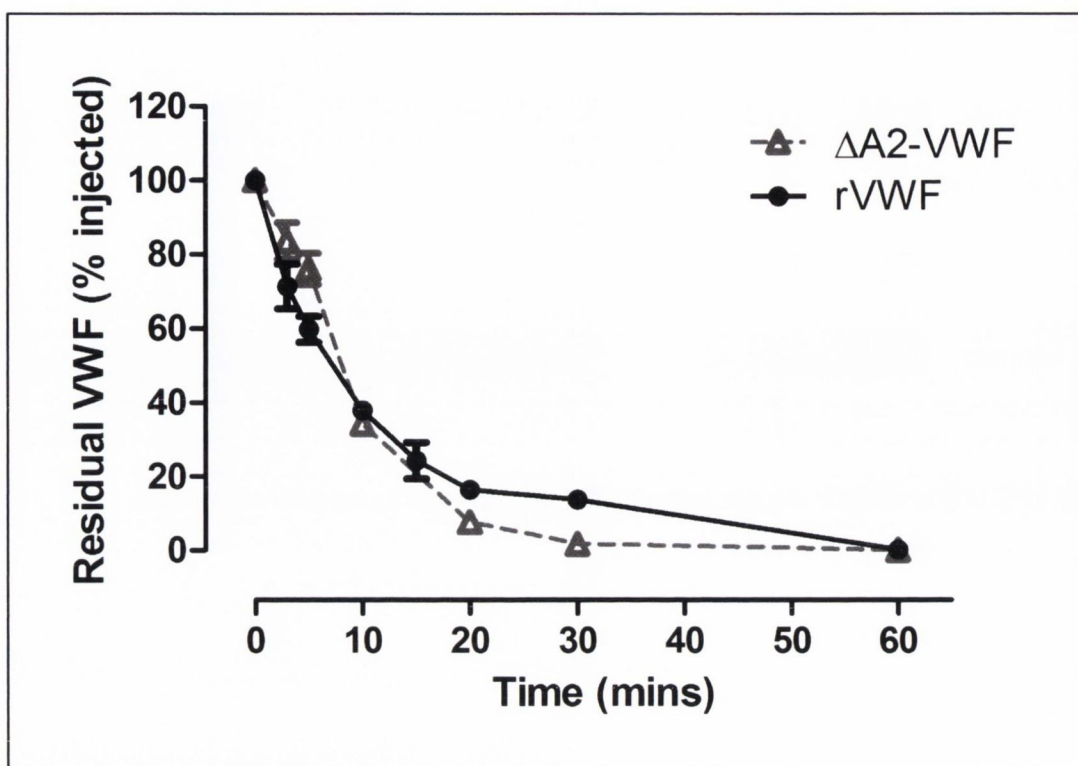


Figure 5.15 Deletion of the A2 domain ($\Delta A2$ -VWF) does not influence the rate of clearance compared to full length rVWF
Data graphed as the mean $\pm$ SEM.

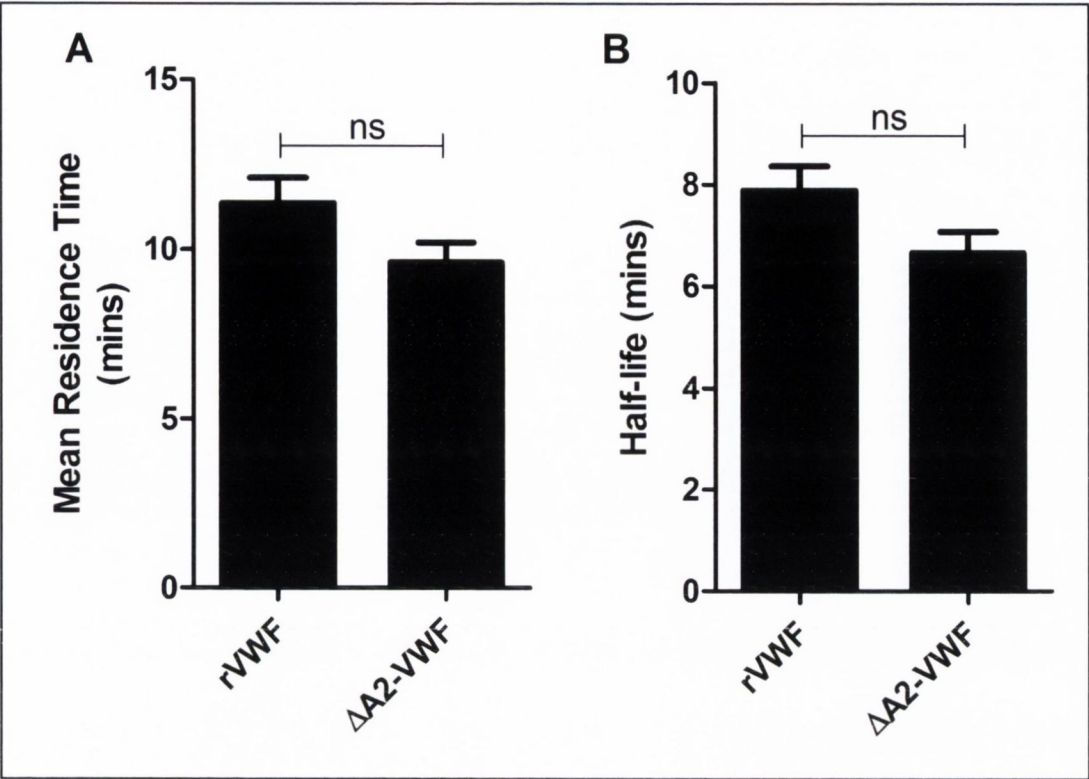


Figure 5.16 Clearance parameters for rVWF and Δ A2-VWF
Data graphed as the mean $\pm$ SEM.

Δ A2-VWF was subjected to PNGase F treatment (PNG Δ A2-VWF) to investigate the role of VWF N-linked glycans outside the A2 domain in regulating VWF survival. The efficacy of the digestion was confirmed by VWF multimer gel analysis and lectin ELISA assessment. PNGase treatment did not reduce VWF capacity to multimerise (data not shown). PNG Δ A2-VWF was administered to VWF^{-/-} mice and clearance assessed as previous. PNG Δ A2-VWF was cleared in a monophasic manner. The calculated clearance parameters, including percentage recovery, $t_{1/2}$ and MRT for PNG Δ A2-VWF compared with Δ A2-VWF are shown below (table 5.4)

	% Recovery (mins)	$T^{1/2}$ (mins)	MRT (mins)
Δ A2-VWF	83.6 $\pm$ 4.9	6.6 $\pm$ 0.4	9.6 $\pm$ 0.5
PNG Δ A2-VWF	96.3 $\pm$ 7.0	6.5 $\pm$ 0.5	9.4 $\pm$ 0.8

Table 5.4 Clearance parameters for Δ A2 and PNG Δ A2 in VWF^{-/-} mice
Values expressed as mean $\pm$ SEM.

The clearance rate for PNG Δ A2-VWF was striking similar to that of untreated Δ A2-VWF (figure 5.17). PNG Δ A2-VWF was cleared with a plasma $t_{1/2}$ of 6.5 $\pm$ 0.5 minutes and a MRT of 9.4 $\pm$ 0.8 minutes (figure 5.18). These values are remarkably comparable to the clearance parameters measured for untreated Δ A2-VWF, 6.6 $\pm$ 0.4 minutes and 9.6 $\pm$ 0.5 minutes ($t_{1/2}$ and MRT, respectively). In contrast PNG-rVWF is cleared in a very rapid manner from the circulation. This suggests a particular role for VWF A2 domain glycans in modulating VWF clearance since removal of N-linked glycans outside the A2 domain does not significantly alter the rate of VWF clearance. Further studies were performed to systematically investigate the two individual N-

glycan sites within the A2 domain of VWF to determine their contribution to VWF plasma survival. This work is discussed in detail in Chapter 6.

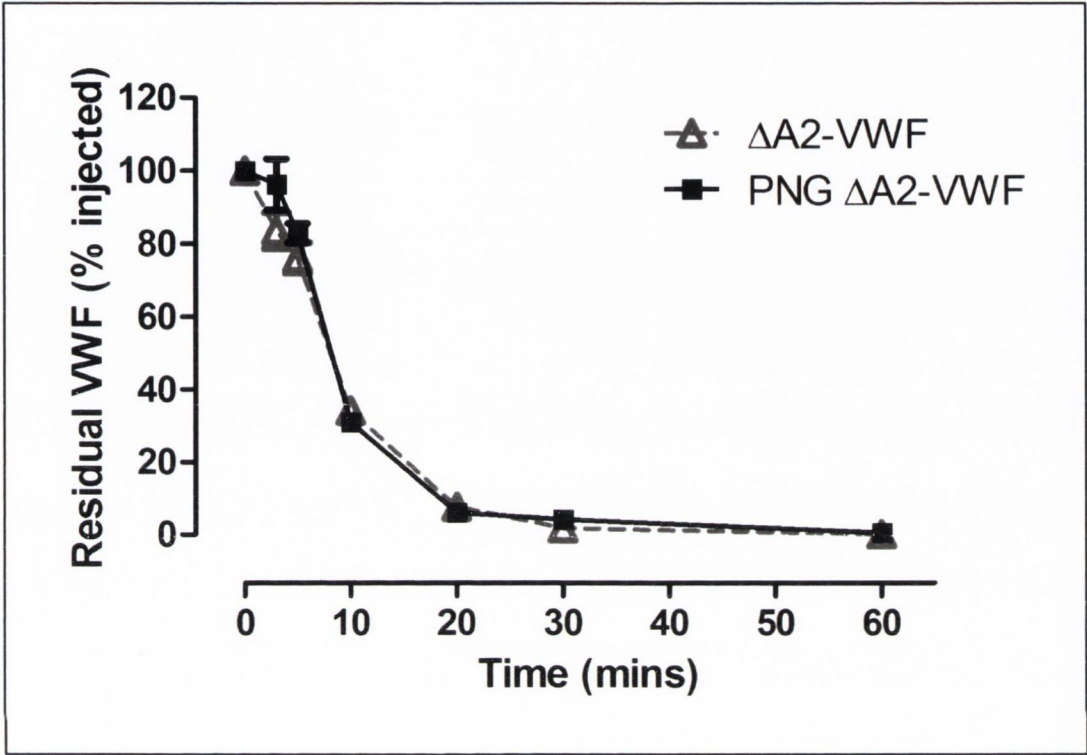


Figure 5.17 The N-linked glycans outside VWF A2 domain do not modulate circulatory clearance
Data graphed as the mean $\pm$ SEM.

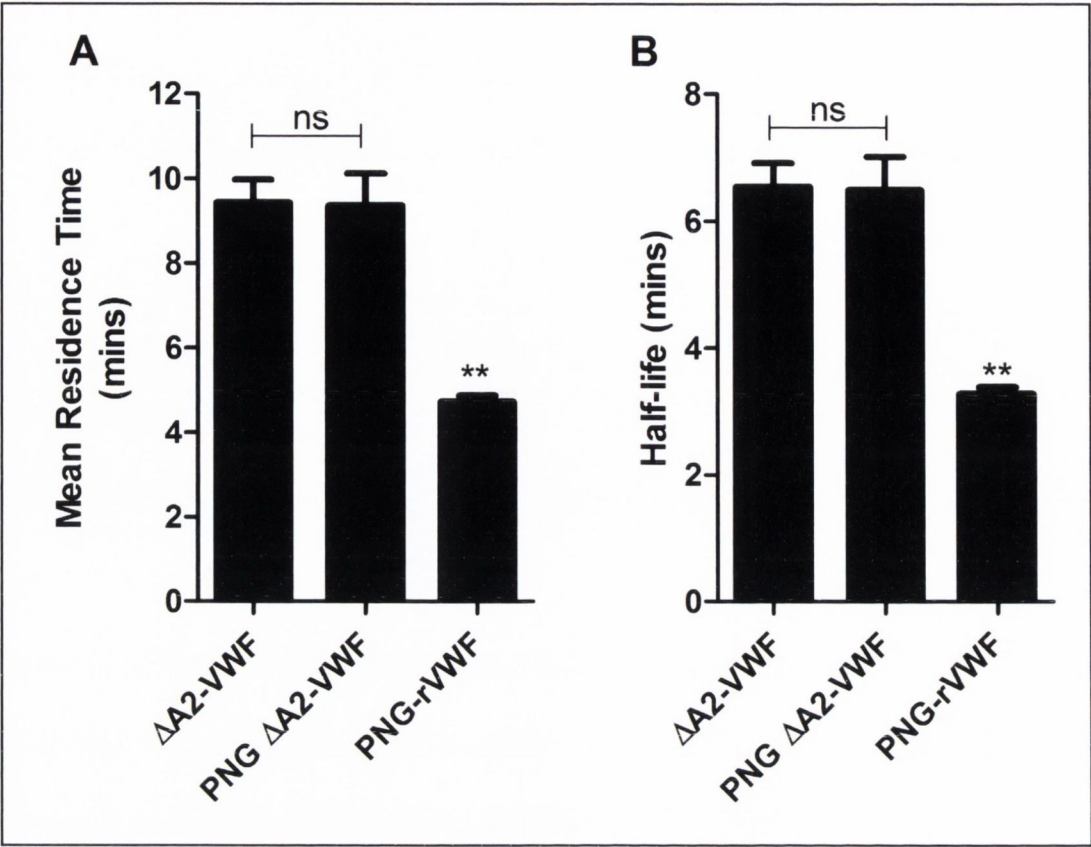


Figure 5.18 Clearance parameters for $\Delta A2$ -VWF, PNG $\Delta A2$ -VWF and PNG-rVWF
Data graphed as the mean $\pm$ SEM.

5.7 Discussion

Much of the recent published data pertaining to VWF clearance is focused on delineating the cellular and receptor basis mediating its uptake *in vivo*. While this is useful in suggesting features of VWF that may be important (for example VWF glycans for lectin based receptors) it does not identify regions and/or specific residues of VWF that define its plasma half-life. Understanding the mechanisms involved in regulating VWF clearance has the potential to enable development of a long-acting molecule to treat patients with VWD. In addition, a long-acting VWF carrier molecule might also significantly advance therapy for patients with haemophilia A by extending FVIII half-life. With this in mind, this investigation sought to further characterise the mode of VWF clearance and specifically to define the domains and features of the protein that are important.

5.7.1 VWF A domains modulate VWF clearance via macrophages

A single publication concluded that the A domains of VWF are important regulators of *in vivo* survival in VWF^{-/-} mice. Consequently we focused our initial attention on the A domains of VWF. Clearance studies in VWF^{-/-} mice demonstrated that monomeric A1A2A3 exhibited a similar rate of clearance to full length multimeric rVWF. The A1A2A3 domains of VWF are central in regulating many VWF interactions. Encoded within this fragment are critical regions for binding to GPIIb α , collagen type I, III, and VI, OPG, PSGPL-1, and ADAMTS13 cleavage. Our data now further suggest that A1A2A3 also contains a sequence involved in mediating VWF removal from the circulation.

Of note, the overall rate of human VWF clearance in VWF^{-/-} mice is markedly more rapid than that observed in humans. The reported $t_{1/2}$ of rVWF in VWF^{-/-} mice ranges from 7.8 minutes to 35 minutes up to 3 hours depending on the cell-line used for VWF expression and the dosage administered (Pruss *et al*, 2011; Lenting *et al*, 2004). These values stand in marked contrast to the $t_{1/2}$ of pd-VWF in humans of approximately 15 hours (Dobrkovska *et al*, 1998). It has been suggested that these differences are partly related to the increased basal metabolic rate of mice. In keeping with this human pd-VWF has a half-life of approximately 8 hours in canine models (Turecek *et al*, 1997).

Given the multimeric nature of VWF, it has been questioned whether differently sized multimers may be cleared at distinct rates, or indeed whether ADAMTS13-mediated proteolysis may contribute to the clearance rate. However, mice on a C57Bl genetic background express a truncated variant of ADAMTS13 lacking the C-terminal TSP-1 and CUB domains. Consequently this variant of ADAMTS13 is not effective in cleaving human VWF (Zhou *et al*, 2007). This confounding factor has made it difficult to assess whether ADAMTS13 contributes to VWF clearance. Notwithstanding these issues, a number of studies have provided evidence to suggest that ADAMTS13-mediated cleavage is not a pre-requisite for VWF clearance. Notably the half-life of murine VWF in ADAMTS13 deficient mice was similar to that of ADAMTS13 sufficient mice (Badirou *et al*, 2010). Nevertheless, other candidate proteases are capable of cleaving VWF, including lymphocyte derived granzymes, plasmin and leukocyte-secreted proteases (De Ceunynck *et al*, 2013). To ensure our infused VWF was not undergoing proteolysis *in vivo* plasma samples were subjected to western blot analysis. No significant evidence of proteolysis was observed.

Cumulatively, these findings confirm an important role for VWF A domains in influencing its clearance in VWF^{-/-} mice, which is in keeping with Lenting *et al.* Further to this, we demonstrate for the first time that macrophages contribute to the clearance of the A domains of VWF *in vivo*. Macrophage depletion using clodronate liposomes significantly prolonged the survival of rVWF and A1A2A3 VWF in our mouse model. Emerging data suggests that macrophages may not only be important in the normal homeostasis of VWF plasma levels but may additionally modulate pathological clearance of VWD mutants. Interestingly, clodronate liposomes have been used to attenuate the rapid clearance associated with VWF in a mouse model of VWD type 2B (Casari *et al*, 2013a). Moreover, work carried out in our lab has described prolonged survival of VWD Vicenza in the presence of clodronate-liposomes in VWF^{-/-} mice (Rawley *et al*, 2015). Collectively, these data therefore define a role for macrophages in mediating both physiological and pathophysiological clearance of VWF *in vivo*. Importantly further work is needed to identify the macrophage receptors involved in this process.

5.7.2 A domain glycosylation regulates binding to THP-1 macrophages

In keeping with previous reports, VWF binding to THP-1 was significantly enhanced in the presence of ristocetin (Castro-Núñez *et al*, 2012). Ristocetin stimulates VWF-GPIb α binding in the absence of shear stress via conformational changes (Dong *et al*, 2001). Moreover, recent data has indicated that ristocetin also induced conformational changes within A2 and thereby enhanced ADAMTS13-mediated proteolysis via enhanced exposure of the cryptic cleavage site (Chen *et al*, 2012). The requirement for unwinding of VWF to facilitate macrophage binding suggests that a

cryptic site may be important in mediating this interaction. Intuitively, the levels of activated unwound VWF, capable of tethering platelets and inducing thrombosis, need to be tightly regulated. Consistent with this, Chen *et al* has described that the exposure of the VWF GPIb α binding site and ADAMTS13 cleavage site are coupled (Chen *et al*, 2012). Thus platelet bound VWF is a preferred substrate for ADAMTS13 proteolysis (Shim *et al*, 2008). VWF interacts with the clearance receptor LRP1 in a shear-dependent manner, suggesting that activated VWF may also be a preferential target for clearance (Castro-Núñez *et al*, 2012; Rastegarlari *et al*, 2012). Furthermore, constitutively active VWD type 2B mutations significantly enhance VWF clearance (Casonato *et al*, 2010).

Interestingly, VWF binding to THP-1 cells in our assays required the presence of divalent manganese ions. The explanation for this critical role of this specific cation remains unclear. Nevertheless, the requirement for manganese is in keeping with work by Pendu *et al* who reported THP-1 cells and primary polymorphonuclear leukocytes bound to immobilized VWF in the presence of cell medium supplemented with MnCl₂ (Pendu *et al*, 2006). Additionally they found the presence of a β 2 integrin antagonist ablated adherence of the cells to VWF. This observation is also in line with data published by Koivunen *et al*, who reported VWF interacts with THP-1 cells in a β 2-integrin dependent manner requiring the presence of manganese cations (Koivunen *et al*, 2001). Importantly VWF contains two Leucine-Leucine-Glycine (LLG) motifs which have been proposed as a consensus integrin binding sequences (Koivunen *et al*, 2001). The A1A2A3 VWF construct used in our study contains one of these LLG amino acid motifs (1482-1484). Moreover, we observed that soluble A1A2A3 VWF bound in a dose-dependent manner to THP-1 macrophages in the presence of MnCl₂. Whether

β 2-integrins are involved in mediating this interaction remains to be determined. Cumulatively, these findings indicate that VWF A domains can interact with macrophages *in vitro* and that this interaction may be important in defining VWF survival *in vivo*.

In further examining features of VWF A domains that may influence this interaction, we identified a critical role for the N-linked glycans. A2 domain glycans constitute key determinants in negatively regulating the interaction between A1A2A3 VWF and macrophages. It has been previously demonstrated that A2 domain glycans of VWF can also influence its interaction with ADAMTS13 via conformational changes in VWF (McKinnon *et al*, 2008). We hypothesise that removal of the A2 glycans may additionally serve to modulate macrophage binding by exposing of critical binding regions.

5.7.3 VWF glycosylation influences its plasma half-life

The presence of N-linked glycans on coagulation factor X (FX) has been shown to be a regulator of its biodistribution in plasma, and a predictor of half-life (Kurdi *et al*, 2012). Moreover, specific glycan determinants of VWF have been shown to modulate its clearance. For example, enzymatic removal of terminal sialic acid residues from VWF glycans markedly reduced plasma half-life when administered in rabbits (Sodetz *et al*, 1977). In keeping with the exposure of sub-terminal galactose sugars, the rapid removal of asialo-VWF was mediated via hepatic lectin receptor ASGPR. The importance of VWF sialylation in regulating plasma clearance is further demonstrated by the RIIS/J inbred mouse strain, where aberrant glycosylation of VWF results in enhanced clearance (Mohlke *et al*, 1999). In this context, and given the importance of

N-linked glycan in modulating macrophage-VWF binding *in vitro*, the role of VWF glycans on *in vivo* clearance was studied. Strikingly, the absence of the N-linked glycans markedly enhanced VWF clearance. Previous work by Badirou *et al* have demonstrated that removal of specific O-linked glycan sites in cluster 1 of VWF also served to enhance VWF clearance in mice (Badirou *et al*, 2012). Collectively, these data suggest that VWF glycosylation can directly modulate its survival via interaction with clearance lectin receptors including ASGPR. However VWF glycosylation also promotes VWF survival by potentially maintaining VWF conformation and/or shielding critical VWF protein clearance sites.

5.7.4 N-linked glycans in the A domains modulate VWF survival in vivo

To elucidate a potential role for VWF A2 domain in influencing VWF clearance a Δ A2-VWF construct was designed. In spite of the removal of approximately 206 amino acids from the protein backbone, the functional features described for this variant remain remarkably intact. Botrocetin induced platelet binding of a Δ A2 variant is normal, as is heparin binding and β 2-glycoprotein I binding (Lankhof *et al*, 1997; Hulstein *et al*, 2007). This suggests that the deletion of A2 domain does not significantly affect the function of the adjacent A1 domain. Moreover, Lankhof *et al*, (1997) also report comparable collagen type III binding between wild-type VWF and Δ A2-VWF suggesting the flanking A3 domain function is also not significantly altered. Consistent with the location of the cleavage site in A2, at position Y1605-M1606, Δ A2-VWF was not sensitive to proteolysis when incubated with normal human plasma (Lankhof *et al*, 1997). Remarkably, our findings demonstrate that removal of the A2 domain of VWF also fails to significantly influence its clearance in VWF^{-/-} mice.

However it has been shown, by examining the clearance of various domain truncations of VWF, that VWF does not possess a simple single clearance site. Various N- and C-terminal truncations of VWF are removed from the circulation in VWF^{-/-} mice. This redundancy most likely means that even in the absence or mutation of important VWF clearance sites, ancillary domains may contribute to regulate VWF plasma levels.

Despite Δ A2-VWF displaying similar clearance parameters of full length VWF, we observed an important role for VWF A2 domain glycans in influencing VWF clearance. In stark contrast to rapid clearance observed with PNG-rVWF, PNG Δ A2-rVWF did not display enhanced clearance. These findings suggest that specific glycans within the A2 domain may be of specific important in regulating the rapid clearance of PNG-rVWF. Moreover the potential role of the glycans in the A2 domain in regulating VWF clearance will be further investigated in Chapter 6.

The work presented in this section highlights an important role for the A domains in contributing to VWF plasma half-life. Moreover, these data demonstrate that the A domains promote macrophage binding both *in vitro* and *in vivo*. Finally the N-linked glycans, specifically those located within VWF A domains, seemingly protect VWF from rapid clearance in plasma. The molecular mechanism through which N-glycans elicit this effect will be further investigated in the next section.

6 Investigating the molecular mechanisms through which VWF glycosylation influences *in vivo* clearance

Accumulating lines of evidence suggest that VWF glycosylation is of direct physiological relevance. VWF glycan structures have been demonstrated to influence platelet adhesion, susceptibility to ADAMTS13 mediated proteolysis and circulatory half-life. Importantly, the molecular mechanisms through which VWF glycans modulate these effects remain unclear. Recently two groups have reported a role for the O-linked glycans of VWF, which are located within cluster 1 at the N-terminal end of the A1 domain, in regulating GPIIb α binding (Nowak *et al*, 2012; Madabhushi *et al*, 2014). Site-directed mutagenesis to eliminate the four O-linked glycans in this cluster resulted in enhanced ristocetin-mediated GPIIb α binding to VWF. Additionally, loss of cluster 1 also increased the ability of VWF to bind to collagen under high shear stress and subsequently mediated enhanced platelet capture under flow.

VWF clearance is regulated by expression of ABO(H) blood group antigens on VWF. Despite the relatively minor change in carbohydrate structure between different blood groups, the presence of ABO(H) structures on VWF glycan has a marked effect on plasma VWF levels. Blood group O individuals have 25% less circulating VWF compared to non-O individuals (Gill *et al*, 1987). Accumulating evidence suggests that expression of ABO(H) blood group determinants modulate VWF survival (Gallinaro *et al*, 2008). Blood group O individuals have a significantly shorter VWF plasma half-life than non-O. In keeping with this group O individuals have significantly higher VWFpp/VWF:Ag ratios consistent with enhanced plasma clearance.

The importance of VWF glycosylation in influencing clearance has also been demonstrated in a number of animal models. Studies in VWF^{-/-} mice have demonstrated a role for VWF O-linked glycans in protecting VWF circulatory survival (Badirou *et al*, 2012). Moreover, genetic inactivation of specific sialyltransferase (ST3Gal-IV) in C57Bl mice results in enhanced exposure of sub-terminal galactose and thus results in a 2-fold reduction in VWF half-life (Ellies *et al*, 2002). Further evidence supporting the importance of VWF glycosylation in regulating plasma clearance has been highlighted in the RIIS/J mouse strain. These inbred mice exhibit aberrant expression of N-acetylgalactosaminyltransferase in EC. This results in aberrant VWF glycosylation, enhanced hepatic clearance and significantly reduced VWF plasma levels (Mohlke *et al*, 1999). Cumulatively these data highlight the major role played by VWF carbohydrate structures in modulating its clearance. Importantly however the biological mechanisms through which glycans contribute to influence clearance *in vivo* remain poorly defined. The ASGPR remains the only definitive lectin receptor mediating VWF clearance *in vivo*. However our observations in Chapter 5 demonstrate PNG-rVWF is cleared from the circulation in a markedly rapid manner in VWF^{-/-} mice. Moreover, our data further suggest that this accelerated clearance is mediated through an ASGPR-independent pathway. Given the key role previously described for macrophages in regulating VWF survival *in vivo* we sought to further investigate the molecular mechanism through which VWF glycans serve to influence its clearance *in vivo*.

6.1 Specific N-linked glycan sites can regulate VWF survival *in vivo*

As previously demonstrated, PNGase treatment of rVWF resulted in enhanced clearance *in vivo*. However PNGase treatment of a ΔA2-VWF did not result in a significantly altered rate of clearance compared to wild-type rVWF. Collectively these findings suggest that glycans within the A2 domain may be of specific important in regulating the rapid clearance of PNG-VWF. The A2 domain contains two complex-type N-linked glycans at position N1515 and N1574. To investigate a potential role for these glycans, initially N1515 was targeted for removal by site-directed mutagenesis (VWF-N1515Q) and expressed in HEK293T cells. For *in vivo* clearance studies, VWF^{-/-} mice were intravenously infused with 30nM of VWF-N1515Q and at appropriate time points mice were anaesthetised and blood collected for ELISA analysis. The calculated clearance parameters, including percentage recovery at 3 minutes, plasma half-life ($t_{1/2}$) and mean residence time (MRT) for rVWF and VWF-N1515Q are shown below (table 6.1)

	% Recovery	$t_{1/2}$ (mins)	MRT (mins)
rVWF	72.0 ± 5.9	7.8 ± 0.4	11.2 ± 0.6
VWF-N1515Q	50.4 ± 1.9	3.7 ± 0.1	5.3 ± 0.2

Table 6.1 Clearance parameters for rVWF and VWF-N1515Q in VWF^{-/-} mice
Values are expressed as mean ± SEM.

Strikingly, VWF-N1515Q was cleared at an accelerated rate compared to rVWF in VWF^{-/-} mice (figure 6.1). Both the $t_{1/2}$ and MRT for VWF-N1515Q (3.7 ± 0.1 minutes and 5.3 ± 0.2 minutes, respectively) were 2-fold reduced compared to rVWF (figure 6.2, $p<0.05$). The clearance rate of VWF-N1515Q occurs in a similarly rapid manner to

PNG-rVWF which is surprising given that the N1515Q mutation only removes one N-linked glycan of 12 that are expressed on monomeric VWF. These data therefore suggest a key role for the glycan chain at N1515 within A2 in influencing VWF clearance *in vivo*.

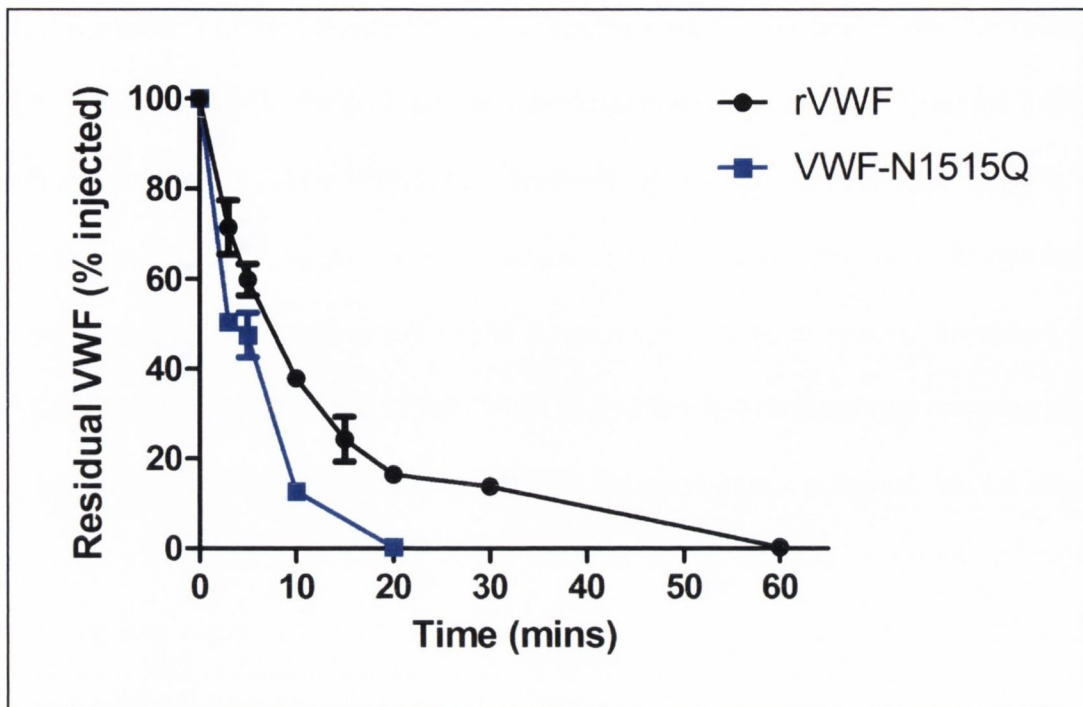


Figure 6.1 VWF-N1515Q accelerates VWF clearance compared to rVWF in $VWF^{-/-}$ mice
Data graphed as the mean $\pm$ SEM.

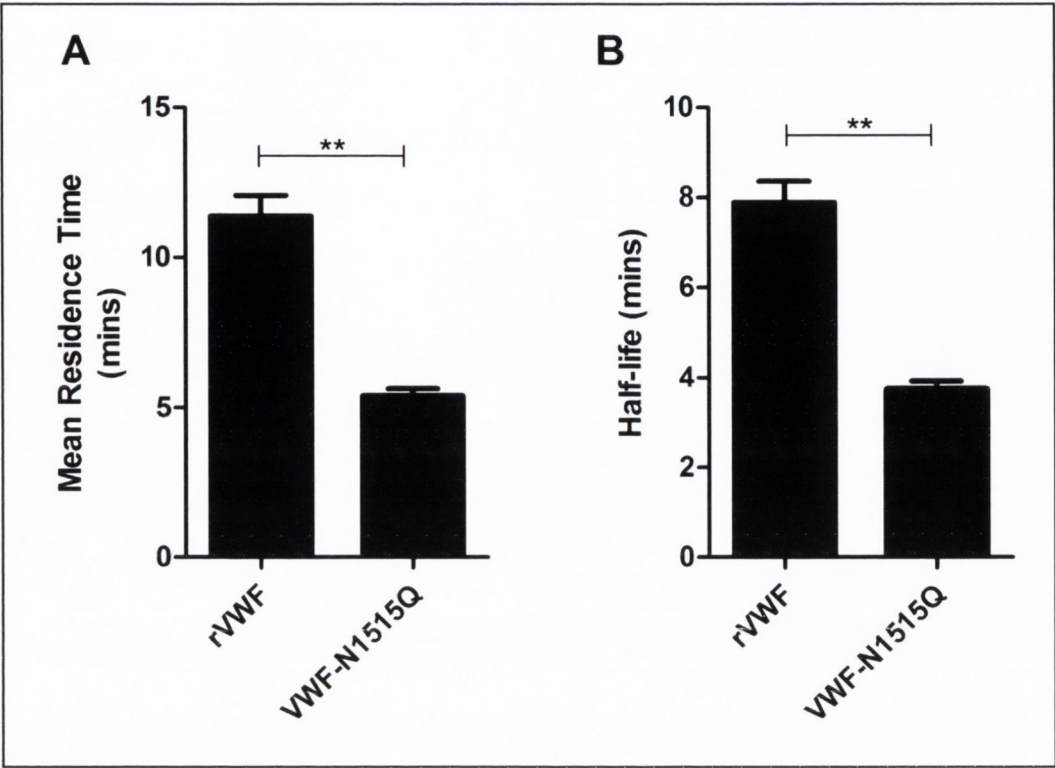


Figure 6.2 Clearance parameters for VWF-N1515Q and rVWF
(A) Mean residence time values
(B) Plasma half-life values for rVWF and N1515Q
Data are graphed as the mean $\pm$ SEM.

6.2 VWF glycosylation can influence macrophage-mediated clearance

To assess the potential contribution of macrophages in modulating the enhanced clearance of VWF-N1515Q, clodronate liposomes were administered to VWF^{-/-} mice. A resting time of 24 hours was necessary to allow sufficient time for depletion of hepatic and splenic macrophages. VWF-N1515Q was infused into the mice and clearance assessed as before. The clearance parameters for VWF-N1515Q in the presence and absence of clodronate liposomes are detailed in table 6.2.

	% Recovery	t _{1/2} (mins)	MRT (mins)
VWF-N1515Q-clodronate	50.4 ± 1.9	3.7 ± 0.1	5.3 ± 0.2
VWF-N1515Q+clodronate	87.5 ± 4.8	15.4 ± 0.9	22.2 ± 1.3

Table 6.2 Clearance parameters for VWF-N1515Q in the presence and absence of clodronate liposomes in VWF^{-/-} mice
Values are expressed as mean ± SEM.

Macrophage depletion served to significantly enhance the recovery of VWF-N1515Q by over 30%. Moreover the calculated t_{1/2} was increased from 3.7 ± 0.1 minutes to 15.4 ± 0.9 minutes (figure 6.4). The MRT of VWF-N1515Q also reflected a 4-fold increase following macrophage depletion. Interestingly we observed that macrophage depletion markedly reversed the enhanced clearance of VWF-N1515Q so that there was no significant difference in residual VWF levels between rVWF and VWF-N1515Q levels (figure 6.5). Collectively these findings depict an important role for macrophages in mediating the rapid clearance observed with glycan variant VWF-N1515Q *in vivo*.

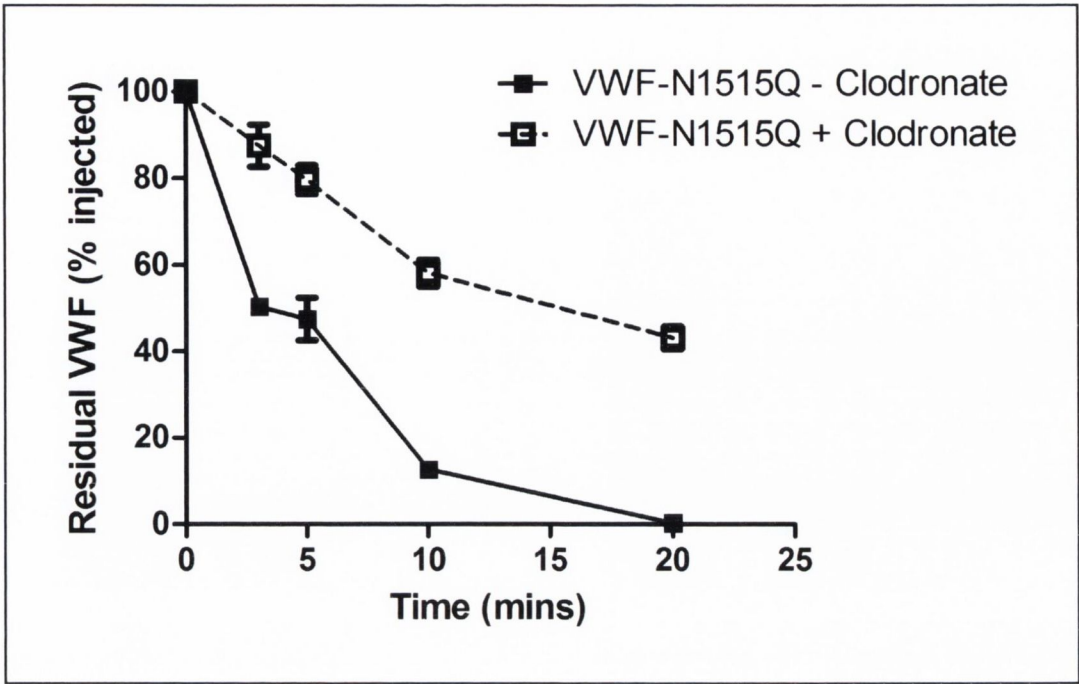


Figure 6.3 Macrophage depletion delays the clearance of VWF-N1515Q VWF^{-/-} mice
Data graphed as the mean $\pm$ SEM.

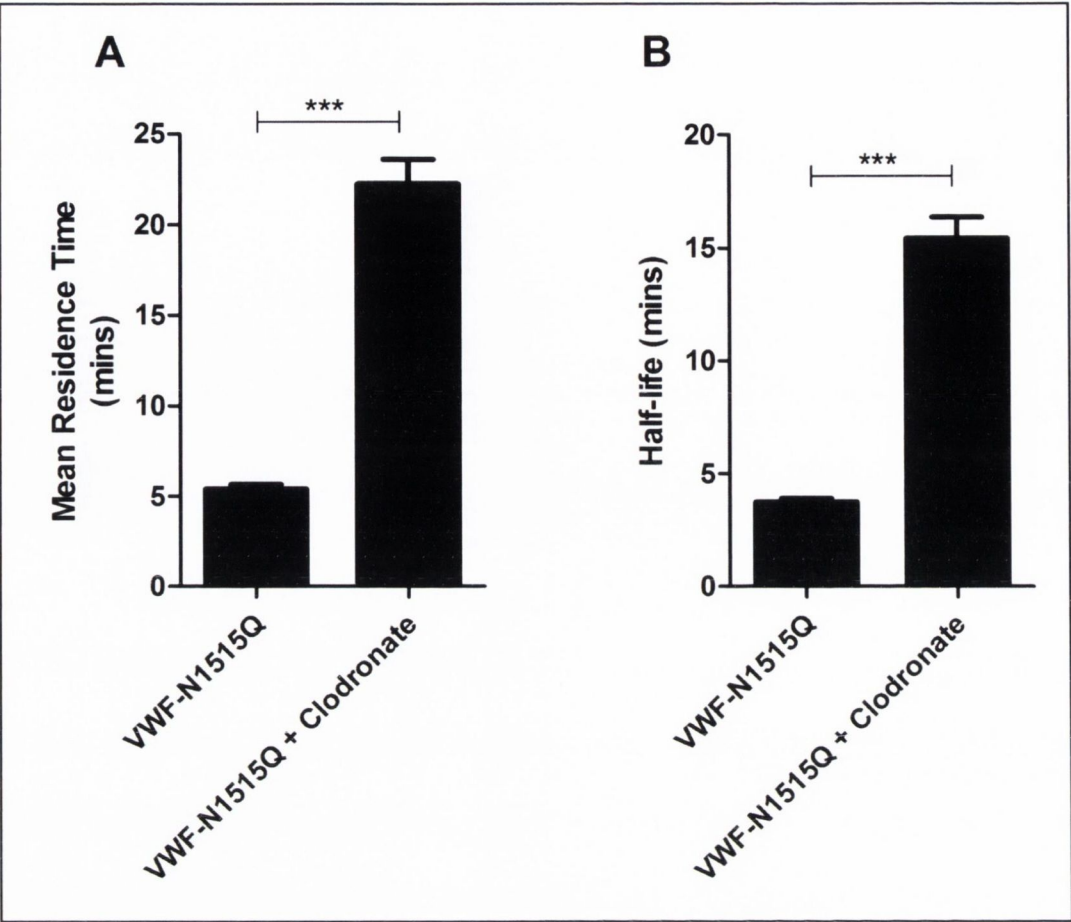


Figure 6.4 Clearance parameters for VWF-N1515Q in the presence and absence of clodronate liposomes

(A) Mean residence time values

(B) Plasma half-life values.

Data are graphed as the mean $\pm$ SEM.

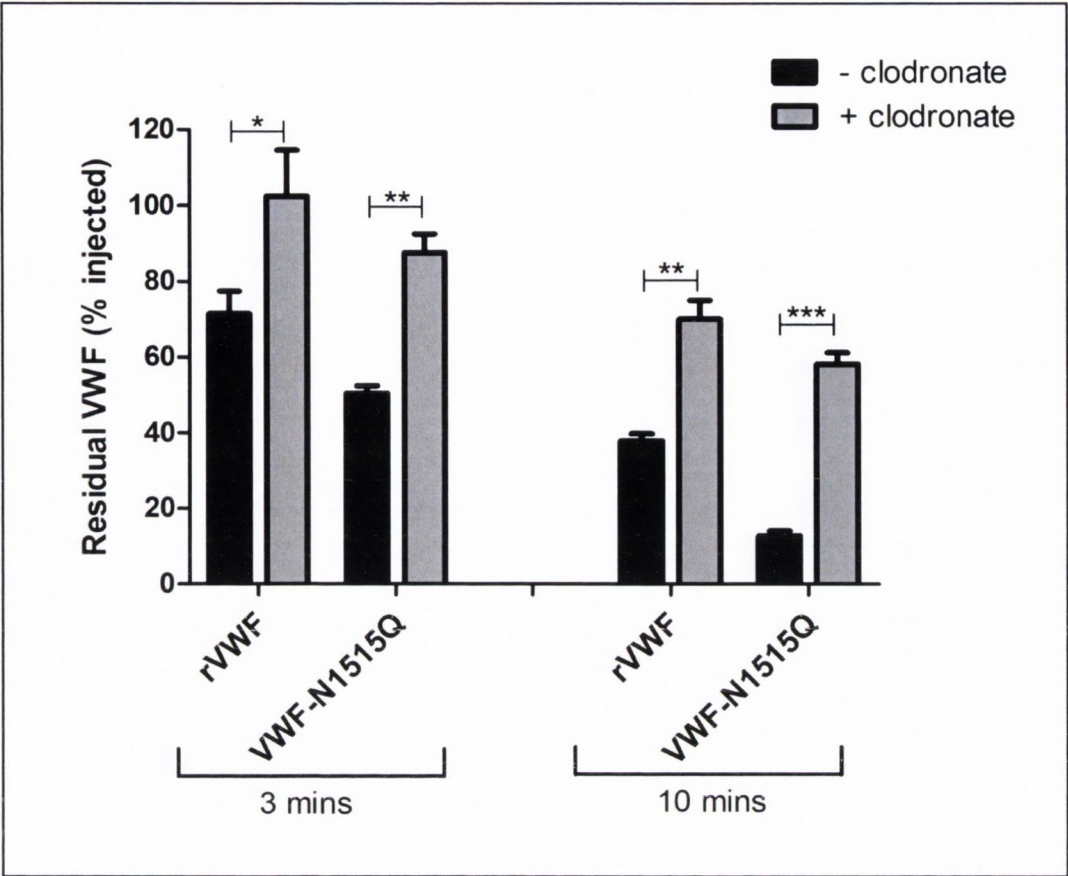


Figure 6.5 Macrophage depletion prolongs the survival of rVWF and VWF-N1515Q to a similar extent
Data graphed as the mean $\pm$ SEM.

6.3 Glycosylation of A1A2A3 VWF can modulate its clearance

We have previously demonstrated that a truncated fragment of VWF comprised of the A1A2A3 domains is cleared at a similar rate to full length rVWF (Chapter 5). Given that the glycan at N1515 resides within the A2 domain of VWF, we further sought to examine if the N1515 glycan could also influence the *in vivo* survival of an A1A2A3 VWF truncated fragment. To this end site-directed mutagenesis was used to eliminate the glycan at N1515 with A1A2A3 VWF (A1A2A3-N1515Q) and clearance examined in VWF^{-/-} mice. The clearance parameters for A1A2A3 VWF and A1A2A3-N1515Q are outlined in table 6.3.

	% Recovery	t _{1/2} (mins)	MRT (mins)
A1A2A3 VWF	82.7 ± 7.0	6.4 ± 0.3	9.2 ± 0.5
A1A2A3-N1515Q	48.8 ± 1.4	3.1 ± 0.05	4.5 ± 0.1

Table 6.3 Clearance parameters for A1A2A3 and A1A2A3-N1515Q in VWF^{-/-} mice
Values are expressed as mean ± SEM.

In keeping with its effect in enhancing clearance of full length VWF, introduction of the N1515Q glycan mutation within the A1A2A3 truncated fragment again resulted in significantly enhanced clearance (figure 6.6). A1A2A3-N1515Q served to reduce both the t_{1/2} and the MRT 2-fold compared with A1A2A3 VWF (figure 6.7). The extent of this accelerated clearance was similar to that seen with VWF-N1515Q in full length rVWF. Cumulatively, these data demonstrate that the enhanced clearance associated with loss of the glycan at position 1515 is modulated through local effects within the A1A2A3 region of VWF.

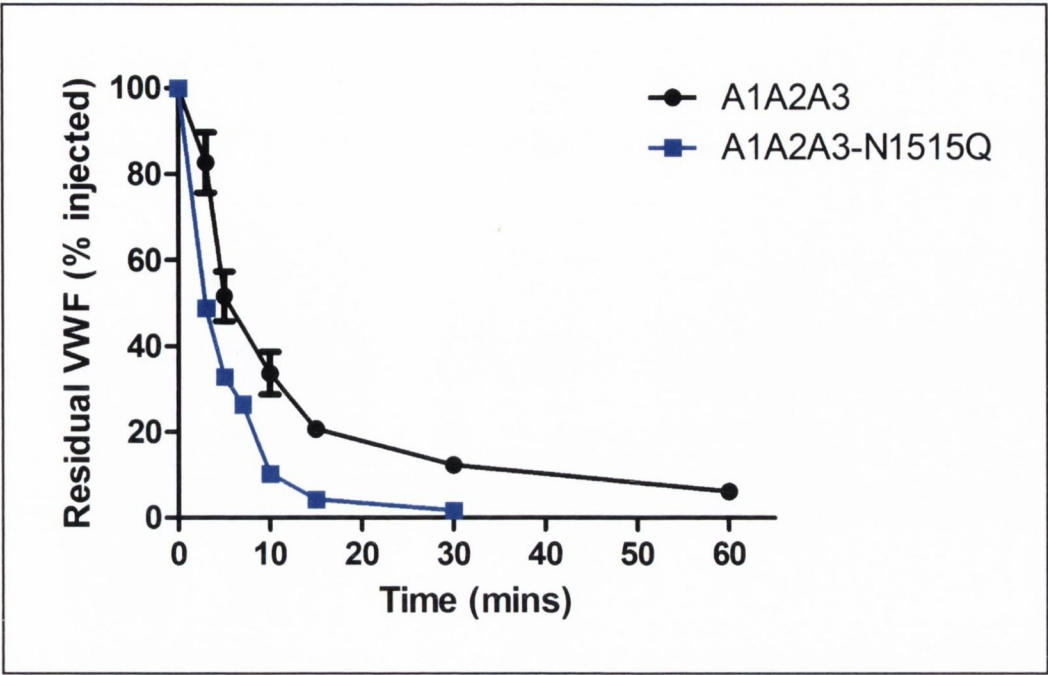


Figure 6.6 N1515Q accelerates the clearance of A1A2A3 in VWF^{-/-} mice
Data graphed as the mean $\pm$ SEM.

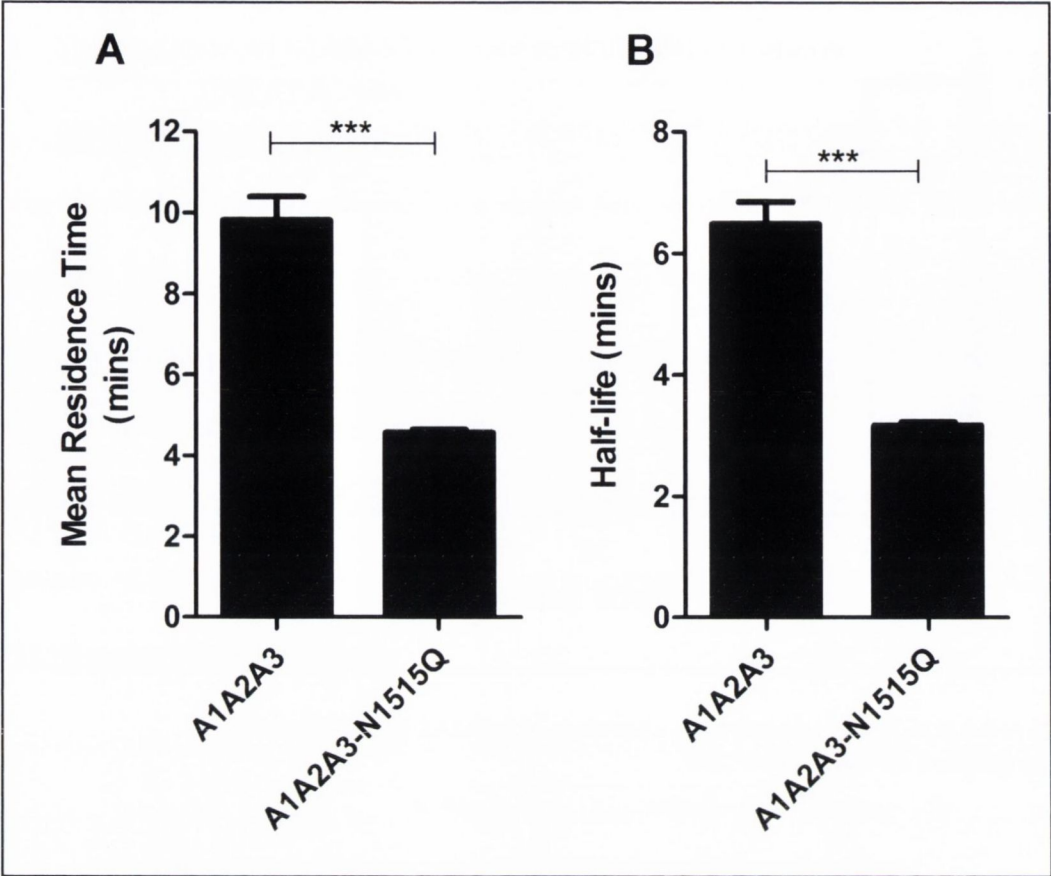


Figure 6.7 Clearance parameters for A1A2A3 VWF and A1A2A3-N1515Q
(A) Mean residence time values
(B) Plasma half-life values
Data are graphed as the mean $\pm$ SEM.

6.4 Specific glycan sites in A1A2A3 can contribute to macrophage-mediated clearance

The enhanced clearance of VWF-N1515Q is mediated through a macrophage-dependent manner. Thus we assessed the contribution of macrophages in regulating the accelerated clearance of an A1A2A3 fragment containing N1515Q. Clodronate-induced macrophage depletion resulted in a significantly prolonged survival of A1A2A3-N1515Q. For example, at 10 minutes, macrophage depletion enhanced the amount of residual VWF from $10.3 \pm 1.4\%$ to $35.4 \pm 4.8\%$ (figure 6.8). Additionally after 15 minutes, A1A2A3-N1515Q survival was improved from $4.3 \pm 0.6\%$, in the absence of clodronate treatment, to $22.1 \pm 1.1\%$ following clodronate administration (figure 6.8, $p < 0.05$). Collectively therefore our data demonstrate that the introduction of the N1515Q mutation in both full length VWF and the truncated A1A2A3 VWF fragment result in enhanced clearance through a macrophage-mediated mechanism.

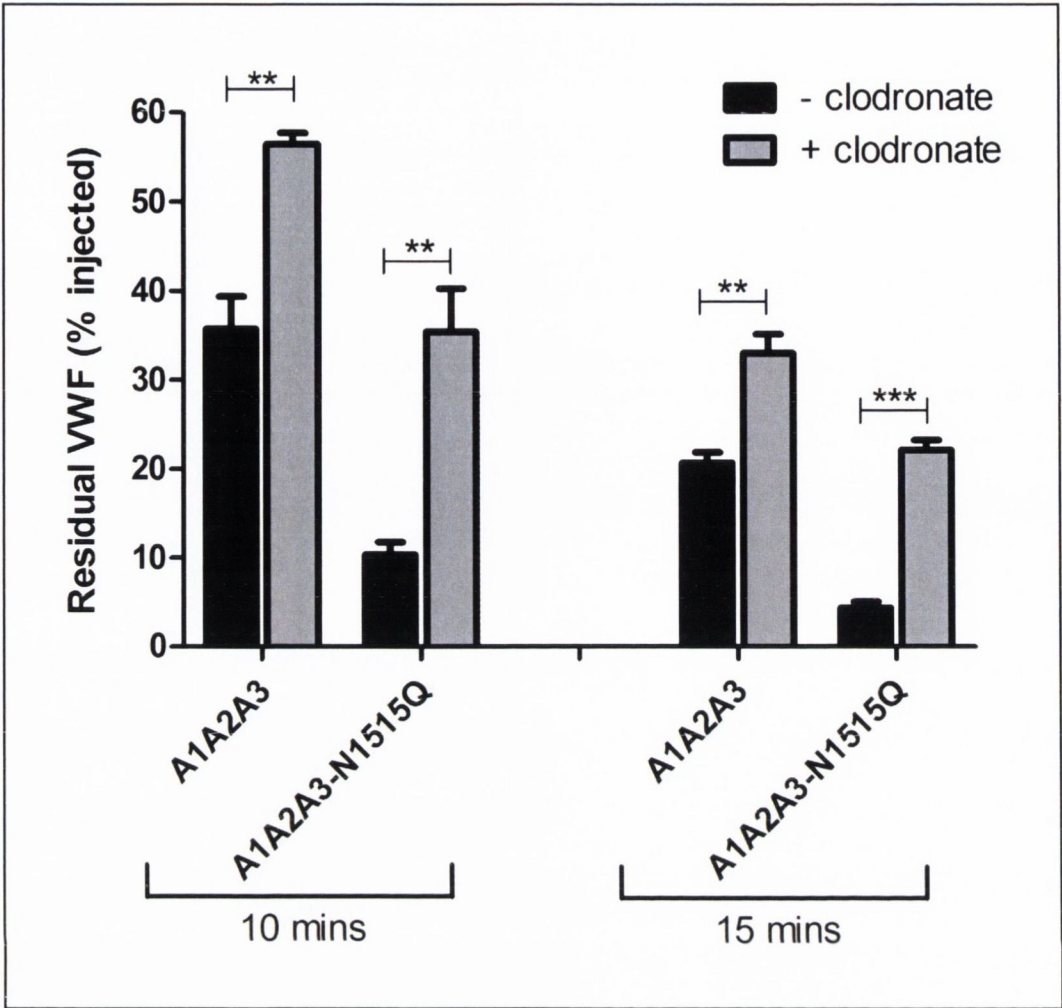


Figure 6.8 Macrophage depletion prolongs the survival of A1A2A3 and A1A2A3-N1515Q VWF in VWF^{-/-} mice
Data graphed as the mean $\pm$ SEM.

6.5 Specific glycan sites in A1A2A3 VWF modulate *in vitro* macrophage

binding

To further examine the biological mechanisms mediating the enhanced clearance of A1A2A3-N1515Q via macrophages we assessed binding to THP-1 macrophages *in vitro*. We examined the binding of A1A2A3 VWF and A1A2A3-N1515Q in the presence or absence of 1mg/mL ristocetin. In the absence of ristocetin, minimal A1A2A3 VWF binding was observed (figure 6.9). In contrast, introduction of the N1515Q glycan mutation resulted in significant spontaneous binding, even in the absence of ristocetin (figure 6.9). The removal of this glycan may result in conformational changes that reveal critical regions in A1A2A3 VWF which promote THP-1 binding. In this context, it is interesting that N1515Q enhances VWF-THP-1 binding to a similar extent to that observed in the presence of ristocetin. Moreover, the binding of A1A2A3-N1515Q was only enhanced to a small degree following addition of ristocetin. As demonstrated in Chapter 5, maximal macrophage binding of A1A2A3 VWF was observed following PNGase treatment. In keeping with this, PNGase treatment of A1A2A3-N1515Q also further increased THP-1 binding (figure 6.9). Taken together our results specify a key role for the VWF glycan at N1515 in regulating *in vitro* macrophage binding but also suggest that the glycan at N1574 may also be important in modulating VWF macrophage binding.

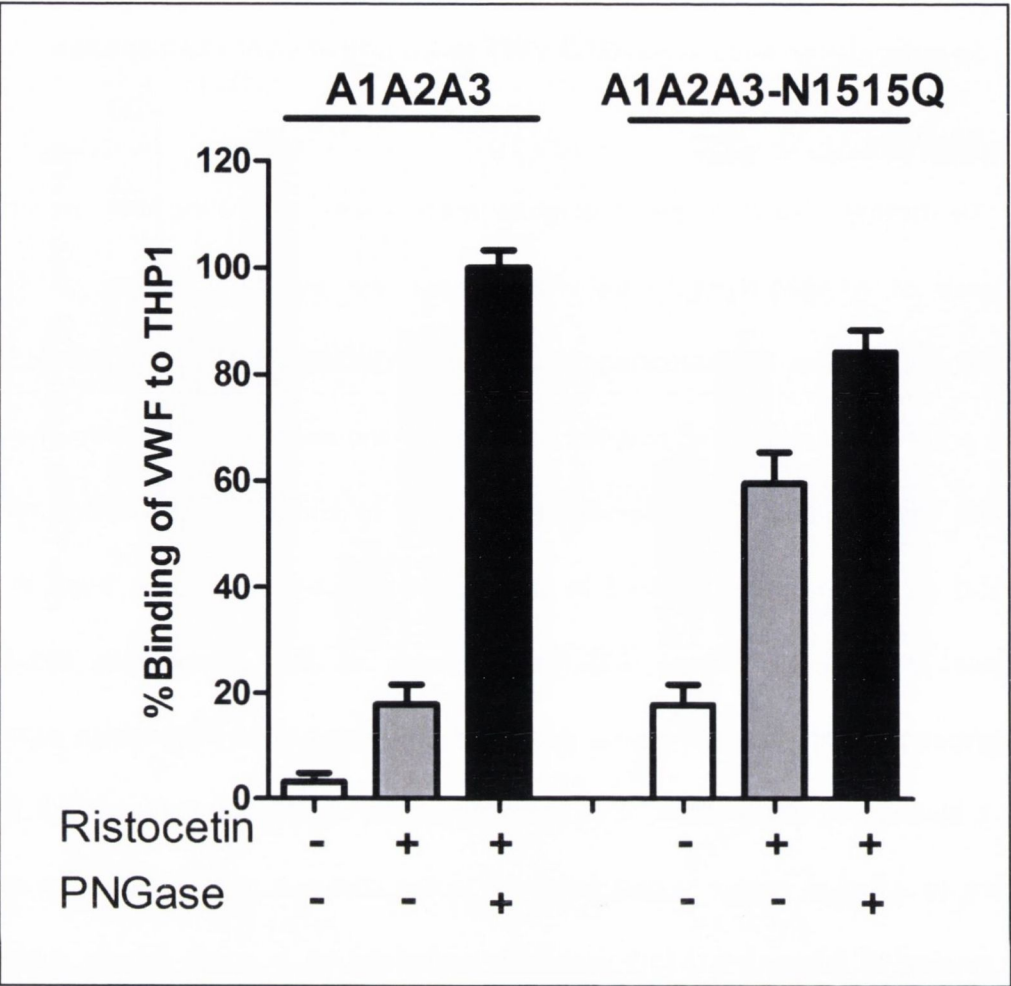


Figure 6.9 N-linked glycans modulate binding of A1A2A3 VWF to THP-1 macrophages *in vitro*
Data are expressed as a percentage of the mean maximum binding $\pm$ SEM.

6.6 Glycosylation of A1A2A3 VWF modulates *in vitro* macrophage binding through conformational changes in A2

McKinnon *et al* have previously demonstrated that PNGase treatment of VWF A2 domain resulted in enhanced ADAMTS13 mediated proteolysis. Moreover PNG-rVWF facilitates ADAMTS13 cleavage in the absence of urea (McKinnon *et al*, 2008). They concluded that the N-linked glycans within the A2 domain may confer stability to the globular structure of the molecule. We sought to investigate if removal of the glycans in the A2 domain may induce conformational changes in VWF promoting macrophage binding.

Within A1A2A3, the A1 and A3 domains are restrained by long range intra-chain disulphide bonds which results in ~186 amino acid loops (figure 6.10). Consequently these domains are limited in their ability to undergo conformational changes under shear stress. This is thought to be essential in order to maintain structural integrity for GPIIb α binding and collagen binding. In contrast the A2 domain is not constrained by a disulphide bridge as this domain only contains a vicinal disulphide bond (C1669-C1670) at the C-terminal end. Thus the A2 domain is considered the least stable VWF domain and the most susceptible to unfolding (Auton *et al*, 2007). This is physiologically relevant as it allows dynamic exposure of the cryptic scissile bond (Y1605-M1606) regulating ADAMTS13 proteolysis. To structurally constrain the A2 domain, in a manner homologous to A1 and A3, we introduced a long range intra-chain disulphide bridge. This was achieved by site-directed mutagenesis of N1493C enabling a disulphide bond between C1493 and C1669. Additionally the free cysteine at C1670 was mutated to serine to allow maximum homology to A1 and A3. We termed this variant

A1A2A3 cysteine clamp (A1A2A3-CC). This VWF mutant has previously been described by Baldauf *et al* who assessed its role in modulating ADAMTS13 proteolysis (Baldauf *et al*, 2009). They found the presence of the long range disulphide bridge within A2 completely abolished ADAMTS13 proteolysis as similarly seen with a Δ A2-VWF variant. To investigate if a structurally confined A2 could modulate macrophage binding and influence the effect of glycosylation on this binding we firstly generated an A1A2A3 VWF variant containing the cysteine clamp (A1A2A3-CC). The ability of A1A2A3-CC to interact with macrophages *in vitro* was then assessed as before.

We observed that similar to wild-type A1A2A3 VWF, A1A2A3-CC bound to THP-1 macrophages in the presence of ristocetin (figure 6.11). Importantly however, although PNGase treatment significantly enhanced the binding of A1A2A3 VWF, PNGase digestion of A1A2A3-CC only resulted in a small increase in macrophage binding. Consequently these findings suggest that the enhanced binding that results from removal of the N-linked glycans in A2 is mediated via unfolding of the A2 domain. Thus, this PNGase effect on macrophage binding is in large part negated in the presence of a structurally constrained A2 domain mutant.

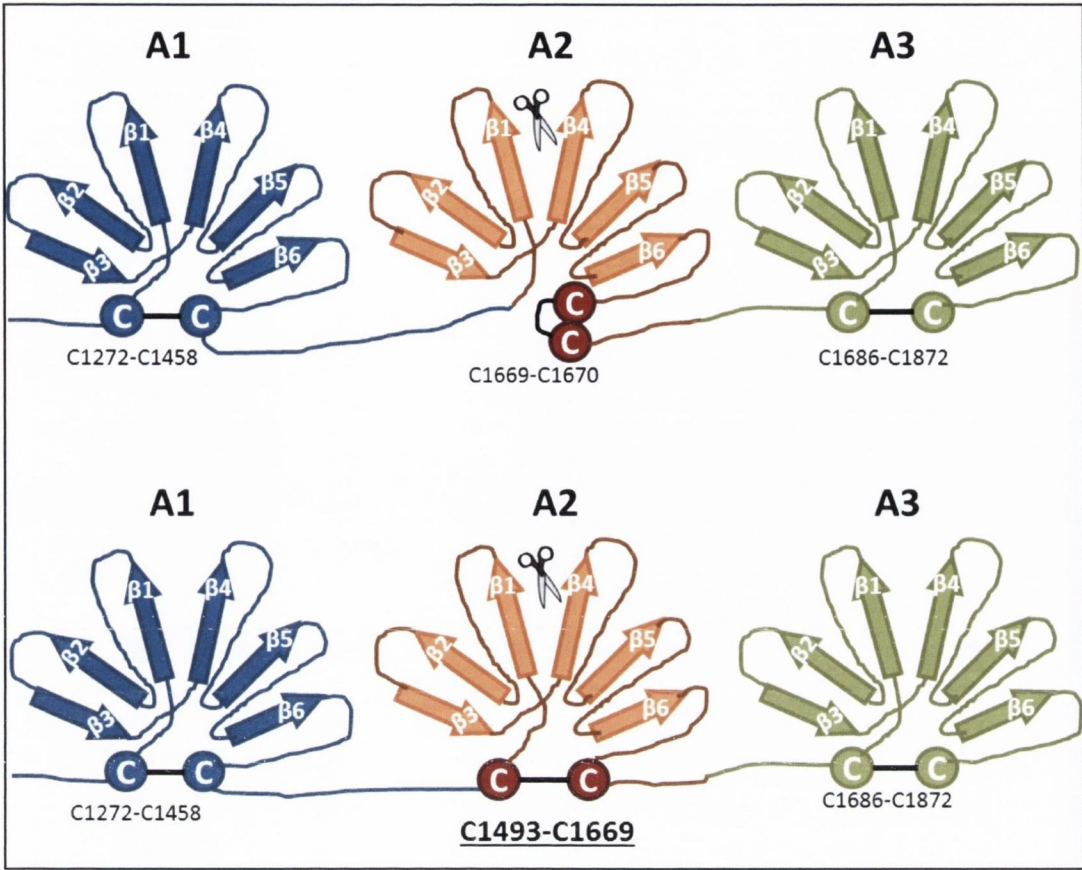


Figure 6.10 A schematic representation of A1A2A3-cysteine clamp variant. The intra-chain disulphide bridges in A1 and A3 are highlighted in blue and green (C1272-C1458 and C1686-C1872). The native A2 domain possesses only a vicinal disulphide bond depicted in red (C1669-C1670). The lower panel illustrates the A1A2A3 cysteine clamp variant with the novel disulphide bridge at C1493-C1669. The variant restricts the unfolding of the A2 domain, preventing ADAMTS13-mediated proteolysis of the cryptic cleavage site within A2 (indicated by the scissors).

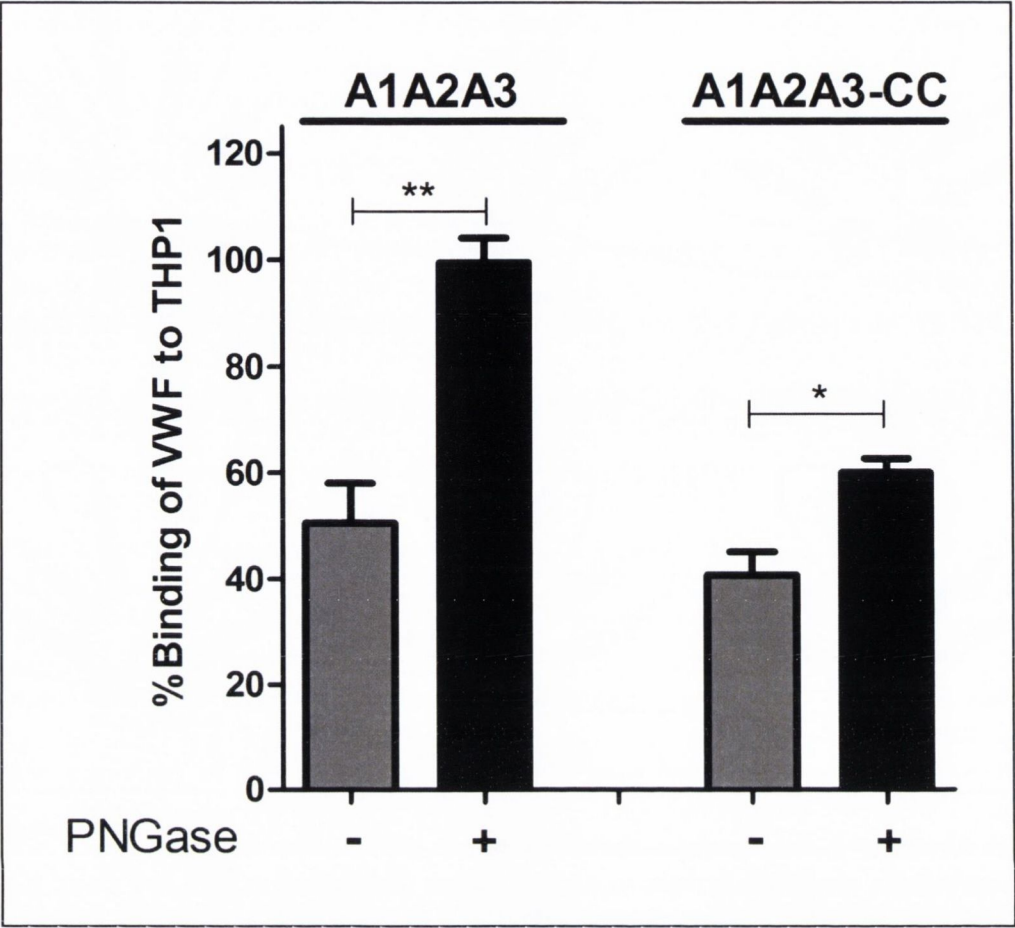


Figure 6.11 A structurally restrained A2 domain (A1A2A3-CC) attenuates binding of PNG-A1A2A3 to macrophages
Data are expressed as a percentage of the mean maximum binding $\pm$ SEM.

6.7 The clearance of A1A2A3 is regulated by N-linked glycan sites inducing unfolding of A2

Since restricting A2 domain unfolding limits the effect of glycosylation on macrophage binding, we further investigated the importance of A2 domain conformation upon macrophage-mediated clearance *in vivo*. As previously demonstrated, A1A2A3-N1515Q is cleared rapidly in VWF^{-/-} mice. The cysteine clamp mutation was therefore introduced into this construct to create A1A2A3-CC-N1515Q. The clearance of A1A2A3-CC-N1515Q was examined alongside A1A2A3-N1515Q and A1A2A3 as controls. The clearance parameters for these variants are outlined in table 6.4.

	% Recovery	t _{1/2} (mins)	MRT (mins)
A1A2A3 VWF	82.7 ± 7.0	6.4 ± 0.3	9.2 ± 0.5
A1A2A3-N1515Q	48.8 ± 1.4	3.1 ± 0.05	4.5 ± 0.1
A1A2A3-CC-N1515Q	85.5 ± 8.8	7.8 ± 0.65	11.3 ± 0.9

Table 6.4 Clearance parameters for A1A2A3 and A1A2A3-N1515Q with and with a cysteine clamp mutation (CC) in VWF^{-/-} mice
Values are expressed as mean ± SEM.

In keeping with our THP-1 *in vitro* binding results, the introduction of the cysteine clamp into A1A2A3 did not significantly influence its rate of clearance *in vivo* compared to wild-type A1A2A3. In contrast, the presence of the cysteine clamp in the A2 domain served to ablate the rapid clearance associated with A1A2A3-N1515Q (figure 6.12). As described in section 6.3, the N1515Q substitution resulted in a 2-fold decrease in the MRT and t_{1/2} of A1A2A3 VWF. In contrast introduction of the cysteine

clamp led to full correction of the MRT and $t_{1/2}$ of A1A2A3-N1515Q VWF to levels comparable with wild-type A1A2A3 (11.3 ± 0.9 minutes for MRT and 7.8 ± 0.65 minutes for $t_{1/2}$ for A1A2A3-CC-N1515Q, figure 6.13). Cumulatively these data confirm our *in vitro* findings and suggest that the glycans within the A2 domain (specifically N1515) influence VWF survival through conformational changes in the A2 domain.

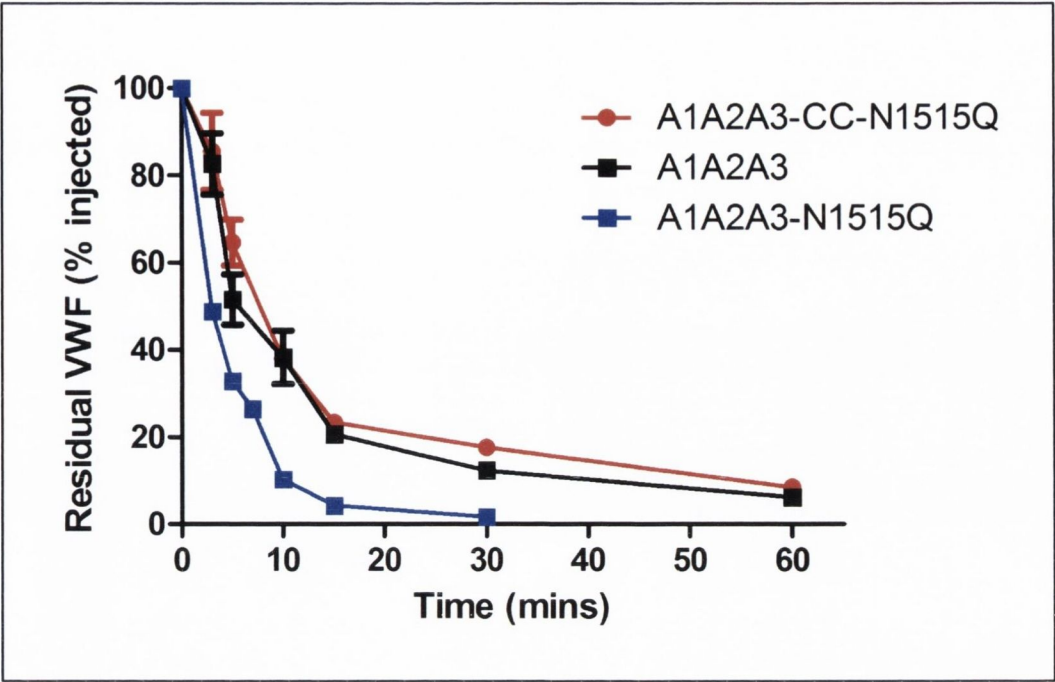


Figure 6.12 A structurally constrained A2 construct (A1A2A3-CC) inhibits the rapid clearance of A1A2A3-N1515Q in VWF^{-/-} mice
Data graphed as the mean $\pm$ SEM, in some cases the error bars are too small to be seen.

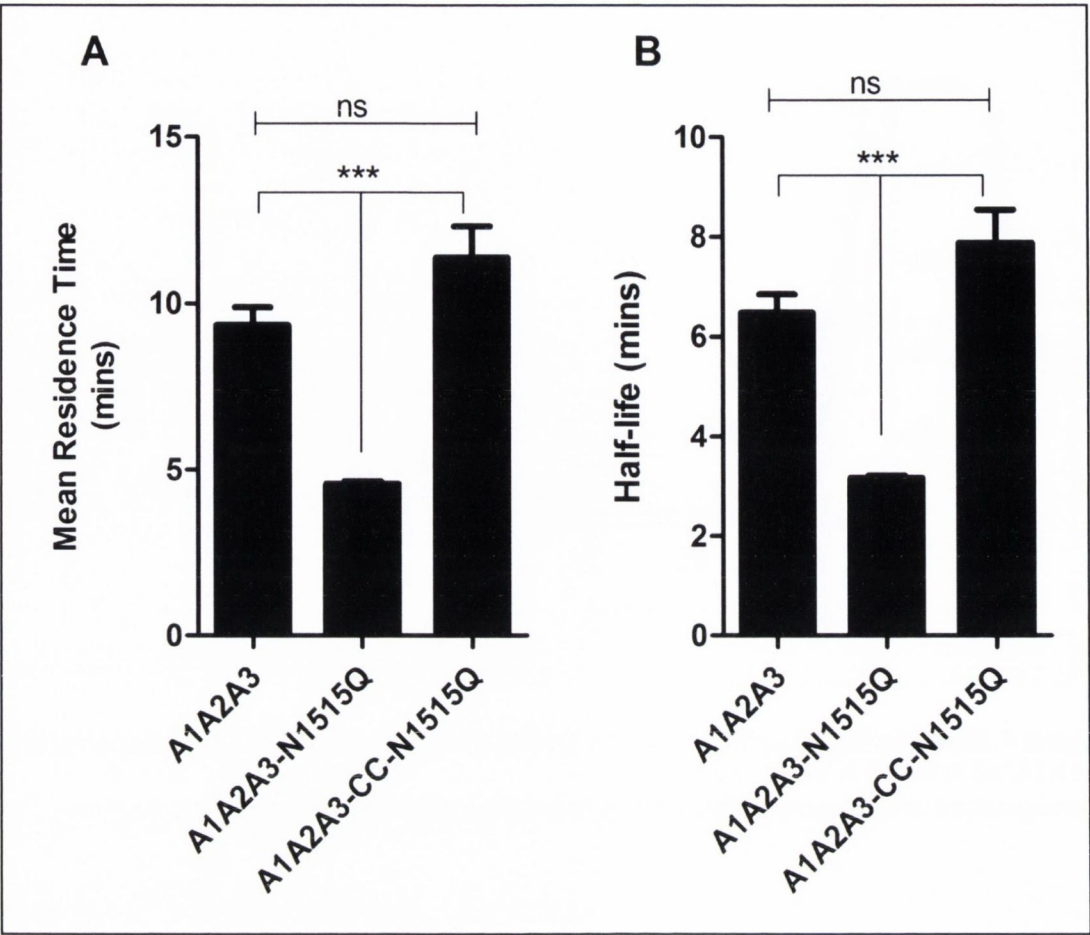


Figure 6.13 Clearance parameters for A1A2A3 VWF, A1A2A3-N1515Q and A1A2A3-CC-N1515Q

(A) Mean residence time values

(B) Plasma half-life values. Data are graphed as the mean $\pm$ SEM.

6.8 N-linked glycosylation and A2 domain conformation influence clearance of full length VWF

To further examine a potential role for the A2 domain conformation in modulating clearance of full length VWF, the cysteine clamp mutation was also inserted into our full length rVWF construct (rVWF-CC) and clearance assessed in VWF^{-/-} mice. The clearance parameters are outlined in table 6.5.

	% Recovery	t _{1/2} (mins)	MRT (mins)
rVWF	72.0 ± 5.9	7.8 ± 0.4	11.2 ± 0.6
rVWF-CC	101.6 ± 3.6	6.5 ± 0.4	9.4 ± 0.5

Table 6.5 Clearance parameters for rVWF and rVWF with the cysteine clamp mutation in A2 (rVWF-CC) in VWF^{-/-} mice
 Values are expressed as mean ± SEM.

The presence of the cysteine clamp mutation in the A2 domain of full length rVWF did not significantly alter the rate of clearance compared to wild-type rVWF (figure 6.14 and 6.15). However the percentage recovery of rVWF-CC at 3 minutes was significantly increased compared to rVWF (101.6 ± 3.6% vs 72.0 ± 5.9%, respectively *p*<0.01). The reason for this enhanced recovery remains unclear, but raises the possibility that A2 domain conformation may be particularly important in regulating the initial phase of VWF clearance *in vivo*. Collectively these results demonstrate that preventing the unfolding of the A2 domain does not influence its rate of clearance in this mouse model.

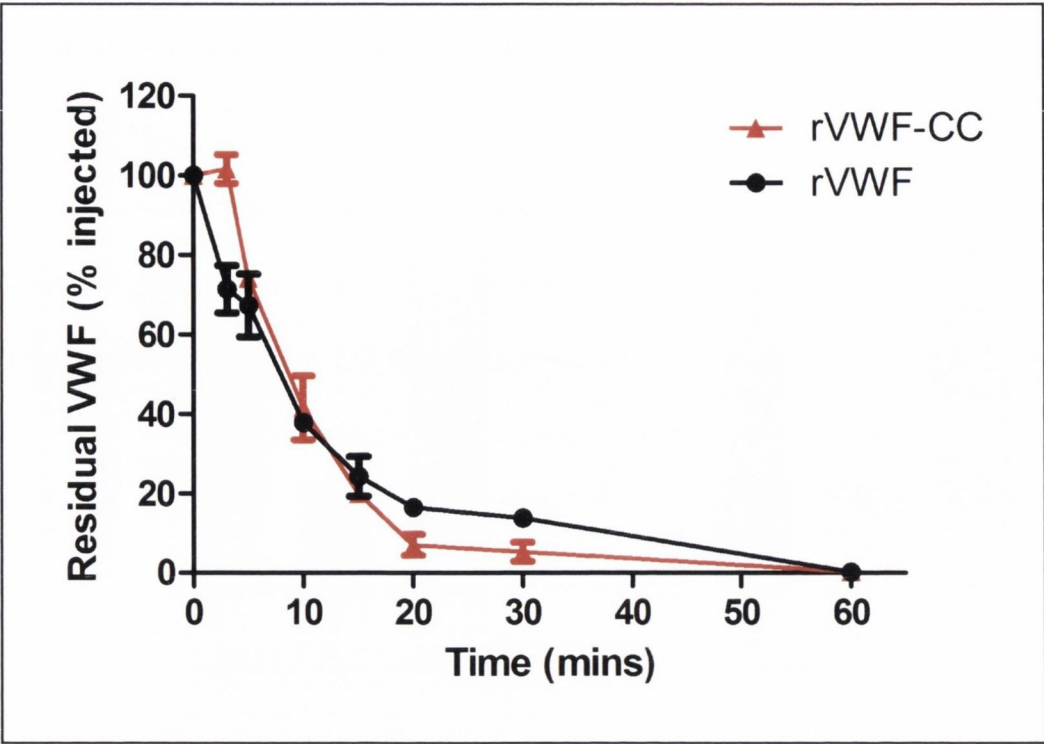


Figure 6.14 A structurally restricted A2 domain does not significantly alter the rate of clearance of full length rVWF in $VWF^{-/-}$ mice
Data graphed as the mean $\pm$ SEM.

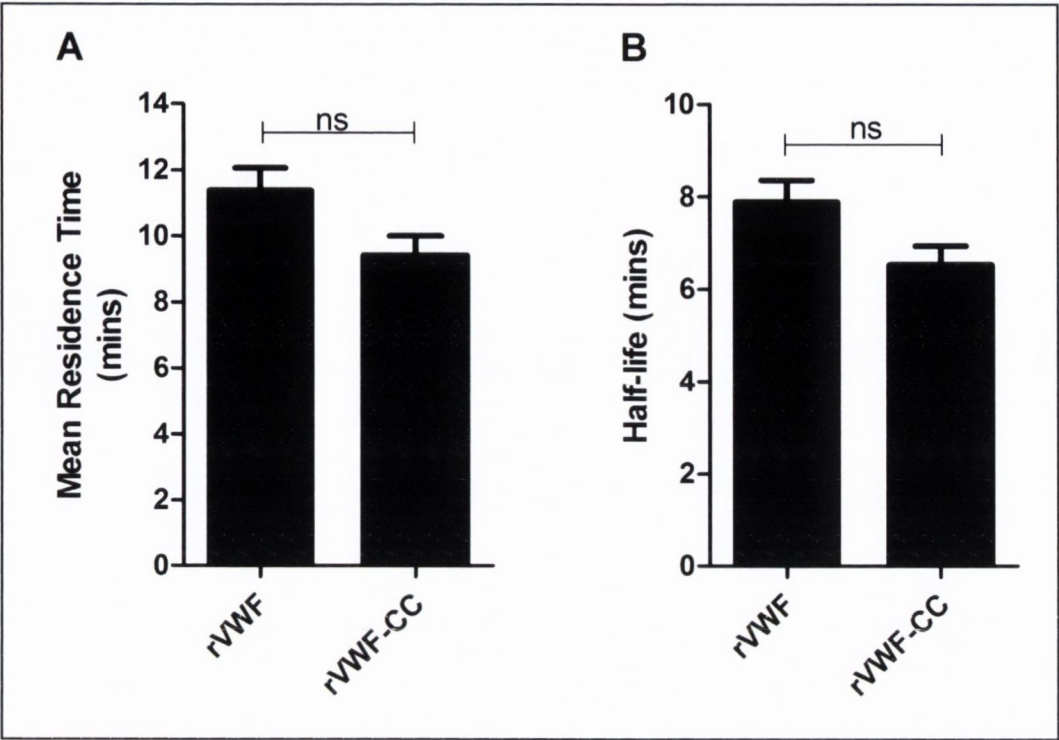


Figure 6.15 Clearance parameters for rVWF compared with rVWF-CC
(A) Mean residence time values
(B) Plasma half-life values
Data are graphed as the mean $\pm$ SEM.

Interestingly, the introduction of the cysteine clamp mutation into full length VWF-N1515Q (VWF-N1515Q-CC) served to significantly prolong survival (figure 6.16). The calculated clearance parameters for VWF-N1515Q with and without the cysteine clamp mutation are shown in table 6.6.

	% Recovery	$t_{1/2}$ (mins)	MRT (mins)
VWF-N1515Q	50.4 ± 1.9	3.7 ± 0.1	5.3 ± 0.2
VWF-N1515Q-CC	100.4 ± 6.4	6.5 ± 0.4	9.3 ± 0.7

Table 6.6 Clearance parameters for N1515Q and CC-N1515 in VWF^{-/-} mice
Values are expressed as mean ± SEM.

The presence of the cysteine clamp within the A2 domain resulted in an approximately 2-fold increase in $t_{1/2}$ from 3.7 ± 0.1 minutes to 6.5 ± 0.4 minutes. Similarly the MRT for VWF-N1515Q was also enhanced 2-fold (5.3 ± 0.2 minutes to 9.3 ± 0.7 minutes) (figure 6.17). These values are comparable with the $t_{1/2}$ and MRT for rVWF. This suggests that restricting the unfolding of the A2 domain completely reverses the accelerated clearance observed with N1515Q in both full length VWF and truncated A1A2A3 VWF. We hypothesise that removal of the glycan at N1515 induces conformational changes in A2 resulting, and thereby leads to enhanced binding to macrophages and reduced circulatory survival *in vivo*.

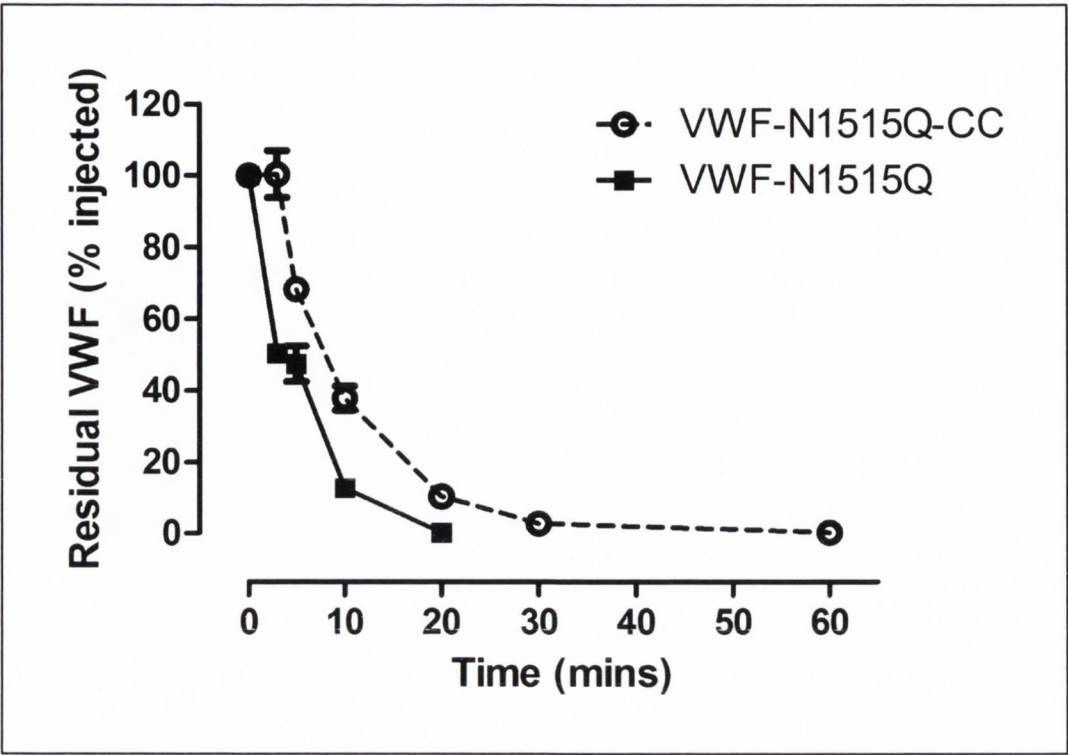


Figure 6.16 Cysteine clamp within A2 inhibits the rapid clearance of VWF-N1515Q in VWF^{-/-} mice
Data graphed as the mean $\pm$ SEM.

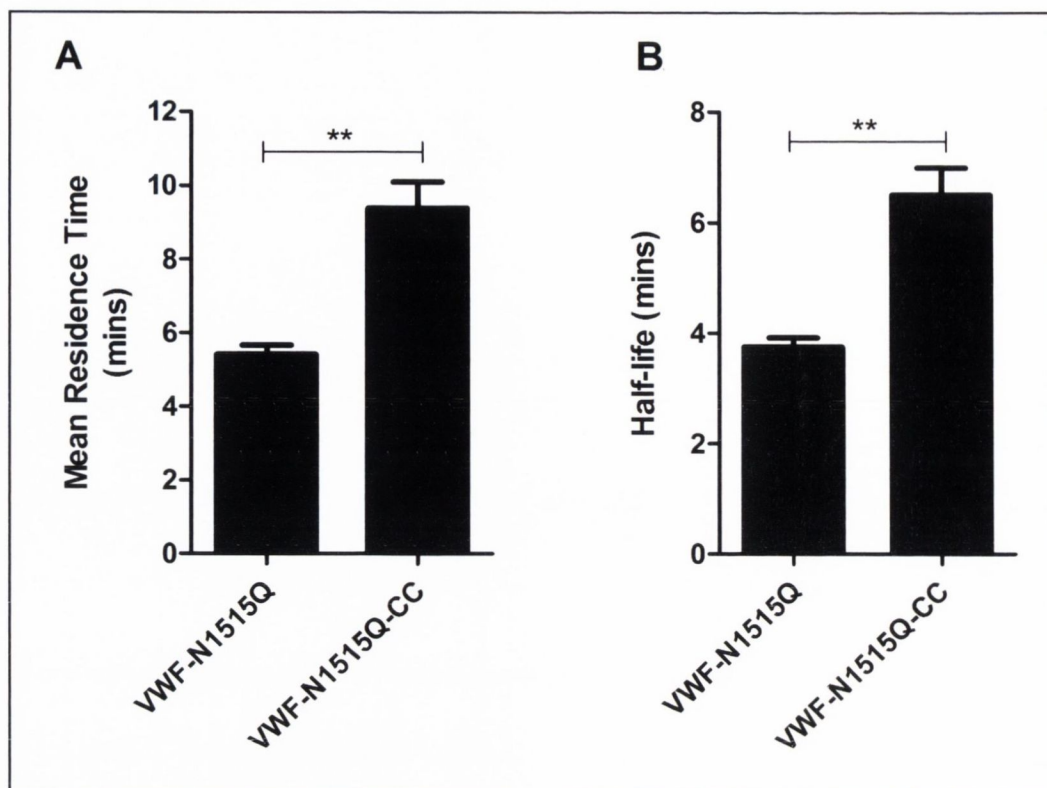


Figure 6.17 Clearance parameters for VWF-N1515Q compared with VWF-N1515Q-CC
(A) Mean residence time values
(B) Plasma half-life values
Data are graphed as the mean $\pm$ SEM.

6.9 Discussion

Our novel findings in Chapter 5 demonstrated that VWF glycosylation is essential in protecting VWF against accelerated clearance *in vivo*. Understanding the mechanism of how VWF glycosylation mediates this effect is important since enhanced plasma clearance of VWF has been shown to play a role in the etiology of both type 1 and type 2 VWD. Further to this, only 50% of VWD type 1 patients with plasma levels 30-50% of normal have mutations in the *VWF* gene. Linkage analysis has demonstrated that causative factors for reduced plasma VWF levels in this cohort lies outside the VWF locus (James *et al*, 2006; Eikenboom *et al*, 2006). Alterations in VWF glycosylation, and/or variation in plasma VWF clearance, constitute potential mechanisms through which these reduced plasma VWF levels could be explained. In humans, this is exemplified by the ABO locus, regulating the clearance of VWF by directly altering its glycosylation.

6.9.1 VWF glycosylation regulates its macrophage-mediated clearance

VWF clearance is markedly accelerated in the absence of the glycan at N1515 within the A2 domain. Mass spectrometry analysis of this glycan site in human pd-VWF has revealed it is occupied by complex type heavily branched (bi- up to tetra-antennary) glycan structures (Canis *et al*, 2012). Moreover N1515 has been shown to carry fucosylated species. Consequently this site is likely to feature ABO(H) antigen determinants in human plasma. Given that the glycan at N1515 protects VWF from rapid clearance *in vivo*, we speculate that ABO(H) determinants expressed at this site in pd-VWF may potentially contribute to regulate ABO-dependent VWF clearance. The

increased branching of the glycan chains due to the presence of ABH antigens may increase the potential shielding effects of the glycan chain, sterically hindering interactive clearance sites in VWF. In support of this, the absence of ABH structures as seen in blood group Bombay VWF results in significantly reduced plasma levels (O'Donnell *et al*, 2005). Moreover Bombay VWF displays enhanced, conformational-dependent, susceptibility to ADAMTS13 proteolysis, suggesting that the absence of ABH antigens allows VWF to adopt a more permissive conformation for cleavage. This may also result in a conformation which facilitates enhanced clearance.

We observed that the rapid clearance of VWF-N1515Q glycan variant was mediated via macrophages. Moreover its effect was localised within the A1A2A3 domains of VWF, since the presence of N1515Q mutation within a truncated A1A2A3 VWF fragment served to enhance its clearance to a similar extent to that of full length rVWF-N1515Q. VWF glycan sites are highly conserved across species suggesting possible functional roles. No patient mutations have been described that result from the direct loss of VWF N-linked glycan sites. Of note Haberichter *et al* have reported a novel patient mutation N1231S, which is a potential N-linked glycan site for VWF. The elevated VWFpp/VWF:Ag ratio associated with this mutation resulted in it being classified as VWD type1C (Haberichter *et al*, 2013). However conflicting reports exist on whether the consensus N-linked glycan sequence at position N1231 is occupied (Carew *et al*, 1990; Canis *et al*, 2012). In contrast Haberichter *et al* have described a patient homozygous for N528S which predicts the introduction of an additional N-linked glycan site within the D2 domain of VWF (Haberichter *et al*, 2010). That patient was classified as type 2A VWD. Importantly, two patients have been described with the mutation S1517R (Castaman *et al*, 2013). This mutation is of particular interest as it

disrupts the consensus sequence for N-linked glycosylation at 1515. Both patients are heterozygous for S1517R substitution but were reported to have plasma VWF:Ag levels as low as 15% of normal.

In addition qualitative changes in VWF glycosylation have also been reported in a cohort of individuals with abnormally low plasma VWF:Ag levels. Lectin analyses have demonstrated that these patients have significant increases in galactose or N-acetylgalactosamine expression on VWF glycans. The increased exposure of these carbohydrates may result in accelerated clearance via lectin receptor ASGPR (Ellies *et al*, 2002). Taken together these findings confirm the importance of both quantitative and qualitative changes to VWF glycosylation *in vivo* and may be of direct clinical significance.

6.9.2 VWF A domain glycosylation influences *in vivo* survival via altered macrophage binding

Macrophages represent the critical mediators underlying the accelerated clearance phenotype of N1515Q *in vivo*. Moreover the effect of this glycan can be localised to the A1A2A3 domains of VWF. Thus, loss of the N1515 glycan significantly enhances VWF binding to macrophages *in vitro* which can occur in the absence of ristocetin. In fact the extent of A1A2A3-N1515Q binding was equivalent to that observed with wild-type A1A2A3 in the presence of ristocetin, suggesting that the N1515Q mutation converts VWF into a conformation more permissive for macrophage interaction. We therefore hypothesise that the N1515 glycan functions to maintain VWF in its globular configuration shielding macrophage interactive sites within VWF. In keeping with this, Mckinnon *et al* described that the removal of the neighbouring

glycan in A2 (N1574Q) promoted conformational changes in A2 and rendered VWF more susceptible to ADAMTS13 proteolysis (McKinnon *et al*, 2008).

Ristocetin is known to induce GPIb α -VWF-dependent platelet aggregation by exposing the cryptic A1 domain in VWF, and serves to mimic the effect of shear stress. Ristocetin has two described binding sites flanking the platelet-binding A1 domain at Cys1237–Pro1251 and Glu1463–Asp1472 (Girma *et al*, 1990). Recently it has been demonstrated that the effect of ristocetin is not limited to the A1 domain. Chen *et al* described a role for ristocetin inducing conformation changes within the adjacent A2 domain, exposing buried methionine residues for oxidation and additionally enhancing ADAMTS13 cleavage (Chen *et al*, 2012). They reported ristocetin also changed the conformation of the A1 domain, but the extent of methionine oxidation in A1 was much less than that observed in the A2 domain. This is consistent with the concept that the A2 domain can undergo extensive unravelling.

A number of putative macrophage receptors for VWF have been described including lectin and non-lectin receptors. For example, LRP1, β 2 integrins, ASGPR and Siglec 5 have been highlighted as candidate macrophage receptors. The relative physiological contribution of these candidate receptors in regulating VWF clearance remains unclear. For example, genetic inactivation of both ASGPR and LRP1 in mice results in only a 1.5-fold increase in plasma VWF levels (Grewal *et al*, 2008; Bovenschen *et al*, 2003). Consequently VWF clearance is likely to be mediated by a number of receptors. The rate of VWF-N1515Q clearance is remarkably similar to that of PNG-rVWF. Moreover the rapid removal of VWF-N1515Q is almost completely corrected following macrophage depletion. We therefore hypothesize that these variants are likely to be cleared via a glycan-independent pathway, possibly involving

non-lectin receptors on macrophages. Casari *et al* have demonstrated a dominant role for macrophages in the clearance of gain of function VWD type 2B mutants (Casari *et al*, 2013a). Moreover additional studies have demonstrated that 2B mutants bind spontaneously to LRP1 *in vitro* in the absence of ristocetin or shear stress (Wohner *et al*, 2013). The enhanced affinity for LRP1 may offer an explanation as to why these mutants are cleared rapidly via macrophages *in vivo*. In light of this data it is tempting to speculate a similar phenomenon may occur with the N1515Q mutation. Further studies will be required to assess if macrophage-LRP1 is important in modulating the enhanced clearance of VWF-N1515Q.

6.9.3 VWF glycosylation in the A domain modulates clearance through conformational changes

In support of our hypothesis that N1515Q mutation induces conformational changes within A1A2A3, *in silico* glycan modelling (section 5.4) revealed the glycans within A2 are both of significant size, and thus capable of exerting considerable shielding effects. However due to the presence of disulphide bridges in A1 and A3, only the A2 domain is predisposed to significant unravelling. Recent thermodynamic studies have demonstrated that the A2 domains unfolds progressively from its C-terminus at an approximate unfolding force of 21pN and has an unfolded contour length of 60-70nm (Ying *et al*, 2010). Studies have described the importance of the vicinal disulphide in A2 stabilisation (Luken *et al*, 2010). Structural analysis demonstrated that the disulphide bond in A2 interacts with the hydrophobic core to stabilise the domain. However under shear stress the bond also regulates the initial unfolding of the C-

terminal helical end of A2 exposing the cryptic ADAMTS13 exosite (figure 6.18) (Luken *et al*, 2010).

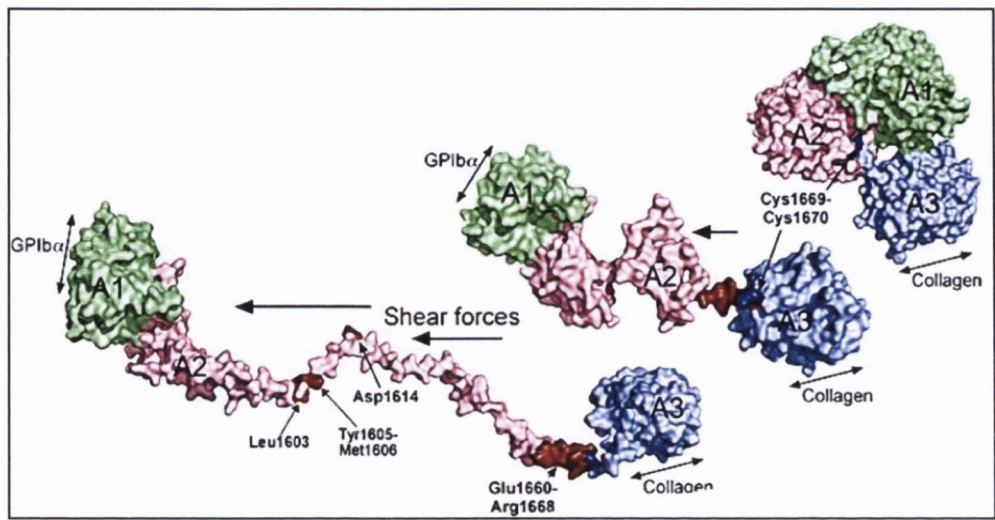


Figure 6.18 Molecular modelling illustration of A1A2A3 VWF
A1, A2 and A3 domains are highlighted in green, pink and blue, respectively. The vicinal disulphide bond in A2 Cys1669-Cys1670 (dark blue) is hidden in the folded A2 domain. Shear forces unravel A2 to expose the face of the A1 domain containing the GPIbα sites, cryptic ADAMTS13 binding and cleavage sites (red). [Adapted from Crawley *et al*, 2011]

To examine the effect of A2 conformation on VWF clearance, a mutant A2 domain resistant to unfolding was constructed. This VWF mutant has previously described to render VWF insensitive to ADAMTS13 cleavage *in vitro* (Baldauf *et al*, 2009). The amino acid residue N1493 was chosen to mutate to a cysteine as molecular modelling revealed that its side chain is in the correct orientation to interact with the corresponding cysteine at C1669. Additionally to remove the remaining unpaired cysteine, C1670 was mutated to serine. This created maximal homology between A1, A2 and A3, each containing a long range intra-chain disulphide loop of approximately 186 amino acids. Work by Luken *et al* has demonstrated that the introduction of this cysteine clamp mutation within A2 and the removal of the native vicinal disulphide

does not disrupt the circular dichromatic spectra of VWF indicating that the overall global structure of the glycoprotein is unaffected (Luken *et al*, 2010).

Constraining the A2 domain did not serve to significantly alter the rate of clearance of either wild-type A1A2A3 VWF or full length rVWF with intact A2 glycans. However, the cysteine clamp mutation had a very significant effect on the survival of glycan variant N1515Q in both of these VWF constructs. The presence of the cysteine clamp resulted in complete inhibition of the rapid clearance observed with A1A2A3-N1515Q and VWF-N1515Q. Our *in vitro* findings suggest that the corrected clearance rate of these glycan variants with the cysteine clamp may be mediated by reduced affinity for macrophages. Enzymatic removal of the glycans within A1A2A3-CC resulted in significantly reduced binding to THP-1 macrophages compared to glycan removal of wild-type A1A2A3. Collectively these data suggest that N1515Q mutation requires conformational changes within the A2 domain and subsequent exposure of critical macrophage binding residues to mediate its effect on VWF survival *in vivo*.

In conclusion, we report the novel observation that VWF A domain glycosylation can influence its survival via macrophage-dependent clearance. In particular the glycan site N1515 plays a crucial role in protecting VWF against clearance by macrophage. Our data suggests that this glycan serves to shield a critical macrophage interactive site within VWF A2 domain. Importantly these glycosylation effects may be clinically relevant given the role of enhanced clearance of VWF in patients with type 1 VWD.

7 Conclusions

VWD is the most common inherited bleeding disorder effecting up to 1% of the general population. The most common type of VWD is caused by a partial quantitative deficiency. Reduced VWF plasma levels can be caused by defects in synthesis and secretion. Alternatively, enhanced circulatory removal of VWF has also been identified as a pathogenic mechanism in type 1 VWD (Haberichter *et al*, 2008). Interestingly, up to 40% of type 1 VWD individuals do not demonstrate a causative mutation within their *VWF* gene (Eikenboom *et al*, 2006). Clearly these findings suggest that other factors, including glycosylation, may play critical roles in modulating plasma VWF levels.

7.1 VWF N-linked glycans and terminal sialic acid critically regulate its interaction with galectin-1

In keeping with the previous report of Saint-Lu *et al*, 2012 we confirmed that galectin-1 bound to pd-VWF in a dose-dependent manner. Systematic modification of the glycosylation profile of VWF demonstrated that this interaction with galectin-1 was principally mediated by the N-linked glycans of VWF. In particular terminal α 2-6 linked sialic acid on VWF glycans served to negatively modulate galectin-1 binding by masking exposure of sub-terminal galactose which is known to constitute a critical galectin-1 ligand. Interestingly, galectin-1 also demonstrated significantly enhanced binding for blood group AB compared to blood group O VWF. In addition to the critical role of N-linked glycans in modulating galectin-1 binding, VWF multimerisation was also found

to be important in regulating galectin-1 binding. Thus HMWM VWF demonstrated significantly enhanced galectin-1 binding compared to LMWM VWF. This enhanced interaction with HMWM likely reflects that fact that these VWF multimers have extensive glycan clustering.

Modification of VWF A domain conformation, with either ristocetin or following introduction of a gain of function type 2B-like VWD mutation, both resulted in markedly enhanced galectin-1 binding. These findings suggest that the A domain conformation and the N-linked glycans expressed in this region at N1515 and N1574 may be particularly important in regulating the VWF/galectin-1 interaction. This hypothesis was supported by site-directed mutagenesis of N1515 and N1574, respectively and also by experiments performed by using domain truncated fragments of VWF. Importantly an A1A2A3 VWF domain truncation was shown to bind to galectin-1. Furthermore, this binding was significantly attenuated with the addition of the D'D3 domains to the A1A2A3 VWF truncation. This observation suggests that the D'D3 domains may play a role in shielding galectin-1 binding sites within A1A2A3.

7.2 Galectin-1 and galectin-3 represent novel ligands for human FVIII and influence its procoagulant function

Given the evidence that FVIII glycans influence its biological properties and since the glycan profile of FVIII and VWF share significant similarities, galectin binding to human FVIII was also investigated. Both galectin-1 and galectin-3 bound to rFVIII in a dose-dependent manner. Once again systematic modification of FVIII glycans demonstrated that the N-linked glycans of FVIII were critical mediators for both

galectin-1 and -3 binding. In addition the O-linked glycans of FVIII also contributed significantly to galectin-3 binding. Commercial FVIII concentrates express differential glycosylation profiles since they are expressed in heterologous cell systems. Consequently it was interesting to note that three different FVIII products exhibited significant differences in galectin binding. In particular, BDD-FVIII studies revealed that galectin-3 binding to FVIII is strongly influenced by the N- and O-glycans within the B domain of FVIII. However a novel role for the two high mannose oligosaccharides within the A1 and C1 domains was also identified in supporting galectin-1 binding. Interestingly, despite the high affinity of both galectin-1 and -3 for purified rFVIII, only galectin-1 was capable of interacting with free FVIII within plasma. Further to this, galectin-1, but not galectin-3 served to reduced FVIII cofactor activity in functional assays. The inhibitory effect of galectin-1 on FVIII activity occurred only at high galectin concentrations. These galectin-1 concentrations were significantly in excess of typical levels reported in normal human plasma. However galectin-1 is expressed in EC and intracellular levels are significantly increased upon EC activation (Lotan *et al*, 1994). Moreover galectin-1 is also known to be expressed on platelets and circulates bound to VWF (Romaniuk *et al*, 2012; Saint-Lu *et al*, 2012). Consequently, increased local concentrations of galectin-1 at sites of vascular injury may function to modulate FVIII biology.

7.3 VWF A domains modulate VWF clearance *in vivo* in a macrophage-mediated manner

In keeping with previous reports on the clearance of human VWF in the VWF^{-/-} mouse model, we observed that a domain fragment consisting of the monomeric A1A2A3 VWF was cleared at the same rate as full length multimeric VWF. Furthermore, chemical depletion of macrophages using clodronate liposomes administration significantly inhibited A1A2A3 clearance *in vivo* to a similar extent as that observed with full length VWF. *In vitro* binding assays utilising THP-1 macrophages were established to further characterise the interaction between VWF and macrophages. Interestingly VWF binding to THP-1 macrophages was significantly enhanced in the presence of manganese ions, indicating a possible role for $\beta 2$ integrins in mediating this interaction. Additionally, co-incubation of VWF with ristocetin significantly increased VWF binding to THP-1 macrophages. Ristocetin is known to induce conformational changes in VWF A1 and A2 domains, enhancing affinity for GPIb α and increasing VWF susceptibility to ADAMTS13 proteolysis (Chen *et al*, 2012). These data indicate a role for VWF A domains in modulating macrophage binding. Consistent with our *in vivo* findings, A1A2A3 VWF bound to THP-1 macrophages in a dose-dependent manner. Cumulatively, these data demonstrate that the A1A2A3 domains of VWF contain receptor-binding sites for macrophage-mediated clearance.

7.4 VWF N-linked glycans within the A2 domain influence macrophage-mediated clearance

The VWF A domains express two large complex-type N-linked glycans at N1515 and N1574. Enzymatic removal of these glycans within A1A2A3 VWF was used to assess their contribution in mediating VWF binding to macrophages. Surprisingly, removal of the N-linked glycans dramatically enhanced VWF binding to macrophages. Similarly enzymatic removal of the N-glycans from full length rVWF (PNG-rVWF) also resulted in markedly increased clearance *in vivo*. We hypothesise that removal of the N-glycans on VWF therefore targets it for rapid macrophage-mediated clearance. Moreover this macrophage mediated clearance is regulated through an ASGPR-independent clearance pathway. Interestingly, removal of the N-glycans in a VWF variant lacking the A2 domain did not enhance the rate of VWF clearance. Collectively these data suggest that the N-glycans within the A2 domain play key roles in regulating VWF clearance.

7.5 Specific VWF N-linked glycan sites within the A2 domain regulate VWF clearance in a conformation-dependent manner

In light on the novel role for VWF A2 domain glycans in influencing macrophage binding and clearance *in vivo*, the role of the specific N-glycans at N1515 and N1574 sites was further investigated using site-directed mutagenesis in full length rVWF. First, the role of the glycan at N1515 was examined (VWF-N1515Q). Removal of this individual glycan site markedly reduced VWF half-life *in vivo*. Moreover introduction of this glycan mutation within an A1A2A3 VWF fragment also served to reduce its half-

life. Importantly, macrophage depletion significantly prolonged the clearance of N1515Q in both full length VWF and truncated A1A2A3 VWF. Cumulatively these data suggest that specific removal of the VWF glycan at N1515 enhances macrophage-mediated clearance of VWF via critical sites localised within the VWF A domains.

To further characterise the role of macrophages, VWF binding to THP-1 macrophages *in vitro* was examined. Interestingly A1A2A3-N1515Q bound spontaneously to THP-1 macrophages without the need for ristocetin. These data suggest that removal of the N1515 glycan within A2 may induce conformational changes within VWF A domains and promote macrophage binding. Interestingly, PNGase treatment of A1A2A3-N1515Q served to further enhance macrophage binding *in vitro* highlighting a potential role for the adjacent glycan at N1574 in also regulating the VWF-macrophage interaction.

A structurally constrained A1A2A3 VWF variant was created by introducing a long range intra-chain disulphide bridge within A2, homologous to the A1 and A3 domains, termed cysteine clamp (CC). While PNGase treatment markedly enhanced A1A2A3 VWF binding to macrophages, PNGase treatment of A1A2A3-CC failed to reach maximal binding. These data suggest that the glycans within the A2 domain regulate the VWF-macrophage interaction through conformational changes in the A2 domain. Consistent with these findings, the presence of a structurally restricted A2 domain in VWF-N1515Q and A1A2A3-N1515Q resulting in prolonged survival *in vivo*. Collectively, these results indicate that the VWF glycan mutant N1515Q induces conformational changes within the A2 domain, potentially exposing critical macrophage binding residues to mediate enhanced clearance of VWF *in vivo*. The N-linked glycans within the A2 domain are important not only for regulating VWF clearance but previous work

has demonstrated a role for the adjacent glycan N1574 in influencing ADAMTS13 cleavage. The specific importance of these two individual N-glycans is likely mediated in part by the dynamic ability of the A2 domain to response to shear stress.

8 Future directions

8.1 Investigating the functional significance of the VWF-galectin interaction

- I. The work outlined herein defines galectin-1 as a high affinity binding partner for VWF. Given the similarities between the respective CRDs of galectin family members it will be of interest to determine if other galectins may also bind to VWF. Interestingly mass spectrometry analysis of platelet lysates has suggested that galectin-8 may constitute another novel binding partner for human VWF (Romaniuk *et al*, 2010).
- II. Our study describes an important role for VWF glycans adjacent to the ADAMTS13 cleavage site in the A2 domain in regulating galectin-1 binding. Moreover Saint-Lu *et al* have previously reported that in the absence of galectin-1 and -3 VWF string formation on the endothelial cell surface was significantly enhanced (Saint-Lu *et al*, 2012). To further investigate the molecular basis through which galectins mediate this effect, the ability of galectins to influence VWF proteolysis by ADAMTS13 under shear will be examined. Additional plasma proteases can also cause VWF proteolysis, including plasmin and leukocyte proteases (Raife *et al*, 2009; Berkowitz *et al*, 1987). Further studies will be required to determine whether galectin binding to VWF may also modulate cleavage by these plasma proteases.

- III. Reduced plasma galectin-1 and galectin-3 levels have been reported in mice deficient in VWF (Saint-Lu *et al*, 2012). To further investigate this observation plasma galectin-1 and -3 levels will also be measured in patients with VWD to assess whether qualitative and/or quantitative changes in VWF levels in humans also influence plasma galectin levels.
- IV. Recent data has suggested that galectin-3 may represent a candidate biomarker for venous thromboembolism in both humans and mice (DeRoo *et al*, 2014). Consequently further studies to determine whether plasma galectin concentrations may vary in patients with elevated VWF:Ag levels would also be of direct clinical relevance.

8.2 Defining the physiological significance of the interaction between FVIII and galectins

Our data reveal that both galectin-1 and -3 constitute novel binding candidates for human FVIII. Moreover, at high concentrations galectin-1 can inhibit FVIII cofactor activity. However several key questions remain to be addressed.

- I. *In vitro* assays suggest a high affinity interaction between galectins and FVIII. Galectin-1 is known to be expressed by liver sinusoidal endothelial cells, which have also recently been demonstrated as the primary site of FVIII production (Shahani *et al*, 2014; Fahs *et al*, 2014). To determine whether

galectin-1 may interact with FVIII within these cells intracellular colocalisation studies will be performed.

- II. Galectins have been shown to complex with VWF within HUVECs and moreover galectins, which lack a typical secretory signal, remain associated with VWF upon secretion (Saint-Lu *et al*, 2012). To assess whether galectins may also associate with FVIII in plasma a coimmunoprecipitation study will be carried out. For these assays plasma from VWD type 3 patients will be used to exclude VWF, since it is known to complex with galectins in plasma. This work may also help to define whether increased local concentrations of galectin-1 with FVIII may occur *in vivo*.
- III. This study demonstrates that galectin-1 can bind and precipitate free FVIII from VWF deficient plasma. However, it is known that once in complex with VWF a number of FVIII interactions are attenuated including binding to LRP1 clearance receptor and CD206 lectin receptor. Consequently it will be necessary to determine whether galectins can bind to FVIII within the FVIII-VWF complex and moreover if the inhibitory effect on FVIII activity observed with galectin-1 can also occur on the FVIII-VWF complex.
- IV. Galectin-1 displays binding preferences for FVIII high mannose oligosaccharides. These glycan chains on FVIII have been proposed to mediate FVIII uptake by antigen presenting cells and T cell activation (Dasgupta *et al*, 2007). Additionally both galectin-1 and galectin-3 displayed

enhanced binding for BHK-rFVIII compared with CHO-rFVIII. Interestingly a recent publication has suggested that a significantly higher incidence of high-titer inhibitor development was observed in a cohort of patients treated with BHK-derived rFVIII compared to CHO-rFVIII (Collins *et al*, 2014). Further studies will be required to assess whether galectins may potentially influence FVIII antigenicity.

- V. Finally given that plasma galectin concentrations are dependent on plasma VWF levels, further studies will be required to determine whether FVIII plasma levels may also influence galectin concentrations. Thus galectin ELISAs will be used to measure plasma levels in haemophilia A patients.

8.3 Defining the role of VWF glycosylation in modulating the clearance of VWF-FVIII complex *in-vivo*

The data presented in this study define a critical role for specific VWF glycans sites in modulating VWF circulatory survival via macrophages. We hypothesise that VWF glycans may influence its clearance through different potential mechanisms. Firstly, VWF glycans can serve as interactive binding ligands for lectin clearance receptors (e.g. ASGPR). Second, the glycans of VWF may impede access to a critical clearance binding site via steric hindrance. Finally, variation in VWF glycosylation may induce conformational changes to shield or expose clearance motifs in the polypeptide

backbone. In this context a number experiments will be required to further define the role of VWF glycans.

- I. Our *in vitro* data presented herein demonstrates an important role for divalent cations and ristocetin in promoting VWF binding to macrophages. This is in keeping with reports that $\beta 2$ integrins and shear stress-induced LRP1 binding are important mediators of VWF clearance (Rastegarlarlari *et al*, 2012). To further investigate the receptor pathways involved, binding of VWF and glycan variants to macrophages will be examined in the presence of $\beta 2$ integrin antagonist NIF and LRP1 antagonist RAP. Furthermore co-infusion studies with RAP and VWF in mice will be used to investigate the potential role of LRP1 *in vivo*.
- II. To further characterise the role of specific VWF domains in mediating interaction with clearance receptor LRP1, SPR assays with purified LRP1 will be established. This will facilitate the assessment of binding kinetics with various VWF domain fragments and may help define the critical residues of VWF involved in LRP1 binding.
- III. The N-linked glycans of VWF are found throughout the length of the glycoprotein. Moreover the glycans are highly conserved across species indicating a functional role. Consequently, the role of each N-glycan in contributing to VWF clearance will be examined systematically. Site-directed mutagenesis of each individual N-glycan site has been performed (cDNAs

were kind gift from Dr. McKinnon). These glycan variants will be expressed, purified and clearance rates assessed in VWF^{-/-} mice.

- IV. Interestingly residues flanking the glycans within the A2 domain been demonstrated to be causative for VWD. Point mutations K1518E and G1573S have been described in patients with VWD type 2A, however no functional studies with these variants have been investigated. Previously it has been documented that VWD type 2A mutations can be associated with increased VWFpp/VWF:Ag ratios and reduced VWF half-life *in vivo* (Schooten *et al*, 2005). Given the proximity to the N-linked sites within A2 it will be of interest to examine the role of K1518E and G1573S mutations in potentially destabilising the nearby glycans at N1515 and N1574 and if these mutations may results in accelerated clearance of VWF *in vivo*.

References

- Altieri, D.C., Morrissey, J.H. & Edgington, T.S. (1988) Adhesive receptor Mac-1 coordinates the activation of factor X on stimulated cells of monocytic and myeloid differentiation: an alternative initiation of the coagulation protease cascade. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 85, 7462–7466.
- Aly, A.M., Higuchi, M., Kasper, C.K., Kazazian, H.H., Antonarakis, S.E. & Hoyer, L.W. (1992) Hemophilia A due to mutations that create new N-glycosylation sites. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 89, 4933–7.
- Auton, M., Cruz, M.A. & Moake, J. (2007) Conformational stability and domain unfolding of the Von Willebrand factor A domains. *Journal Of Molecular Biology*, 366, 986–1000.
- Auton, M., Sowa, K.E., Behymer, M. & Cruz, M.A. (2012) N-terminal flanking region of A1 domain in von Willebrand factor stabilizes structure of A1A2A3 complex and modulates platelet activation under shear stress. *Journal Of Biological Chemistry*, 287, 14579–14585.
- Auton, M., Sowa, K.E., Smith, S.M., Sedlák, E., Vijayan, K.V. & Cruz, M.A. (2010) Destabilization of the A1 domain in von Willebrand factor dissociates the A1A2A3 tri-domain and provokes spontaneous binding to glycoprotein Ib α and platelet activation under shear stress. *Journal Of Biological Chemistry*, 285, 22831–22839.
- Badirou, I., Kurdi, M., Legendre, P., Rayes, J., Bryckaert, M., Casari, C., Lenting, P.J., Christophe, O.D. & Denis, C. V (2012) In vivo analysis of the role of O-Glycosylations of von Willebrand factor. *PLoS ONE*, 7, e37508.
- Badirou, I., Kurdi, M., Rayes, J., Legendre, P., Christophe, O.D., Lenting, P.J. & Denis, C. V (2010) von Willebrand factor clearance does not involve proteolysis by ADAMTS-13. *Journal Of Thrombosis And Haemostasis : JTH*, 8, 2338–40.
- Baenziger, J.U. & Maynard, Y. (1980) Human hepatic lectin. Physiochemical properties and specificity. *The Journal Of Biological Chemistry*, 255, 4607–13.
- Baldauf, C., Schneppenheim, R., Stacklies, W., Obser, T., Pieconka, A., Schneppenheim, S., Budde, U., Zhou, J. & Gräter, F. (2009) Shear-induced unfolding activates von Willebrand factor A2 domain for proteolysis. *Journal Of Thrombosis And Haemostasis : JTH*, 7, 2096–105.
- Baud'huin, M., Duplomb, L., Téletchéa, S., Charrier, C., Maillason, M., Fouassier, M. & Heymann, D. (2009) Factor VIII-von Willebrand factor complex inhibits osteoclastogenesis and controls cell survival. *The Journal Of Biological Chemistry*, 284, 31704–13.
- Beacham, D.A., Wise, R.J., Turci, S.M. & Handin, R.I. (1992) Selective inactivation of the Arg-Gly-Asp-Ser (RGDS) binding site in von Willebrand factor by site-directed mutagenesis. *The Journal Of Biological Chemistry*, 267, 3409–15.
- Berkowitz, S.D., Dent, J., Roberts, J., Fujimura, Y., Plow, E.F., Titani, K., Ruggeri, Z.M. & Zimmerman, T.S. (1987) Epitope mapping of the von Willebrand factor subunit distinguishes fragments present in normal and type IIA von Willebrand disease from those generated by plasmin. *Journal Of Clinical Investigation*, 79, 524–531.
- Bhende, Y.M., Deshpande, C.K., Bhatia, H.M., Sanger, R., Race, R.R., Morgan, W.T. & Watkins, W.M. (2008) A 'new' blood-group character related to the ABO system. 1952. *The National Medical Journal Of India*, 21, 3 p.
- Bian, C.F., Zhang, Y., Sun, H., Li, D.F. & Wang, D.C. (2011) Structural basis for distinct binding properties of the human galectins to Thomsen-Friedenreich antigen. *PloS One*, 6, e25007.
- Bonfanti, R., Furie, B.C., Furie, B. & Wagner, D.D. (1989) PADGEM (GMP140) is a component of Weibel-Palade bodies of human endothelial cells. *Blood*, 73, 1109–12.
- Bongers, T.N., de Maat, M.P.M., van Goor, M.L.P.J., Bhagwanbali, V., van Vliet, H.H.D.M., Gómez García, E.B., Dippel, D.W.J. & Leebeek, F.W.G. (2006) High von Willebrand factor

- levels increase the risk of first ischemic stroke: influence of ADAMTS13, inflammation, and genetic variability. *Stroke; A Journal Of Cerebral Circulation*, 37, 2672–7.
- Borchiellini, A., Fijnvandraat, K., ten Cate, J.W., Pajkrt, D., van Deventer, S.J., Pasterkamp, G., Meijer-Huizinga, F., Zwart-Huinink, L., Voorberg, J. & van Mourik, J.A. (1996) Quantitative analysis of von Willebrand factor propeptide release in vivo: effect of experimental endotoxemia and administration of 1-deamino-8-D-arginine vasopressin in humans. *Blood*, 88, 2951–2958.
- Bork, P. (1993) The modular architecture of a new family of growth regulators related to connective tissue growth factor. *FEBS Letters*, 327, 125–130.
- Bovenschen, N., Herz, J., Grimbergen, J.M., Lenting, P.J., Havekes, L.M., Mertens, K. & van Vlijmen, B.J.M. (2003) Elevated plasma factor VIII in a mouse model of low-density lipoprotein receptor-related protein deficiency. *Blood*, 101, 3933–9.
- Bovenschen, N., Rijken, D.C., Havekes, L.M., Van Vlijmen, B.J.M. & Mertens, K. (2005) The B domain of coagulation factor VIII interacts with the asialoglycoprotein receptor. *Journal Of Thrombosis And Haemostasis JTH*, 3, 1257–1265.
- Bowen, D. (2003) An influence of ABO blood group on the rate of proteolysis of von Willebrand factor by ADAMTS13. *Journal Of Thrombosis And Haemostasis*, 33–40.
- Bridges, K., Harford, J., Ashwell, G. & Klausner, R.D. (1982) Fate of receptor and ligand during endocytosis of asialoglycoproteins by isolated hepatocytes. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 79, 350–354.
- Brinkman-Van Der Linden, E.C.M. & Varki, A. (2000) New aspects of siglec binding specificities, including the significance of fucosylation and of the sialyl-Tn epitope. *Journal Of Biological Chemistry*, 275, 8625–8632.
- Brown, S.A., Collins, P.W. & Bowen, D.J. (2002) Heterogeneous detection of A-antigen on von Willebrand factor derived from platelets, endothelial cells and plasma. *Thrombosis And Haemostasis*, 87, 990–6.
- Canis, K., McKinnon, T.A.J., Nowak, A., Haslam, S.M., Panico, M., Morris, H.R., Laffan, M.A. & Dell, A. (2012) Mapping the N-glycome of human von Willebrand factor. *The Biochemical Journal*, 447, 217–28.
- Canis, K., McKinnon, T.A.J., Nowak, A., Panico, M., Morris, H.R., Laffan, M. & Dell, A. (2010) The plasma von Willebrand factor O-glycome comprises a surprising variety of structures including ABH antigens and disialosyl motifs. *Journal Of Thrombosis And Haemostasis : JTH*, 8, 137–45.
- Carew, J.A., Browning, P.J. & Lynch, D.C. (1990) Sulfation of von Willebrand factor. *Blood*, 76, 2530–2539.
- Casari, C., Du, V., Wu, Y.P., Kauskot, A., de Groot, P.G., Christophe, O.D., Denis, C. V, de Laat, B. & Lenting, P.J. (2013a) Accelerated uptake of VWF/platelet complexes in macrophages contributes to VWD type 2B-associated thrombocytopenia. *Blood*, 122, 2893–902.
- Casari, C., Lenting, P.J., Wohner, N., Christophe, O.D. & Denis, C. V (2013b) Clearance of von Willebrand factor. *Journal Of Thrombosis And Haemostasis : JTH*, 11 Suppl 1, 202–11.
- Casonato, A., Gallinaro, L., Cattini, M.G., Pontara, E., Padriani, R., Bertomoro, A., Daidone, V. & Pagnan, A. (2010) Reduced survival of type 2B von Willebrand factor, irrespective of large multimer representation or thrombocytopenia. *Haematologica*, 95, 1366–1372.
- Casonato, A., Pontara, E., Sartorello, F., Cattini, M.G., Sartori, M.T., Padriani, R. & Girolami, A. (2002) Reduced von Willebrand factor survival in type Vicenza von Willebrand disease. 99, 180–184.
- Castaman, G., Giacomelli, S. & Coppola, A. (2013) A de novo type 2A von Willebrand factor mutation (Ser1517Arg). *Journal Of Thrombosis And Haemostasis*, 11, 1–1322.
- Castaman, G., Lethagen, S., Federici, A.B., Tosoetto, A., Goodeve, A., Budde, U., Batlle, J., Meyer, D., Mazurier, C., Fressinaud, E., Goudemand, J., Eikenboom, J., Schneppenheim, R., Ingerslev, J., Vorlova, Z., Habart, D., Holmberg, L., Pasi, J., Hill, F., Peake, I., et al (2008) Response to desmopressin is influenced by the genotype and phenotype in type 1 von

- Willebrand disease (VWD): results from the European Study MCMDM-1VWD. *Blood*, 111, 3531–9.
- Castro-Núñez, L., Bloem, E., Boon-Spijker, M.G., van der Zwaan, C., van den Biggelaar, M., Mertens, K. & Meijer, A.B. (2013) Distinct roles of Ser-764 and Lys-773 at the N terminus of von Willebrand factor in complex assembly with coagulation factor VIII. *The Journal Of Biological Chemistry*, 288, 393–400.
- Castro-Núñez, L., Dienava-Verdoold, I., Herczenik, E., Mertens, K. & Meijer, A.B. (2012) Shear stress is required for the endocytic uptake of the factor VIII-von Willebrand factor complex by macrophages. *Journal Of Thrombosis And Haemostasis : JTH*, 10, 1929–37.
- Cederfur, C., Salomonsson, E., Nilsson, J., Halim, A., Oberg, C.T., Larson, G., Nilsson, U.J. & Leffler, H. (2008) Different affinity of galectins for human serum glycoproteins: galectin-3 binds many protease inhibitors and acute phase proteins. *Glycobiology*, 18, 384–94.
- De Ceunynck, K., De Meyer, S.F. & Vanhoorelbeke, K. (2013) Unwinding the von Willebrand factor strings puzzle. *Blood*, 121, 270–7.
- Chen, J., Ling, M., Fu, X., López, J. a & Chung, D.W. (2012) Simultaneous exposure of sites in von Willebrand factor for glycoprotein Ib binding and ADAMTS13 cleavage: studies with ristocetin. *Arteriosclerosis, Thrombosis, And Vascular Biology*, 32, 2625–30.
- Collins, P.W., Palmer, B.P., Chalmers, E.A., Hart, D.P., Liesner, R., Rangarajan, S., Talks, K., Williams, M. & Hay, C.R.M. (2014) Factor VIII brand and the incidence of factor VIII inhibitors in previously untreated UK children with severe hemophilia A, 2000-2011. *Blood*, 124, 3389–97.
- Connolly, D.T., Townsend, R.R., Kawaguchi, K., Bell, W.R. & Lee, Y.C. (1982) Binding and endocytosis of cluster glycosides by rabbit hepatocytes. Evidence for a short-circuit pathway that does not lead to degradation. *Journal Of Biological Chemistry*, 257, 939–945.
- Cornish, A.L., Freeman, S., Forbes, G., Ni, J., Zhang, M., Cepeda, M., Gentz, R., Augustus, M., Carter, K.C. & Crocker, P.R. (1998) Characterization of siglec-5, a novel glycoprotein expressed on myeloid cells related to CD33. *Blood*, 92, 2123–2132.
- Cramer, E.M., Meyer, D., le Menn, R. & Breton-Gorius, J. (1985) Eccentric localization of von Willebrand factor in an internal structure of platelet alpha-granule resembling that of Weibel-Palade bodies. *Blood*, 66, 710–713.
- Crawley, J.T.B., Lane, D.A., Woodward, M., Rumley, A. & Lowe, G.D.O. (2008) Evidence that high von Willebrand factor and low ADAMTS-13 levels independently increase the risk of a non-fatal heart attack. *Journal Of Thrombosis And Haemostasis : JTH*, 6, 583–8.
- Cruz, M.A., Yuan, H., Lee, J.R., Wise, R.J. & Handin, R.I. (1995) Interaction of the von Willebrand factor (vWF) with collagen. Localization of the primary collagen-binding site by analysis of recombinant vWF a domain polypeptides. *Journal Of Biological Chemistry*, 270, 10822–10827.
- Daigneault, M., Preston, J. a, Marriott, H.M., Whyte, M.K.B. & Dockrell, D.H. (2010) The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. *PloS One*, 5, e8668.
- Dasgupta, S., Navarrete, A.M., Bayry, J., Delignat, S., Wootla, B., André, S., Christophe, O., Nascimbeni, M., Jacquemin, M., Martinez-Pomares, L., Geijtenbeek, T.B.H., Moris, A., Saint-Remy, J.M., Kazatchkine, M.D., Kaveri, S. V & Lacroix-Desmazes, S. (2007) A role for exposed mannosylations in presentation of human therapeutic self-proteins to CD4+ T lymphocytes. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 104, 8965–70.
- Denis, C., Methia, N., Frenette, P.S., Rayburn, H., Ullman-Culleré, M., Hynes, R.O. & Wagner, D.D. (1998) A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 95, 9524–9529.

- Denis, C. V, André, P., Saffaripour, S. & Wagner, D.D. (2001) Defect in regulated secretion of P-selectin affects leukocyte recruitment in von Willebrand factor-deficient mice. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 98, 4072–4077.
- DeRoo, E.P., Wroblewski, S.K., Shea, E.M., Al-Khalil, R.K., Hawley, A.E., Henke, P.K., Myers, D.D., Wakefield, T.W. & Diaz, J.A. (2014) The role of galectin-3 and galectin-3-binding protein in venous thrombosis. *Blood*.
- Diamond, M.S., Staunton, D.E., De Fougerolles, A.R., Stacker, S.A., Garcia-Aguilar, J., Hibbs, M.L. & Springer, T.A. (1990) ICAM-1 (CD54): A counter-receptor for Mac-1 (CD11b/CD18). *Journal Of Cell Biology*, 111, 3129–3139.
- Diaz, S.L., Padler-Karavani, V., Ghaderi, D., Hurtado-Ziola, N., Yu, H., Chen, X., Brinkman-Van der Linden, E.C.M., Varki, A. & Varki, N.M. (2009) Sensitive and specific detection of the non-human sialic Acid N-glycolylneuraminic acid in human tissues and biotherapeutic products. *PloS One*, 4, e4241.
- Dobrkovska, a, Krzenski, U. & Chediak, J.R. (1998) Pharmacokinetics, efficacy and safety of Humate-P® in von Willebrand disease. *Haemophilia*, 4, 33–39.
- Dong, J.F., Berndt, M.C., Schade, A., McIntire, L. V, Andrews, R.K. & López, J.A. (2001) Ristocetin-dependent, but not botrocetin-dependent, binding of von Willebrand factor to the platelet glycoprotein Ib-IX-V complex correlates with shear-dependent interactions. *Blood*, 97, 162–8.
- Dumas, J.J., Kumar, R., McDonagh, T., Sullivan, F., Stahl, M.L., Somers, W.S. & Mosyak, L. (2004) Crystal structure of the wild-type von Willebrand factor A1-glycoprotein Ibalph complex reveals conformation differences with a complex bearing von Willebrand disease mutations. *The Journal Of Biological Chemistry*, 279, 23327–34.
- Eikenboom, J., Federici, A.B., Dirven, R.J., Castaman, G., Rodeghiero, F., Budde, U., Schneppenheim, R., Batlle, J., Canciani, M.T., Goudemand, J., Peake, I., Goodeve, A. & MCMDM-1VWD Study Group (2013) VWF propeptide and ratios between VWF, VWF propeptide, and FVIII in the characterization of type 1 von Willebrand disease. *Blood*.
- Eikenboom, J., Van Marion, V., Putter, H., Goodeve, A., Rodeghiero, F., Castaman, G., Federici, A.B., Batlle, J., Meyer, D., Mazurier, C., Goudemand, J., Schneppenheim, R., Budde, U., Ingerslev, J., Vorlova, Z., Habart, D., Holmberg, L., Lethagen, S., Pasi, J., Hill, F., et al (2006) Linkage analysis in families diagnosed with type 1 von Willebrand disease in the European study, molecular and clinical markers for the diagnosis and management of type 1 VWD. *Journal Of Thrombosis And Haemostasis : JTH*, 4, 774–782.
- Ellies, L.G., Ditto, D., Levy, G.G., Wahrenbrock, M., Ginsburg, D., Varki, A., Le, D.T. & Marth, J.D. (2002) Sialyltransferase ST3Gal-IV operates as a dominant modifier of hemostasis by concealing asialoglycoprotein receptor ligands. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 99, 10042–7.
- Emsley, J., Cruz, M., Handin, R. & Liddington, R. (1998) Crystal Structure of the von Willebrand Factor A1 Domain and Implications for the Binding of Platelet Glycoprotein Ib. *Journal Of Biological Chemistry*, 273, 10396–10401.
- Fahs, S.A., Hille, M.T., Shi, Q., Weiler, H. & Montgomery, R.R. (2014) A conditional knockout mouse model reveals endothelial cells as the principal and possibly exclusive source of plasma factor VIII. *Blood*, 123, 3706–13.
- Fay, P.J., Coumans, J. V & Walker, F.J. (1991) von Willebrand factor mediates protection of factor VIII from activated protein C-catalyzed inactivation. *The Journal Of Biological Chemistry*, 266, 2172–7.
- Federici, A.B., Elder, J.H., de Marco, L., Ruggeri, Z.M. & Zimmerman, T.S. (1984) Carbohydrate moiety of von Willebrand factor is not necessary for maintaining multimeric structure and ristocetin cofactor activity but protects from proteolytic degradation. *Journal Of Clinical Investigation*, 74, 2049–2055.

- Feng, S., Liang, X., Cruz, M. a, Vu, H., Zhou, Z., Pemmaraju, N., Dong, J.F., Kroll, M.H. & Afshar-Kharghan, V. (2013) The interaction between factor H and Von Willebrand factor. *PLoS One*, 8, e73715.
- Fernandez, M.F., Ginsberg, M.H., Ruggeri, Z.M., Batlle, F.J. & Zimmerman, T.S. (1982) Multimeric structure of platelet factor VIII/von Willebrand factor: the presence of larger multimers and their reassociation with thrombin-stimulated platelets. *Blood*, 60, 1132–1138.
- Feys, H.B., Anderson, P.J., Vanhoorelbeke, K., Majerus, E.M. & Sadler, J.E. (2009) Multi-step binding of ADAMTS-13 to von Willebrand factor. *Journal Of Thrombosis And Haemostasis : JTH*, 7, 2088–95.
- Fiedler, U., Scharpfenecker, M., Koidl, S., Hegen, A., Grunow, V., Schmidt, J.M., Kriz, W., Thurston, G. & Augustin, H.G. (2004) The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. *Blood*, 103, 4150–6.
- Folsom, A.R., Rosamond, W.D., Shahar, E., Cooper, L.S., Aleksic, N., Nieto, F.J.J., Rasmussen, M.L. & Wu, K.K. (1999) Prospective study of markers of hemostatic function with risk of ischemic stroke. The Atherosclerosis Risk in Communities (ARIC) Study Investigators. *Circulation*, 100, 736–742.
- Foster, P.A., Fulcher, C.A., Marti, T., Titani, K. & Zimmerman, T.S. (1987) A major factor VIII binding domain resides within the amino-terminal 272 amino acid residues of von Willebrand factor. *Journal Of Biological Chemistry*, 262, 8443–8450.
- Gallinaro, L., Cattini, M.G., Sztukowska, M., Padriani, R., Sartorello, F., Pontara, E., Bertomoro, A., Daidone, V., Pagnan, A. & Casonato, A. (2008) A shorter von Willebrand factor survival in O blood group subjects explains how ABO determinants influence plasma von Willebrand factor. *Blood*, 111, 3540–5.
- Gill, J.C., Endres-Brooks, J., Bauer, P.J., Marks, W.J. & Montgomery, R.R. (1987) The effect of ABO blood group on the diagnosis of von Willebrand disease. *Blood*, 69, 1691–1695.
- Ginsburg, D., Handin, R.I., Bonthron, D.T., Donlon, T.A., Bruns, G.A., Latt, S.A. & Orkin, S.H. (1985) Human von Willebrand factor (vWF): isolation of complementary DNA (cDNA) clones and chromosomal localization. *Science (New York, N.Y.)*, 228, 1401–1406.
- Girma, J.P., Takahashi, Y., Yoshioka, A., Diaz, J. & Meyer, D. (1990) Ristocetin and botrocetin involve two distinct domains of von Willebrand factor for binding to platelet membrane glycoprotein Ib. *Thrombosis And Haemostasis*, 64, 326–332.
- Goodeve, A., Eikenboom, J., Castaman, G., Rodeghiero, F., Federici, A.B., Batlle, J., Meyer, D., Mazurier, C., Goudemand, J., Schneppenheim, R., Budde, U., Ingerslev, J., Habart, D., Vorlova, Z., Holmberg, L., Lethagen, S., Pasi, J., Hill, F., Hashemi Sotah, M., Baronciani, L., et al (2007) Phenotype and genotype of a cohort of families historically diagnosed with type 1 von Willebrand disease in the European study, Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand Disease (MCMDM-1VWD). *Blood*, 109, 112–21.
- Gralnick, H.R., Collier, B.S. & Sultan, Y. (1976) Carbohydrate deficiency of the factor VIII/von Willebrand factor Protein in von Willebrand's disease variants. *Science (New York, N.Y.)*, 192, 56–9.
- Grewal, P.K., Uchiyama, S., Ditto, D., Varki, N., Le, D.T., Nizet, V. & Marth, J.D. (2008) The Ashwell receptor mitigates the lethal coagulopathy of sepsis. *Nature Medicine*, 14, 648–55.
- Haberichter, S.L., Balistreri, M., Christopherson, P., Morateck, P., Gavazova, S., Bellissimo, D.B., Manco-Johnson, M.J., Gill, J.C. & Montgomery, R.R. (2006) Assay of the von Willebrand factor (VWF) propeptide to identify patients with type 1 von Willebrand disease with decreased VWF survival. *Blood*, 108, 3344–51.

- Haberichter, S.L., Budde, U., Obser, T., Schneppenheim, S., Wermes, C. & Schneppenheim, R. (2010) The mutation N528S in the von Willebrand factor (VWF) propeptide causes defective multimerization and storage of VWF. *Blood*, 115, 4580–7.
- Haberichter, S.L., Castaman, G., Budde, U., Peake, I., Goodeve, A., Rodeghiero, F., Federici, A.B., Batlle, J., Meyer, D., Mazurier, C., Goudemand, J., Eikenboom, J., Schneppenheim, R., Ingerslev, J., Vorlova, Z., Habart, D., Holmberg, L., Lethagen, S., Pasi, J., Hill, F.G.H., et al (2008) Identification of type 1 von Willebrand disease patients with reduced von Willebrand factor survival by assay of the VWF propeptide in the European study: molecular and clinical markers for the diagnosis and management of type 1 VWD (MCMDM-1VWD). *Blood*, 111, 4979–85.
- Haberichter, S.L., Jakab, D.A. & Jacobi, P.M. (2013) Upstream Mechanisms Causing Type 1C Von Willebrand Disease (VWD): Contribution Of Defective Von Willebrand Factor (VWF) Multimerization, Regulated Storage, and Secretion. *Blood*, 122, 3571.
- Harrison, P. & Cramer, E.M. (1993) Platelet alpha-granules. *Blood Reviews*, 7, 52–62.
- Harvey, D.J., Wing, D.R., Küster, B. & Wilson, I.B. (2000) Composition of N-linked carbohydrates from ovalbumin and co-purified glycoproteins. *Journal Of The American Society For Mass Spectrometry*, 11, 564–71.
- Hirabayashi, J., Hashidate, T., Arata, Y., Nishi, N., Nakamura, T., Hirashima, M., Urashima, T., Oka, T., Futai, M., Muller, W.E.G., Yagi, F. & Kasai, K. (2002) Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. *Biochimica Et Biophysica Acta*, 1572, 232–54.
- Hironaka, T., Furukawa, K., Esmon, P.C., Fournel, M.A., Sawada, S., Kato, M., Minaga, T. & Kobata, A. (1992) Comparative study of the sugar chains of factor VIII purified from human plasma and from the culture media of recombinant baby hamster kidney cells. *The Journal Of Biological Chemistry*, 267, 8012–20.
- Hoffmeister, K.M., Josefsson, E.C., Isaac, N.A., Clausen, H., Hartwig, J.H. & Stossel, T.P. (2003) Glycosylation restores survival of chilled blood platelets. *Science (New York, N.Y.)*, 301, 1531–4.
- Howard, M.A., Montgomery, D.C. & Hardisty, R.M. (1974) Factor-VIII-related antigen in platelets. *Thrombosis Research*, 4, 617–624.
- Hoylaerts, M.F., Yamamoto, H., Nuyts, K., Vreys, I., Deckmyn, H. & Vermeylen, J. (1997) von Willebrand factor binds to native collagen VI primarily via its A1 domain. *The Biochemical Journal*, 324 (Pt 1), 185–191.
- Huang, J., Roth, R., Heuser, J.E. & Sadler, J.E. (2009) Integrin alpha(v)beta(3) on human endothelial cells binds von Willebrand factor strings under fluid shear stress. *Blood*, 113, 1589–97.
- Huang, R.H., Wang, Y., Roth, R., Yu, X., Purvis, A.R., Heuser, J.E., Egelman, E.H. & Sadler, J.E. (2008) Assembly of Weibel-Palade body-like tubules from N-terminal domains of von Willebrand factor. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 105, 482–7.
- Huizinga, E.G., Martijn van der Plas, R., Kroon, J., Sixma, J.J. & Gros, P. (1997) Crystal structure of the A3 domain of human von Willebrand factor: implications for collagen binding. *Structure (London, England : 1993)*, 5, 1147–56.
- Hulstein, J.J.J., Lenting, P.J., de Laat, B., Derksen, R.H.W.M., Fijnheer, R. & de Groot, P.G. (2007) beta2-Glycoprotein I inhibits von Willebrand factor dependent platelet adhesion and aggregation. *Blood*, 110, 1483–91.
- Ii, M., Kurata, H., Itoh, N., Yamashina, I. & Kawasaki, T. (1990) Molecular cloning and sequence analysis of cDNA encoding the macrophage lectin specific for galactose and N-acetylgalactosamine. *J. Biol. Chem.*, 265, 11295–11298.
- Ikeda, Y., Handa, M., Kawano, K., Kamata, T., Murata, M., Araki, Y., Anbo, H., Kawai, Y., Watanabe, K. & Itagaki, I. (1991) The role of von Willebrand factor and fibrinogen in

- platelet aggregation under varying shear stress. *The Journal Of Clinical Investigation*, 87, 1234–40.
- Jaffe, E.A., Hoyer, L.W. & Nachman, R.L. (1974) Synthesis of von Willebrand factor by cultured human endothelial cells. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 71, 1906–9.
- James, P.D., Paterson, A.D., Notley, C., Cameron, C., Hegadorn, C., Tinlin, S., Brown, C., O'Brien, L., Leggo, J. & Lillicrap, D. (2006) Genetic linkage and association analysis in type 1 von Willebrand disease: results from the Canadian type 1 VWD study. *Journal Of Thrombosis And Haemostasis : JTH*, 4, 783–92.
- Kagami, K., Williams, S., Horne, M. & Gralnick, H. (2000) A preliminary analysis of platelet von Willebrand factor oligosaccharides. *Nagoya Journal Of Medical Science*, 63, 51–6.
- Kanaji, S., Fahs, S. a, Shi, Q., Haberichter, S.L. & Montgomery, R.R. (2012) Contribution of platelet vs. endothelial VWF to platelet adhesion and hemostasis. *Journal Of Thrombosis And Haemostasis : JTH*, 10, 1646–52.
- Kaufman, R.J., Wasley, L.C. & Dorner, A.J. (1988) Synthesis, processing, and secretion of recombinant human factor VIII expressed in mammalian cells. *The Journal Of Biological Chemistry*, 263, 6352–62.
- Kaufmann, J.E., Oksche, A., Wollheim, C.B., Günther, G., Rosenthal, W. & Vischer, U.M. (2000) Vasopressin-induced von Willebrand factor secretion from endothelial cells involves V2 receptors and cAMP. *Journal Of Clinical Investigation*, 106, 107–116.
- Koivunen, E., Ranta, T. maria, Annila, A., Taube, S., Uppala, A., Jokinen, M., van Willigen, G., Ihanus, E. & Gahmberg, C.G. (2001) Inhibition of beta(2) integrin-mediated leukocyte cell adhesion by leucine-leucine-glycine motif-containing peptides. *The Journal Of Cell Biology*, 153, 905–16.
- Koppelman, S.J., van Hoeij, M., Vink, T., Lankhof, H., Schiphorst, M.E., Damas, C., Vlot, A.J., Wise, R., Bouma, B.N. & Sixma, J.J. (1996) Requirements of von Willebrand factor to protect factor VIII from inactivation by activated protein C. *Blood*, 87, 2292–2300.
- Kosloski, M.P., Miclea, R.D. & Balu-Iyer, S. V (2009) Role of glycosylation in conformational stability, activity, macromolecular interaction and immunogenicity of recombinant human factor VIII. *The AAPS Journal*, 11, 424–431.
- Koster, T., Blann, A.D., Briët, E., Vandenbroucke, J.P. & Rosendaal, F.R. (1995) Role of clotting factor VIII in effect of von Willebrand factor on occurrence of deep-vein thrombosis. *Lancet*, 345, 152–155.
- Krishnamoorthy, L., Bess, J.W., Preston, A.B., Nagashima, K. & Mahal, L.K. (2009) HIV-1 and microvesicles from T cells share a common glycome, arguing for a common origin. *Nature Chemical Biology*, 5, 244–250.
- Kurdi, M., Cherel, G., Lenting, P.J., Denis, C. V & Christophe, O.D. (2012) Coagulation factor X interaction with macrophages through its N-glycans protects it from a rapid clearance. *PloS One*, 7, e45111.
- De la Fuente, B., Kasper, C.K., Rickles, F.R. & Hoyer, L.W. (1985) Response of patients with mild and moderate hemophilia A and von Willebrand's disease to treatment with desmopressin. *Annals Of Internal Medicine*, 103, 6–14.
- Lankhof, H., Damas, C., Schiphorst, M.E., Ijsseldijk, M.J., Bracke, M., Furlan, M., Tsai, H.M., de Groot, P.G., Sixma, J.J. & Vink, T. (1997) von Willebrand factor without the A2 domain is resistant to proteolysis. *Thrombosis And Haemostasis*, 77, 1008–13.
- Lenting, P.J., Casari, C., Christophe, O.D. & Denis, C. V (2012) von Willebrand factor: the old, the new and the unknown. *Journal Of Thrombosis And Haemostasis : JTH*, 10, 2428–37.
- Lenting, P.J., Neels, J.G., van den Berg, B.M., Clijsters, P.P., Meijerman, D.W., Pannekoek, H., van Mourik, J. a, Mertens, K. & van Zonneveld, a J. (1999) The light chain of factor VIII comprises a binding site for low density lipoprotein receptor-related protein. *The Journal Of Biological Chemistry*, 274, 23734–9.

- Lenting, P.J., Westein, E., Terraube, V., Ribba, A.S., Huizinga, E.G., Meyer, D., de Groot, P.G. & Denis, C. V (2004) An experimental model to study the in vivo survival of von Willebrand factor. Basic aspects and application to the R1205H mutation. *The Journal Of Biological Chemistry*, 279, 12102–9.
- Li, F., Li, C.Q., Moake, J.L., López, J.A. & McIntire, L. V (2004) Shear Stress-Induced Binding of Large and Unusually Large von Willebrand Factor to Human Platelet Glycoprotein Iba. *Annals Of Biomedical Engineering*, 32, 961–969.
- Lillis, A.P., Van Duyn, L.B., Murphy-Ullrich, J.E. & Strickland, D.K. (2008) LDL receptor-related protein 1: unique tissue-specific functions revealed by selective gene knockout studies. *Physiological Reviews*, 88, 887–918.
- Lollar, P. (1991) The association of factor VIII with von Willebrand factor. *Mayo Clinic Proceedings*, 66, 524–34.
- Lollar, P., Hill-Eubanks, D.C. & Parker, C.G. (1988) Association of the factor VIII light chain with von Willebrand factor. *The Journal Of Biological Chemistry*, 263, 10451–5.
- Lotan, R., Belloni, P.N., Tressler, R.J., Lotan, D., Xu, X.C. & Nicolson, G.L. (1994) Expression of galectins on microvessel endothelial cells and their involvement in tumour cell adhesion. *Glycoconjugate Journal*, 11, 462–468.
- Luken, B.M., Winn, L.Y.N., Emsley, J., Lane, D.A. & Crawley, J.T.B. (2010) The importance of vicinal cysteines, C1669 and C1670, for von Willebrand factor A2 domain function. *Blood*, 115, 4910–3.
- Macher, B.A. & Galili, U. (2008) The Galalpha1,3Galbeta1,4GlcNAc-R (alpha-Gal) epitope: a carbohydrate of unique evolution and clinical relevance. *Biochimica Et Biophysica Acta*, 1780, 75–88.
- Madabhushi, S.R., Zhang, C., Kelkar, A., Dayananda, K.M. & Neelamegham, S. (2014) Platelet GpIba binding to von Willebrand Factor under fluid shear: contributions of the D'D3-domain, A1-domain flanking peptide and O-linked glycans. *Journal Of The American Heart Association*, 3, e001420.
- Mancuso, D.J., Tuley, E.A., Westfield, L.A., Lester-Mancuso, T.L., Le Beau, M.M., Sorace, J.M. & Sadler, J.E. (1991) Human von Willebrand factor gene and pseudogene: structural analysis and differentiation by polymerase chain reaction. *Biochemistry*, 30, 253–69.
- Marti, T., Rösselet, S.J., Titani, K. & Walsh, K.A. (1987) Identification of disulfide-bridged substructures within human von Willebrand factor. *Biochemistry*, 26, 8099–8109.
- Marx, I., Christophe, O.D., Lenting, P.J., Rupin, A., Vallez, M.O., Verbeuren, T.J. & Denis, C. V (2008) Altered thrombus formation in von Willebrand factor-deficient mice expressing von Willebrand factor variants with defective binding to collagen or GPIIb/IIIa. *Blood*, 112, 603–9.
- Matsui, T., Titani, K. & Mizuochi, T. (1992) Structures of the asparagine-linked oligosaccharide chains of human von Willebrand factor. Occurrence of blood group A, B, and H(O) structures. *Journal Of Biological Chemistry*, 267, 8723–8731.
- Mayadas, T.N. & Wagner, D.D. (1989) In vitro multimerization of von Willebrand factor is triggered by low pH. Importance of the propeptide and free sulfhydryls. *The Journal Of Biological Chemistry*, 264, 13497–13503.
- Mayadas, T.N. & Wagner, D.D. (1992) Vicinal cysteines in the prosequence play a role in von Willebrand factor multimer assembly. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 89, 3531–3535.
- Mazsaroff, I., Yu, W., Kelley, B.D. & Vath, J.E. (1997) Quantitative comparison of global carbohydrate structures of glycoproteins using lc-ms and in-source fragmentation. *Analytical Chemistry*, 69, 2517–2524.
- McGrath, R.T., van den Biggelaar, M., Byrne, B., O'Sullivan, J.M., Rawley, O., O'Kennedy, R., Voorberg, J., Preston, R.J.S. & O'Donnell, J.S. (2013) Altered glycosylation of platelet-derived von Willebrand factor confers resistance to ADAMTS13 proteolysis. *Blood*, 122, 4107–10.

- McGrath, R.T., McKinnon, T.A.J., Byrne, B., O’Kennedy, R., Terraube, V., McRae, E., Preston, R.J.S., Laffan, M. a & O’Donnell, J.S. (2010) Expression of terminal alpha2-6-linked sialic acid on von Willebrand factor specifically enhances proteolysis by ADAMTS13. *Blood*, 115, 2666–73.
- McKinnon, T.A.J., Chion, A.C.K., Millington, A.J., Lane, D.A. & Laffan, M.A. (2008) N-linked glycosylation of VWF modulates its interaction with ADAMTS13. *Blood*, 111, 3042–9.
- McKinnon, T.A.J., Goode, E.C., Birdsey, G.M., Nowak, A.A., Chan, A.C.K., Lane, D.A. & Laffan, M.A. (2010) Specific N-linked glycosylation sites modulate synthesis and secretion of von Willebrand factor. *Blood*, 116, 640–648.
- Medzihradszky, K.F., Besman, M.J. & Burlingame, a L. (1997) Structural characterization of site-specific N-glycosylation of recombinant human factor VIII by reversed-phase high-performance liquid chromatography-electrospray ionization mass spectrometry. *Analytical Chemistry*, 69, 3986–94.
- Meitinger, T., Meindl, A., Bork, P., Rost, B., Sander, C., Haasemann, M. & Murken, J. (1993) Molecular modelling of the Norrie disease protein predicts a cystine knot growth factor tertiary structure. *Nature Genetics*, 5, 376–380.
- Michaux, G., Pullen, T.J., Haberichter, S.L. & Cutler, D.F. (2006) P-selectin binds to the D’-D3 domains of von Willebrand factor in Weibel-Palade bodies. *Blood*, 107, 3922–3924.
- Mohlke, K.L., Nichols, W.C., Westrick, R.J., Novak, E.K., Cooney, K. a, Swank, R.T. & Ginsburg, D. (1996) A novel modifier gene for plasma von Willebrand factor level maps to distal mouse chromosome 11. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 93, 15352–7.
- Mohlke, K.L., Purkayastha, a a, Westrick, R.J., Smith, P.L., Petryniak, B., Lowe, J.B. & Ginsburg, D. (1999) Mvwf, a dominant modifier of murine von Willebrand factor, results from altered lineage-specific expression of a glycosyltransferase. *Cell*, 96, 111–120.
- Mohri, H., Fujimura, Y., Shima, M., Yoshioka, A., Houghten, R.A., Ruggeri, Z.M. & Zimmerman, T.S. (1988) Structure of the von Willebrand factor domain interacting with glycoprotein Ib. *Journal Of Biological Chemistry*, 263, 17901–17904.
- Morange, P.E., Tregouet, D.A., Frere, C., Saut, N., Pellegrina, L., Alessi, M.C., Visvikis, S., Tiret, L. & Juhan-Vague, I. (2005) Biological and genetic factors influencing plasma factor VIII levels in a healthy family population: Results from the stanislas cohort. *British Journal Of Haematology*, 128, 91–99.
- Moussalli, M., Pipe, S.W., Hauri, H. peter, Nichols, W.C., Ginsburg, D. & Kaufman, R.J. (1999) ER to Golgi Trafficking of Coagulation Factors V and VIII. *The Journal Of Biological Chemistry*, 274, 32539–32542.
- Nachman, R., Levine, R. & Jaffe, E.A. (1977) Synthesis of factor VIII antigen by cultured guinea pig megakaryocytes. *Journal Of Clinical Investigation*, 60, 914–921.
- Nichols, W.C., Seligsohn, U., Zivelin, A., Terry, V.H., Hertel, C.E., Wheatley, M.A., Moussalli, M.J., Hauri, H.P., Ciavarella, N., Kaufman, R.J. & Ginsburg, D. (1998) Mutations in the ER-Golgi intermediate compartment protein ERGIC-53 cause combined deficiency of coagulation factors V and VIII. *Cell*, 93, 61–70.
- Nossent, A.Y., Van Marion, V., Van Tilburg, N.H., Rosendaal, F.R., Bertina, R.M., Van Mourik, J.A. & Eikenboom, H.C.J. (2006) von Willebrand factor and its propeptide: The influence of secretion and clearance on protein levels and the risk of venous thrombosis. *Journal Of Thrombosis And Haemostasis*, 4, 2556–2562.
- Nowak, A.A., Canis, K., Riddell, A., Laffan, M.A. & McKinnon, T.A.J. (2012) O-linked glycosylation of von Willebrand factor modulates the interaction with platelet receptor glycoprotein Ib under static and shear stress conditions. *Blood*, 120, 214–22.
- Nowak, A.A., McKinnon, T.A.J., Hughes, J.M., Chion, A.C.K. & Laffan, M.A. (2014) The O-linked glycans of human von Willebrand factor modulate its interaction with ADAMTS-13. *Journal Of Thrombosis And Haemostasis : JTH*, 12, 54–61.

- O'Donnell, J. & Laffan, M. (2001) Elevated plasma factor VIII levels--a novel risk factor for venous thromboembolism. *Clin Lab*, 47, 1–6.
- O'Donnell, J.S., McKinnon, T.A.J., Crawley, J.T.B., Lane, D.A. & Laffan, M.A. (2005) Bombay phenotype is associated with reduced plasma-VWF levels and an increased susceptibility to ADAMTS13 proteolysis. *Blood*, 106, 1988–1991.
- Orstavik, K.H., Magnus, P., Reisner, H., Berg, K., Graham, J.B. & Nance, W. (1985) Factor VIII and factor IX in a twin population. Evidence for a major effect of ABO locus on factor VIII level. *American Journal Of Human Genetics*, 37, 89–101.
- Pacienza, N., Pozner, R.G., Bianco, G.A., D'Atri, L.P., Croci, D.O., Negrotto, S., Malaver, E., Gómez, R.M., Rabinovich, G.A. & Schattner, M. (2008) The immunoregulatory glycan-binding protein galectin-1 triggers human platelet activation. *The FASEB Journal : Official Publication Of The Federation Of American Societies For Experimental Biology*, 22, 1113–1123.
- Padilla, A., Moake, J.L., Bernardo, A., Ball, C., Wang, Y., Arya, M., Nolasco, L., Turner, N., Berndt, M.C., Anvari, B., López, J.A. & Dong, J.F. (2004) P-selectin anchors newly released ultralarge von Willebrand factor multimers to the endothelial cell surface. *Blood*, 103, 2150–2156.
- Papaioannou, V.E. & Fox, J.G. (1993) Efficacy of tribromoethanol anesthesia in mice. *Laboratory Animal Science*, 43, 189–92.
- Pareti, F.I., Niiya, K., McPherson, J.M. & Ruggeri, Z.M. (1987) Isolation and characterization of two domains of human von Willebrand factor that interact with fibrillar collagen types I and III. *The Journal Of Biological Chemistry*, 262, 13835–13841.
- Park, E.I., Mi, Y., Unverzagt, C., Gabius, H.J. & Baenziger, J.U. (2005) The asialoglycoprotein receptor clears glycoconjugates terminating with sialic acid alpha 2,6GalNAc. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 102, 17125–17129.
- Pegon, J.N., Kurdi, M., Casari, C., Odouard, S., Denis, C. V, Christophe, O.D. & Lenting, P.J. (2012) Factor VIII and von Willebrand factor are ligands for the carbohydrate-receptor Siglec-5. *Haematologica*, 97, 1855–63.
- Pendu, R., Terraube, V., Christophe, O.D., Gahmberg, C.G., de Groot, P.G., Lenting, P.J. & Denis, C. V (2006) P-selectin glycoprotein ligand 1 and beta2-integrins cooperate in the adhesion of leukocytes to von Willebrand factor. *Blood*, 108, 3746–52.
- Perkins, S.J., Smith, K.F., Williams, S.C., Haris, P.I., Chapman, D. & Sim, R.B. (1994) The secondary structure of the von Willebrand factor type A domain in factor B of human complement by Fourier transform infrared spectroscopy. Its occurrence in collagen types VI, VII, XII and XIV, the integrins and other proteins by averaged structure pr. *Journal Of Molecular Biology*, 238, 104–119.
- Pipe, S.W., Morris, J.A., Shah, J. & Kaufman, R.J. (1998) Differential interaction of coagulation factor VIII and factor V with protein chaperones calnexin and calreticulin. *The Journal Of Biological Chemistry*, 273, 8537–44.
- Pittman, D.D., Alderman, E.M., Tomkinson, K.N., Wang, J.H., Giles, A.R. & Kaufman, R.J. (1993) Biochemical, immunological, and in vivo functional characterization of B-domain-deleted factor VIII. *Blood*, 81, 2925–2935.
- Pruss, C.M., Golder, M., Bryant, A., Hegadorn, C. a, Burnett, E., Laverty, K., Sponagle, K., Dhala, A., Notley, C., Haberichter, S. & Lillicrap, D. (2011) Pathologic mechanisms of type 1 VWD mutations R1205H and Y1584C through in vitro and in vivo mouse models. *Blood*, 117, 4358–66.
- Purvis, A.R., Gross, J., Dang, L.T., Huang, R.H., Kapadia, M., Townsend, R.R. & Sadler, J.E. (2007) Two Cys residues essential for von Willebrand factor multimer assembly in the Golgi. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 104, 15647–52.

- Rabinovich, G.A., Toscano, M.A., Jackson, S.S. & Vasta, G.R. (2007) Functions of cell surface galectin-glycoprotein lattices. *Current Opinion In Structural Biology*, 17, 513–20.
- Raife, T.J., Cao, W., Atkinson, B.S., Bedell, B., Montgomery, R.R., Lentz, S.R., Johnson, G.F. & Zheng, X.L. (2009) Leukocyte proteases cleave von Willebrand factor at or near the ADAMTS13 cleavage site. *Blood*, 114, 1666–1674.
- Rand, J.H., Badimon, L., Gordon, R.E., Uson, R.R. & Fuster, V. (1987) Distribution of von Willebrand factor in porcine intima varies with blood vessel type and location. *Arteriosclerosis, Thrombosis, And Vascular Biology*, 7, 287–291.
- Rastegarlar, G., Pegon, J.N., Casari, C., Odouard, S., Navarrete, A.M., Saint-Lu, N., van Vlijmen, B.J., Legendre, P., Christophe, O.D., Denis, C. V & Lenting, P.J. (2012) Macrophage LRP1 contributes to the clearance of von Willebrand factor. *Blood*, 119, 2126–34.
- Rawley, O., O'Sullivan, J.M., Chion, A., Keyes, S., Lavin, M., van Rooijen, N., Brophy, T.M., Fallon, P., Preston, R.J.S. & O'Donnell, J.S. (2015) von Willebrand factor arginine 1205 substitution results in accelerated macrophage-dependent clearance in vivo. *Journal Of Thrombosis And Haemostasis*, n/a–n/a.
- Repešé, Y., Dasgupta, S., Navarrete, A.M., Delignat, S., Kaveri, S. V & Lacroix-Desmazes, S. (2012) Mannose-sensitive receptors mediate the uptake of factor VIII therapeutics by human dendritic cells. *Journal Of Allergy And Clinical Immunology*, 129, 1172–1173.
- Romaniuk, M. a, Croci, D.O., Lapponi, M.J., Tribulatti, M. V, Negrotto, S., Poirier, F., Campetella, O., Rabinovich, G. a & Schattner, M. (2012) Binding of galectin-1 to $\alpha\text{IIb}\beta_3$ integrin triggers 'outside-in' signals, stimulates platelet activation, and controls primary hemostasis. *FASEB Journal : Official Publication Of The Federation Of American Societies For Experimental Biology*, 26, 2788–98.
- Romaniuk, M.A., Tribulatti, M.V., Cattaneo, V., Lapponi, M.J., Molinas, F.C., Campetella, O. & Schattner, M. (2010) Human platelets express and are activated by galectin-8. *The Biochemical Journal*, 432, 535–47.
- Romijn, R.A.P., Bouma, B., Wuyster, W., Gros, P., Kroon, J., Sixma, J.J. & Huizinga, E.G. (2001) Identification of the Collagen-binding Site of the von Willebrand Factor A3-domain. *Journal Of Biological Chemistry*, 276, 9985–9991.
- Van Rooijen, N. & Hendriks, E. (2010) Liposomes for specific depletion of macrophages from organs and tissues. *Methods In Molecular Biology (Clifton, N.J.)*, 605, 189–203.
- Van Rooijen, N., Kors, N. & Kraal, G. (1989) Macrophage subset repopulation in the spleen: differential kinetics after liposome-mediated elimination. *Journal Of Leukocyte Biology*, 45, 97–104.
- Van Rooijen, N., Sanders, A. & van den Berg, T.K. (1996) Apoptosis of macrophages induced by liposome-mediated intracellular delivery of clodronate and propamidine. *Journal Of Immunological Methods*, 193, 93–9.
- Rydz, N., Swystun, L.L., Notley, C., Paterson, A.D., Riches, J.J., Sponagle, K., Boonyawat, B., Montgomery, R.R., James, P.D. & Lillicrap, D. (2013) The C-type lectin receptor CLEC4M binds, internalizes, and clears von Willebrand factor and contributes to the variation in plasma von Willebrand factor levels. *Blood*, 121, 5228–37.
- Sadler, J.E., Budde, U., Eikenboom, J.C.J., Favaloro, E.J., Hill, F.G.H., Holmberg, L., Ingerslev, J., Lee, C. a, Lillicrap, D., Mannucci, P.M., Mazurier, C., Meyer, D., Nichols, W.L., Nishino, M., Peake, I.R., Rodeghiero, F., Schneppenheim, R., Ruggeri, Z.M., Srivastava, a, Montgomery, R.R., et al (2006) Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. *Journal Of Thrombosis And Haemostasis JTH*, 4, 2103–2114.
- Saenko, E.L. & Scandella, D. (1995) A mechanism for inhibition of factor VIII binding to phospholipid by von Willebrand factor. *The Journal Of Biological Chemistry*, 270, 13826–13833.

- Saenko, E.L. & Scandella, D. (1997) The acidic region of the factor VIII light chain and the C2 domain together form the high affinity binding site for von Willebrand factor. *Journal Of Biological Chemistry*, 272, 18007–18014.
- Saenko, E.L., Yakhyayev, a V, Mikhailenko, I., Strickland, D.K. & Sarafanov, a G. (1999) Role of the low density lipoprotein-related protein receptor in mediation of factor VIII catabolism. *The Journal Of Biological Chemistry*, 274, 37685–92.
- Saint-Lu, N., Oortwijn, B.D., Pegon, J.N., Odouard, S., Christophe, O.D., de Groot, P.G., Denis, C. V & Lenting, P.J. (2012) Identification of galectin-1 and galectin-3 as novel partners for von Willebrand factor. *Arteriosclerosis, Thrombosis, And Vascular Biology*, 32, 894–901.
- Samor, B., Michalski, J.C., Mazurier, C., Goudemand, M., De Waard, P., Vliegenthart, J.F., Strecker, G. & Montreuil, J. (1989) Primary structure of the major O-glycosidically linked carbohydrate unit of human von Willebrand factor. *Glycoconjugate Journal*, 6, 263–270.
- Savage, B., Almus-Jacobs, F. & Ruggeri, Z.M. (1998) Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. *Cell*, 94, 657–66.
- Savage, B., Saldívar, E. & Ruggeri, Z.M. (1996) Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell*, 84, 289–97.
- Schooten, C.J., Tjernberg, P., Westein, E., Terraube, V., Castaman, G., Mourik, J.A., Hollestelle, M.J., Vos, H.L., Bertina, R.M., Berg, H.M., Eikenboom, J.C.J., Lenting, P.J. & Denis, C. V (2005) Cysteine-mutations in von Willebrand factor associated with increased clearance. *Journal Of Thrombosis And Haemostasis : JTH*, 3, 2228–2237.
- Van Schooten, C.J., Shahbazi, S., Groot, E., Oortwijn, B.D., van den Berg, H.M., Denis, C. V & Lenting, P.J. (2008) Macrophages contribute to the cellular uptake of von Willebrand factor and factor VIII in vivo. *Blood*, 112, 1704–12.
- Schwarz, F. & Aebi, M. (2011) Mechanisms and principles of N-linked protein glycosylation. *Current Opinion In Structural Biology*, 21, 576–82.
- Shahani, T., Covens, K., Lavend’homme, R., Jazouli, N., Sokal, E., Peerlinck, K. & Jacquemin, M. (2014) Human liver sinusoidal endothelial cells but not hepatocytes contain factor VIII. *Journal Of Thrombosis And Haemostasis : JTH*, 12, 36–42.
- Shahbazi, S., Lenting, P.J., Fribourg, C., Terraube, V., Denis, C. V & Christophe, O.D. (2007) Characterization of the interaction between von Willebrand factor and osteoprotegerin. *Journal Of Thrombosis And Haemostasis : JTH*, 5, 1956–62.
- Shi, Q., Kuether, E.L., Schroeder, J.A., Fahs, S.A. & Montgomery, R.R. (2012) Intravascular recovery of VWF and FVIII following intraperitoneal injection and differences from intravenous and subcutaneous injection in mice. *Haemophilia : The Official Journal Of The World Federation Of Hemophilia*, 18, 639–46.
- Shim, K., Anderson, P.J., Tuley, E.A., Wiswall, E. & Sadler, J.E. (2008) Platelet-VWF complexes are preferred substrates of ADAMTS13 under fluid shear stress. *Blood*, 111, 651–657.
- Siedlecki, C.A., Lestini, B.J., Kottke-Marchant, K.K., Eppell, S.J., Wilson, D.L. & Marchant, R.E. (1996) Shear-dependent changes in the three-dimensional structure of human von Willebrand factor. *Blood*, 88, 2939–2950.
- Simon, D.I., Chen, Z., Xu, H., Li, C.Q., Dong, J.F., McIntire, L. V, Ballantyne, C.M., Zhang, L., Furman, M.I., Berndt, M.C. & López, J.A. (2000) Platelet glycoprotein Iba1 is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). *The Journal Of Experimental Medicine*, 192, 193–204.
- Sixma, J.J., Schiphorst, M.E., Verweij, C.L. & Pannekoek, H. (1991) Effect of deletion of the A1 domain of von Willebrand factor on its binding to heparin, collagen and platelets in the presence of ristocetin. *European Journal Of Biochemistry / FEBS*, 196, 369–75.
- Smith, N.L., Chen, M.H., Dehghan, A., Strachan, D.P., Basu, S., Soranzo, N., Hayward, C., Rudan, I., Sabater-Lleal, M., Bis, J.C., de Maat, M.P.M., Rumley, A., Kong, X., Yang, Q., Williams, F.M.K., Vitart, V., Campbell, H., Mälarstig, A., Wiggins, K.L., Van Duijn, C.M., et al (2010) Novel associations of multiple genetic loci with plasma levels of factor VII, factor VIII, and

- von Willebrand factor: The CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) Consortium. *Circulation*, 121, 1382–92.
- Sodetz, J.M., Pizzo, S. V & McKee, P.A. (1977) Relationship of sialic acid to function and in vivo survival of human factor VIII/von Willebrand factor protein. *The Journal Of Biological Chemistry*, 252, 5538–5546.
- Souto, J.C., Almasy, L., Muniz-Diaz, E., Soria, J.M., Borrell, M., Bayen, L., Mateo, J., Madoz, P., Stone, W., Blangero, J. & Fontcuberta, J. (2000) Functional effects of the ABO locus polymorphism on plasma levels of von Willebrand factor, factor VIII, and activated partial thromboplastin time. *Arterioscler Thromb Vasc Biol*, 20, 2024–2028.
- Spiess, M. & Lodish, H.F. (1985) Sequence of a second human asialoglycoprotein receptor: conservation of two receptor genes during evolution. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 82, 6465–6469.
- Spijkers, P.P.E.M., Da Costa Martins, P., Westein, E., Gahmberg, C.G., Zwaginga, J.J. & Lenting, P.J. (2005) LDL-receptor-related protein regulates α_2 -integrin-mediated leukocyte adhesion. *Blood*, 105, 170–177.
- Sporn, L.A., Marder, V.J. & Wagner, D.D. (1989) Differing polarity of the constitutive and regulated secretory pathways for von Willebrand factor in endothelial cells. *The Journal Of Cell Biology*, 108, 1283–9.
- Steer, C.J. & Ashwell, G. (1980) Studies on a mammalian hepatic binding protein specific for asialoglycoproteins. Evidence for receptor recycling in isolated rat hepatocytes. *Journal Of Biological Chemistry*, 255, 3008–3013.
- Stoddart, J.H., Andersen, J. & Lynch, D.C. (1996) Clearance of normal and type 2A von Willebrand factor in the rat. *Blood*, 88, 1692–9.
- Sweeney, J.D., Novak, E.K., Reddington, M., Takeuchi, K.H. & Swank, R.T. (1990) The RIIIS/J inbred mouse strain as a model for von Willebrand disease. *Blood*, 76, 2258–2265.
- Thijssen, V.L., Hulsmans, S. & Griffioen, A.W. (2008) The galectin profile of the endothelium: altered expression and localization in activated and tumor endothelial cells. *The American Journal Of Pathology*, 172, 545–53.
- Thornton, B.P., Větvicka, V., Pitman, M., Goldman, R.C. & Ross, G.D. (1996) Analysis of the sugar specificity and molecular location of the beta-glucan-binding lectin site of complement receptor type 3 (CD11b/CD18). *Journal Of Immunology (Baltimore, Md. : 1950)*, 156, 1235–1246.
- Tirado, I., Mateo, J., Soria, J.M., Oliver, A., Martínez-Sánchez, E., Vallvé, C., Borrell, M., Urrutia, T. & Fontcuberta, J. (2005) The ABO blood group genotype and factor VIII levels as independent risk factors for venous thromboembolism. *Thrombosis And Haemostasis*, 93, 468–474.
- Tischer, A., Cruz, M.A. & Auton, M. (2013) The linker between the D3 and A1 domains of vWF suppresses A1-GPIIb α catch bonds by site-specific binding to the A1 domain. *Protein Science : A Publication Of The Protein Society*, 22, 1049–59.
- Titani, K., Kumar, S., Takio, K., Ericsson, L.H., Wade, R.D., Ashida, K., Walsh, K.A., Chopek, M.W., Sadler, J.E. & Fujikawa, K. (1986) Amino acid sequence of human von Willebrand factor. *Biochemistry*, 25, 3171–3184.
- Toole, J.J., Pittman, D.D., Orr, E.C., Murtha, P., Wasley, L.C. & Kaufman, R.J. (1986) A large region (approximately equal to 95 kDa) of human factor VIII is dispensable for in vitro procoagulant activity. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 83, 5939–5942.
- Turecek, P.L., Gritsch, H., Pichler, L., Auer, W., Fischer, B., Mitterer, A., Mundt, W., Schlokot, U., Dorner, F., Brinkman, H.J., van Mourik, J.A. & Schwarz, H.P. (1997) In vivo characterization of recombinant von Willebrand factor in dogs with von Willebrand disease. *Blood*, 90, 3555–3567.
- Turecek, P.L., Mitterer, A., Matthiessen, H.P., Gritsch, H., Varadi, K., Siekmann, J., Schnecker, K., Plaimauer, B., Kaliwoda, M., Purtscher, M., Woehrer, W., Mundt, W., Muchitsch, E.M.,

- Suiter, T., Ewenstein, B., Ehrlich, H.J. & Schwarz, H.P. (2009) Development of a plasma- and albumin-free recombinant von Willebrand factor. *Hämostaseologie*, 29 Suppl 1, S32–8.
- Valladeau, J., Duvert-Frances, V., Pin, J.J., Kleijmeer, M.J., Ait-Yahia, S., Ravel, O., Vincent, C., Vega, F., Helms, A., Gorman, D., Zurawski, S.M., Zurawski, G., Ford, J. & Saeland, S. (2001) Immature human dendritic cells express asialoglycoprotein receptor isoforms for efficient receptor-mediated endocytosis. *Journal Of Immunology (Baltimore, Md. : 1950)*, 167, 5767–74.
- Varki, A. (2009) Multiple changes in sialic acid biology during human evolution. In *Glycoconjugate Journal* pp 231–245.
- Vlot, A.J., Koppelman, S.J., van den Berg, M.H., Bouma, B.N. & Sixma, J.J. (1995) The affinity and stoichiometry of binding of human factor VIII to von Willebrand factor. *Blood*, 85, 3150–3157.
- Voorberg, J., Fontijn, R., Calafat, J., Janssen, H., van Mourik, J.A. & Pannekoek, H. (1993) Biogenesis of von Willebrand factor-containing organelles in heterologous transfected CV-1 cells. *The EMBO Journal*, 12, 749–58.
- Wagner, D.D. (1990) Cell biology of von Willebrand factor. *Annual Review Of Cell Biology*, 6, 217–246.
- Wagner, D.D., Fay, P.J., Sporn, L.A., Sinha, S., Lawrence, S.O. & Marder, V.J. (1987) Divergent fates of von Willebrand factor and its propolypeptide (von Willebrand antigen II) after secretion from endothelial cells. *Proceedings Of The National Academy Of Sciences*, 84, 1955–1959.
- Wagner, D.D., Mayadas, T. & Marder, V.J. (1986) Initial glycosylation and acidic pH in the Golgi apparatus are required for multimerization of von Willebrand factor. *The Journal Of Cell Biology*, 102, 1320–1324.
- Wagner, D.D., Saffaripour, S., Bonfanti, R., Sadler, J.E., Cramer, E.M., Chapman, B. & Mayadas, T.N. (1991) Induction of specific storage organelles by von Willebrand factor propolypeptide. *Cell*, 64, 403–13.
- Wang, A., Liu, F., Dong, N., Ma, Z., Zhang, J., Su, J., Zhao, Y. & Ruan, C. (2010) Thrombospondin-1 and ADAMTS13 competitively bind to VWF A2 and A3 domains in vitro. *Thrombosis Research*, 126, e260–e265.
- Weiss, H.J., Sussman, I.I. & Hoyer, L.W. (1977) Stabilization of factor VIII in plasma by the von Willebrand factor. Studies on posttransfusion and dissociated factor VIII and in patients with von Willebrand's disease. *The Journal Of Clinical Investigation*, 60, 390–404.
- Wight, J. & Paisley, S. (2003) The epidemiology of inhibitors in haemophilia A: a systematic review. *Haemophilia : The Official Journal Of The World Federation Of Hemophilia*, 9, 418–435.
- Williams, S.B., McKeown, L.P., Krutzsch, H., Hansmann, K. & Gralnick, H.R. (1994) Purification and characterization of human platelet von Willebrands factor. *British Journal Of Haematology*, 197, 582–591.
- Wise, R., Dorner, A., Krane, M., Pittman, D. & Kaufman, R. (1991) The role of von Willebrand factor multimers and propeptide cleavage in binding and stabilization of factor VIII. *J. Biol. Chem.*, 266, 21948–21955.
- Wohner, N., Casari, C., Christophe, O.D., Denis, C. V & and Lenting, P.J. (2013) Enhanced binding of von Willebrand factor mutants to LRP1 may explain their reduced circulatory half-life. *Journal Of Thrombosis And Haemostasis*, 11, 1–84.
- Wright, S.D., Weitz, J.I., Huang, A.J., Levin, S.M., Silverstein, S.C. & Loike, J.D. (1988) Complement receptor type three (CD11b/CD18) of human polymorphonuclear leukocytes recognizes fibrinogen. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 85, 7734–7738.

- Yago, T., Lou, J., Wu, T., Yang, J., Miner, J.J., Coburn, L., López, J.A., Cruz, M.A., Dong, J. fei, Mcintire, L. V, Mcever, R.P. & Zhu, C. (2008) Platelet glycoprotein Ib α forms catch bonds with human WT vWF but not with type 2B von Willebrand disease vWF. *Blood*, 118, 3195–3207.
- Yamamoto, F. (2000) Molecular genetics of ABO. *Vox Sanguinis*, 78 Suppl 2, 91–103.
- Yamamoto, K., de Waard, V., Fearn, C. & Loskutoff, D.J. (1998) Tissue distribution and regulation of murine von Willebrand factor gene expression in vivo. *Blood*, 92, 2791–801.
- Ying, J., Ling, Y., Westfield, L.A., Sadler, J.E. & Shao, J.Y. (2010) Unfolding the A2 domain of von Willebrand factor with the optical trap. *Biophysical Journal*, 98, 1685–93.
- Zanardelli, S., Chion, A.C.K., Groot, E., Lenting, P.J., McKinnon, T.A.J., Laffan, M.A., Tseng, M. & Lane, D.A. (2009) A novel binding site for ADAMTS13 constitutively exposed on the surface of globular VWF. *Blood*, 114, 2819–2828.
- Zannettino, A.C.W., Holding, C.A., Diamond, P., Atkins, G.J., Kostakis, P., Farrugia, A., Gamble, J., To, L.B., Findlay, D.M. & Haynes, D.R. (2005) Osteoprotegerin (OPG) is localized to the Weibel-Palade bodies of human vascular endothelial cells and is physically associated with von Willebrand factor. *Journal Of Cellular Physiology*, 204, 714–723.
- Zhang, Q., Zhou, Y.F., Zhang, C.Z., Zhang, X., Lu, C. & Springer, T.A. (2009) Structural specializations of A2, a force-sensing domain in the ultralarge vascular protein von Willebrand factor. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 106, 9226–9231.
- Zheng, X., Chung, D., Takayama, T.K., Majerus, E.M., Sadler, J.E. & Fujikawa, K. (2001) Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura. *The Journal Of Biological Chemistry*, 276, 41059–63.
- Zhou, M., Dong, X., Baldauf, C., Chen, H., Zhou, Y., Springer, T.A., Luo, X., Zhong, C., Gräter, F. & Ding, J. (2011a) A novel calcium-binding site of von Willebrand factor A2 domain regulates its cleavage by ADAMTS13. *Blood*, 117, 4623–4631.
- Zhou, W., Bouhassira, E.E. & Tsai, H.M. (2007) An IAP retrotransposon in the mouse ADAMTS13 gene creates ADAMTS13 variant proteins that are less effective in cleaving von Willebrand factor multimers. *Blood*, 110, 886–93.
- Zhou, Y.F., Eng, E.T., Nishida, N., Lu, C., Walz, T. & Springer, T.A. (2011b) A pH-regulated dimeric bouquet in the structure of von Willebrand factor. *The EMBO Journal*, 30, 4098–111.
- Zhou, Y.F., Eng, E.T., Zhu, J., Lu, C., Walz, T. & Springer, T. a (2012) Sequence and structure relationships within von Willebrand factor. *Blood*, 120, 449–58.
- Zimmerman, T.S., Voss, R. & Edgington, T.S. (1979) Carbohydrate of the factor VIII/von Willebrand factor in von Willebrand's disease. *The Journal Of Clinical Investigation*, 64, 1298–302.

Appendix I Abstracts leading to poster presentations

1. IDENTIFICATION OF GALECTIN-1 AND GALECTIN-3 AS NOVEL BINDING PARTNERS FOR FACTOR VIII

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

Background

Recent studies have demonstrated that galectin-1 and galectin-3 can bind Von Willebrand Factor (VWF) in plasma and influence VWF-dependent thrombosis formation. Interestingly, the glycan determinants expressed on human FVIII are similar in structure to those on VWF. Although FVIII glycans are important in regulating its biology, little is known regarding FVIII-lectin interactions. In this study, we report for the first time that galectins also directly interact with FVIII and modulate its activity.

Materials and Methods

Galectin-1/-3 were His-tagged and expressed in *E-coli*. Different purified commercial concentrates of recombinant FVIII (rFVIII) were utilised. FVIII glycosylation profiles were systematically modified using specific exoglycosidases. Galectin-FVIII interactions were characterised using immunosorbant assays and surface plasmon resonance (SPR).

Results

Galectin-1 and -3 bound to rFVIII in a dose-dependent manner with high affinity. Removal of rFVIII N-linked glycans significantly reduced galectin-1 and galectin-3 binding ($8.6 \pm 1\%$, $30.3 \pm 3\%$, respectively). In addition, galectin-3 binding was further attenuated by combined removal of N- and O-glycans.

FVIII concentrates are manufactured in heterologous cell systems and are subsequently characterised by the presence of non-human glyco-epitopes. In keeping with these differential glycosylation profiles, both galectin-1/-3 displayed distinct binding affinities for various FVIII concentrates.

The majority of galectin-3 binding is mediated by FVIII B-domain glycans. Interestingly, FVIII high mannose oligosaccharides, which have been previously shown to modulate FVIII immunogenicity *in vitro*, supported a significant proportion of galectin-1.

Galectin-1 was found to directly bind to and precipitate FVIII from plasma. Galectin-1 also served to negatively modulate FVIII functional activity as measured in one-stage clotting assays and reduce intrinsic FXa generation. Conversely, no effect on FVIII function was observed for galectin-3.

Conclusions

Galectin-1 and -3 are novel binding partners for FVIII, binding in a glycan-dependent manner. The ability of differentially glycosylated rFVIII concentrates to interact with these lectins *in vivo* may have implications for understanding the importance of FVIII glycosylation and how it influences function.

8th Bari Interaction Conference, 2014.

2. 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

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*These authors contributed equally to this work.

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 immunosorbant 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 ($13\pm1\%$ and $57\pm2\%$, $p<0.001$). Moreover, removal of both N- and O-linked glycans (PNGase F and O-glycosidase treatment) further attenuated galectin-3 binding ($21\pm1\%$, $p<0.0001$). 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 $\alpha 2$ -3,6,8,9 neuraminidase markedly enhanced binding to galectin-1 and galectin-3 ($231\pm6\%$ and $136\pm6\%$, $p<0.05$). 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 ($914 \pm 50\%$ and $205 \pm 23\%$, $p < 0.05$). Moreover, both galectins bound to the VWD type 2B mimic R1450E with significantly enhanced affinity ($165 \pm 10\%$ and $117 \pm 4\%$, $p < 0.05$). 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 ($23.86 \pm 3\%$, $p < 0.0001$).

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.

3. 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 α 2-3,6,8,9 neuraminidase (Neu-VWF) markedly enhanced binding to galectin-1 ($231\pm6\%$, $p<0.0001$). Similarly, removal of terminal sialic acid also increased binding to galectin-3, albeit to a lesser extent ($136\pm6\%$, $p<0.05$). 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 ($51\pm5\%$ and $52\pm6\%$ 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 ($13\pm1\%$ and $57\pm2\%$, $p<0.001$). Moreover, combined PNGase F and O-glycosidase digestions further attenuated galectin-3 binding ($21\pm1\%$, $p<0.001$), 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 ($146\pm 8\%$ and $483\pm 19\%$; $p<0.01$). 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 $\alpha 1-2$ 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 ($\sim 50\%$), 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 ($72\pm 6\%$ and $67\pm 7\%$ versus pdVWF; $p<0.05$).

53rd American Society of Haematology Annual Meeting, 2011.

4. 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.

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INTRODUCTION

Von Willebrand factor (VWF) is a large multimeric 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 ($K_d=4.7$ 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 ($\alpha 2$ -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 $\alpha 2$ -6 linked sialic acid) play critical roles in regulating these interactions.

References:

- [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 II: Abstracts leading to oral presentations

1. N-Linked glycans within A1A2A3 domains of VWF play a critical role in modulating macrophage-mediated clearance.

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Abstract

Enhanced plasma clearance of von Willebrand factor (VWF) plays an important role in the etiology of both type 1 and type 2 VWD. Nevertheless, although significant progress has been achieved in understanding the structure and functional properties of VWF, the mechanism(s) responsible for modulating VWF clearance from the plasma remain poorly understood. Accumulating recent data suggests that hepatic and splenic macrophages play key roles in modulating VWF clearance. A number of putative macrophage receptors for VWF have been also been described, including LRP1, β 2-integrins and Siglec-5. In addition, it is well recognised that variation in VWF glycan expression significantly influences its clearance rate. In particular, terminal ABO(H) blood group determinants which are predominantly expressed on the N-linked glycans of human VWF significantly modulate its rate of clearance. Critically however, the molecular mechanisms through which specific macrophage receptors interact with particular regions of the complex VWF glycoprotein have not been defined.

To investigate the role of VWF glycans and specific VWF domains in regulating VWF clearance, we expressed and purified a series of recombinant VWF variants and truncations with/without specific glycan sites. In addition, VWF glycosylation was modified using specific exoglycosidase digestions. Subsequently, recombinant VWF variants and glycoforms thereof were intravenously injected into VWF^{-/-} mice, and plasma VWF clearance rates determined by ELISA. VWF-macrophage interactions were also quantified *in vitro* using phorbol ester-differentiated monocytic THP-1 cells and primary human monocytes in a HighContentAnalysis Imaging system.

In keeping with previous reports, we observed that clearance of a truncated VWFA1A2A3 fragment in VWF^{-/-} mice was very similar to that of full length wild-type (WT-) VWF (VWFA1A2A3; $t_{1/2}$ = 6.3 min versus rWT-VWF; $t_{1/2}$ = 7.9 min). Furthermore, chemical depletion of macrophages using clodronate liposomes administration significantly inhibited A1A2A3 clearance *in vivo* (1.7-fold at 10 min time point) to a similar extent to that observed with full length VWF. In vitro binding experiments confirmed that A1A2A3 bound to differentiated THP-1 cells in a dose- and time-dependent manner. Interestingly, this binding was significantly enhanced in the presence of ristocetin. Cumulatively, these data demonstrate that the A1A2A3

domains of VWF contain a critical receptor-binding site for macrophage-mediated clearance.

Interestingly, we observed that the half-life of infused human plasma-derived VWF and recombinant VWF expressed in HEK293T cells in VWF^{-/-} mice were significantly different. Furthermore, treatment with PNGase F to completely remove N-linked glycan structures markedly enhanced the clearance of full length VWF ($t_{1/2}$ 2.1 min; $p < 0.05$). Collectively, these findings highlight the essential roles played by N-glycans in regulating VWF survival. Two N-linked glycan sites are located within A1A2A3 at N1515 and N1574. Importantly, we found that PNGase digestion of A1A2A3 resulted in markedly enhanced macrophage binding *in vitro*. Consequently we hypothesized that the two N-glycans located within the A2 domain might be important in regulating VWF clearance by macrophages. Targeted disruption of these individual N-glycan sites by site-directed mutagenesis (A1A2A3-N1515Q and N1574Q respectively) resulted in significantly enhanced macrophage binding *in vitro* compared to wild-type A1A2A3. Furthermore, following tail vein infusion in VWF^{-/-} mice, full length VWFN1515Q and VWFN1574Q both demonstrated markedly reduced half-lives compared to wild-type VWF (VWFN1515Q; $t_{1/2}$ = 3.7 min, VWFN1574Q; $t_{1/2}$ = 5.5 min). Finally, introduction of the N1515Q into truncated A1A2A3 also served to significantly enhance plasma half-life, (A1A2A3N1515Q-VWF; $t_{1/2}$ = 3.1 min versus A1A2A3-VWF; $t_{1/2}$ = 6.3 min).

In conclusion, our novel data identify a crucial role of the VWF A domains in regulating macrophage-mediated VWF clearance. In addition, we further demonstrate that the N-linked glycan structures located at N1515 and N1574 within the A2 domain play specific roles in protecting VWF against *in vivo* clearance by macrophages. Given the role of enhanced clearance of VWF in patients with type I VWD, these findings are of direct clinical importance.

56th American Society of Haematology Annual Meeting, 2014.

Acknowledgement at the Best of ASH symposium.

2. N-LINKED GLYCANS WITHIN VON WILLEBRAND FACTOR A1A2A3 DOMAINS PLAY A CRITICAL ROLE IN MODULATING VWF CIRCULATORY CLEARANCE VIA MACROPHAGES

Jamie O'Sullivan, Alain Chion, Gudmundur Bergsson, Orla Rawley, Niamh O' Regan, Sean Keyes, Teresa Brophy, James S. O'Donnell.

Haemostasis Research Group, Trinity College Dublin.

Introduction

The biosynthesis and functional properties of VWF have been well characterised, however the molecular mechanisms underlying VWF circulatory clearance remains poorly defined. Moreover, emerging evidence indicates that accelerated clearance represents a pathophysiological mechanism in VWD.

In-vivo studies have demonstrated that liver and splenic macrophages play a key role in mediating cellular uptake and clearance of VWF from circulation. Additionally VWF glycosylation, including ABO sugars expressed predominantly on N-glycans, are critical determinants of VWF survival *in vivo*.

Methodology

We constructed a series of VWF truncated fragments with/without key glycan sites. Subsequently, these recombinant VWF variants were intravenously injected into VWF^{-/-} mice, and plasma VWF clearance rates determined by ELISA. VWF-macrophage interactions were also quantified *in vitro* using differentiated monocytic THP-1 cells.

Results

Firstly, we observed that clearance of a truncated VWF-A1A2A3 fragment in VWF^{-/-} mice was similar to that of full length wild-type VWF (WT-VWF). Furthermore, chemical depletion of macrophages using clodronate liposomes significantly inhibited both VWF-A1A2A3 and WT-VWF clearance *in vivo* to a similar extent. *In vitro* binding experiments confirmed that VWF-A1A2A3 bound to differentiated THP-1 macrophages in a dose-dependent manner. Collectively, these data demonstrate that the A1A2A3 domains of VWF contain a critical receptor-binding site for macrophage-mediated clearance.

We observed that the clearance rates of plasma-derived (pd) and recombinant (r) VWF are markedly different. However, removal of the N-glycans population on both pd-VWF and r-VWF significantly reduces their half-lives such that the clearance rates of both are comparable. Cumulatively, these data indicate that the N-linked glycans expressed on VWF play a critical role in regulating plasma clearance.

Two N-linked glycan sites are located within A1A2A3 at N1515 and N1574. Importantly, we observed that enzymatic removal of these glycans resulted in markedly enhanced macrophage binding of VWF-A1A2A3 *in vitro*. Consequently we hypothesized that these N-glycans may be important in regulating VWF clearance by macrophages. Indeed, site-directed mutagenesis of N1515Q within VWF-A1A2A3 significantly reduced its half-life compared to wild-type A1A2A3. Moreover, macrophage depletion *in-vivo* inhibited this accelerated clearance.

Conclusions

These data demonstrate a crucial role of the VWF A domains in regulating macrophage-mediated VWF clearance. In addition, we identify that the N-linked glycans within the A2 domain play specific roles in protecting VWF against *in vivo* clearance by macrophages. These data provide insight into the complex mechanism(s) of VWF clearance from circulation and highlight that variation in VWF glycosylation represents a key regulator of VWF survival.

Haematology Association Ireland, Annual Meeting 2014.

Oral presentation, President's Symposium.

Winner: President's Prize, Best Overall Abstract.

3. 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.

Haemostasis Research Group, Trinity College Dublin.

During biosynthesis, Factor VIII (FVIII) undergoes complex post-translational modification including significant glycosylation. Consequently each FVIII molecule can contain 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 (K_d of 0.11 ± 0.02 nM for Gal-1 and 0.21 ± 0.1 nM 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 ($8.6 \pm 1\%$; $p < 0.0001$). In contrast, PNG-FVIII retained significant ability to bind Gal-3 ($30.3 \pm 3\%$; $p < 0.0001$). However, combined FVIII digestion with both PNGase F and O glycosidase further attenuated Gal-3 binding ($16.5 \pm 2\%$; $p < 0.05$). 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 ($42 \pm 1\%$ and $26 \pm 0.8\%$; $p < 0.0001$). 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 ($107 \pm 2\%$ and $124 \pm 1\%$; $p < 0.05$). These findings are consistent with the fact that the N-linked glycans of BHK-derived FVIII express galactose $\alpha 1-3$ 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 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 (0.5-17 μ M) 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 20 μ M, 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.

55th American Society of Haematology Annual Meeting, 2013.

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

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Haemostasis Research Group, Institute of Molecular Medicine, St. James's Hospital, Trinity College Dublin, Ireland.

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.

5. IDENTIFICATION OF GALECTIN-1 AND GALECTIN-3 AS NOVEL BINDING PARTNERS FOR FACTOR VIII

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

Human FVIII is extensively glycosylated, with 25 N- and 6 O-linked glycans, which are essential for its activity. However, the molecular mechanism through which these sugars modulate FVIII biology is not defined. Recently it's been demonstrated that the plasma carbohydrate-binding galectin (Gal) -1 and -3 can bind VWF and influence its function. In this study, we demonstrate that galectins also interact with procoagulant FVIII and modulate its activity.

Gal-1 and -3 were expressed in *E-coli* and purified by nickel chromatography. Recombinant FVIII (rFVIII) was purified from different commercial concentrates. FVIII glycosylation was modified using specific exoglycosidases. Interactions were characterised using immunosorbant assays and surface plasmon resonance (SPR).

Gal-1 and Gal-3 bound to rFVIII in a dose-dependent manner with unusually high affinities. Removal of FVIII N-linked glycans ablated Gal-1 binding but had a less marked effect on Gal-3 ($8.6 \pm 1\%$, $30.3 \pm 3\%$; $p < 0.0001$). However, combined removal of N- and O- FVIII glycans further attenuated Gal-3 binding ($16.5 \pm 2\%$). In keeping with their differential glycosylation profiles, different commercial FVIII concentrates displayed distinct affinities for both galectins. Finally, preincubation of FVIII with Gal-1 ($0.5\text{-}17\mu\text{M}$) resulted in a prolonged one-stage clotting assay (58 ± 0.2 sec vs. 26 ± 3 sec, $p < 0.001$). Conversely, no effect was observed for Gal-3, suggesting these galectins may have differential effects on FVIII biology.

We identify Gal-1 and Gal-3 as novel ligands for FVIII. Both the N- and O-linked carbohydrates of FVIII contribute to galectin binding. Furthermore, Gal-1 binding can modulate the procoagulant activity of FVIII.

Haematology Association Ireland, Annual Meeting 2013.
Best Oral presentation.

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

Rawley O, O'Sullivan J, O'Regan N, Chion A, Brophy T, Jenkins PV, O'Donnell JS.

Haemostasis Research Group, Institute of Molecular Medicine, St. James's Hospital, Trinity College Dublin, Ireland.

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 ($21 \pm 1\%$, $p < 0.001$). Further enzymatic modification of N- and O-linked sugars demonstrated that loss of N-linked capping sialic acid significantly enhanced galectin-3 binding ($136 \pm 6\%$, $p < 0.05$). Additionally, preferential binding to blood group AB vs group O VWF ($483 \pm 19\%$, vs $116 \pm 7\%$, $p < 0.0001$) was observed. Interestingly, targeted removal of individual N-linked sugars 1515 and 1574 within the VWF A2 domain led to dramatically reduced binding ($24 \pm 3\%$, and $44 \pm 3\%$). 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.

7. GALECTIN-1 BINDING TO VWF IN NORMAL PLASMA: CRITICAL ROLES FOR SPECIFIC TERMINAL GLYCAN DETERMINANTS AND AMINO ACID RESIDUES ADJACENT TO THE ADAMTS13 CLEAVAGE SITE.

O'Sullivan J, Rawley O, O'Regan N, Chion A, Brophy T, Jenkins PV, O'Donnell JS.

Haemostasis Research Group, Institute of Molecular Medicine, St. James's Hospital, Trinity College Dublin, Ireland.

Introduction

Physiological thrombus formation is normally regulated in part by ADAMTS13 proteolysis of VWF multimers. Emerging data suggest that this proteolysis may be promoted by galectin binding to VWF. We therefore sought to characterise the molecular basis through which galectin-1 interacts with VWF.

Methods

Plasma-derived (pd-VWF) was purified from commercial Haemate P by gel filtration. VWF glycosylation profile was modified using exoglycosidases. Recombinant VWF and a series of VWF mutants were expressed in HEK293T cells. Recombinant galectin-1 was transiently expressed in competent *E-coli* cells and purified by nickel chromatography.

Results

Removal of VWF N-linked glycan structures resulted in an $86\pm1\%$ ($p<0.0001$) loss in galectin-1 binding. Elimination of terminal sialic acid from VWF glycans enhanced galectin-1 binding ($231\pm6\%$ $p<0.0001$). In contrast, specific removal of O-glycan sialylation, or indeed entire O-linked glycosylation, had no significant effect on galectin-1 binding. In addition, site-directed mutagenesis of two fucosylated N-linked glycans sites, N1515A and N1574A, located in close proximity to the ADAMTS13 cleavage site, resulted in markedly attenuated galectin-1 binding (32.43% and 32.47%). Finally, recombinant VWF containing a classical Type 2B mutation (R1450E) demonstrated significantly increased galectin-1 binding. Similarly, VWF pre-incubation with ristocetin (which influences VWF A1A2A3 conformation) also resulted in enhanced galectin-1 interaction ($914\pm4\%$ $p<0.0001$).

Conclusions

Recent data have clearly demonstrated that in normal plasma, VWF circulates in complex with galectins. Our novel data demonstrate that VWF conformation, and the N-linked sugars expressed on VWF, both play critical roles in regulating binding to galectin-1, and thus in turn defining VWF susceptibility to ADAMTS13 proteolysis.

Haematology Association Ireland, Annual Meeting 2012.

Appendix III Publications

Brief Report

THROMBOSIS AND HEMOSTASIS

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

Rachel T. McGrath,¹ Maartje van den Biggelaar,² Barry Byrne,³ Jamie M. O'Sullivan,¹ Orla Rawley,¹ Richard O'Kennedy,³ Jan Voorberg,² Roger J. S. Preston,⁴ and James S. O'Donnell^{1,5}

¹Haemostasis Research Group, Institute of Molecular Medicine, Trinity Centre for Health Sciences, St James's Hospital, Trinity College Dublin, Ireland; ²Department of Plasma Proteins, Sanquin Research, Amsterdam, The Netherlands; ³Biomedical Diagnostics Institute and Applied Biochemistry Group, Dublin City University, Ireland; ⁴National Children's Research Centre, Our Lady's Children's Hospital, Crumlin, Dublin, Ireland; and ⁵National Centre for Hereditary Coagulation Disorders, St James's Hospital, Dublin, Ireland.

Key Points

- Platelet-VWF exists as a distinct natural glycoform.
- Platelet-VWF is resistant to ADAMTS13 proteolysis.

Platelet-von Willebrand factor (VWF) is stored within α -granules and accounts for ~20% of total VWF in platelet-rich plasma. This platelet-VWF pool is distinct from plasma-VWF and is enriched in high molecular weight multimers (HMWM). Previous studies have described significant functional discrepancies between platelet-VWF and plasma-VWF; however, the molecular basis of these differences is not well understood. We have characterized terminal glycan expression on platelet-VWF. Our findings demonstrate that platelet-VWF exists as a distinct natural glycoform. In particular, *N*-linked sialylation is

markedly reduced (>50%) compared with plasma-VWF. Moreover, unlike plasma-VWF, platelet-VWF does not express AB blood group determinants, although precursor H antigen expression is similar to that on plasma-VWF. Because of this differential glycosylation, platelet-VWF exhibits specific resistance to ADAMTS13 proteolysis. Thus platelet activation at sites of vascular injury results in the release of high local concentrations of HMWM platelet-VWF that is more resistant to ADAMTS13, thereby facilitating platelet-plug formation. (*Blood*. 2013;122(25):4107-4110)

Introduction

Von Willebrand factor (VWF) is a large multimeric sialoglycoprotein that plays critical roles in normal hemostasis. Besides circulating in plasma, significant amounts of VWF are also stored within the α -granules of platelets.^{1,2} This platelet-VWF is synthesized during megakaryocytopoiesis and accounts for 10% to 20% of the total VWF present in normal platelet-rich plasma.²⁻⁴ Importantly, the platelet-VWF pool is distinct from plasma-VWF and is enriched in hemostatically active high molecular weight multimers (HMWM).^{5,6} Consequently, high local concentrations of HMWM platelet-VWF are released from α -granules at sites of vascular injury following platelet activation. Accumulating data from in vitro and in vivo studies suggest that both plasma-VWF and platelet-VWF play critical roles in primary hemostasis.⁷⁻¹⁰ Furthermore, reduced platelet-VWF levels have also been implicated in mediating the pathogenesis of bleeding disorders including von Willebrand disease and essential thrombocythemia.^{3,6}

Previous studies have described several important functional differences between platelet-VWF and plasma-VWF. In particular, despite the fact that platelet-VWF is enriched in HMWM, it binds to platelet GpIb α with significantly lower affinity compared with plasma-VWF.¹¹ In contrast, platelet-VWF demonstrates significantly enhanced binding to both GpIIb/IIIa and heparin.¹¹ The molecular mechanisms responsible for these differences have not been defined, but are likely to relate to variations in the posttranslational modification of platelet-VWF (synthesized within megakaryocytes) as

opposed to plasma-VWF (synthesized within endothelial cells). The *N*- and *O*-linked glycosylation profiles of plasma-VWF have been characterized in detail.^{12,13} The glycan profile of platelet-VWF has not been elucidated. However, preliminary data suggest that these structures may differ significantly from those expressed on plasma-VWF.^{11,14} In recent studies, we and others have clearly demonstrated the key role played by VWF glycans in regulating proteolysis by ADAMTS13.¹⁵⁻¹⁸ Importantly, expression of both ABO blood group antigens and terminal sialic acid on VWF were shown to be of critical importance.^{15,16,18} In this study, we have characterized terminal carbohydrate expression on platelet-VWF. Our findings demonstrate that platelet-VWF exists as a distinct natural glycoform, with a particular marked reduction in *N*-linked sialic acid expression compared with plasma-VWF. Moreover, as a result of this difference in posttranslational modification, platelet-VWF exhibits specific resistance to ADAMTS13 proteolysis.

Study design

Isolation and purification of platelet- and plasma-VWF

Platelets were isolated from platelet-rich plasma as previously described.¹⁹ Platelets were then lysed by performing repeated snap freeze-thaw cycles in the presence of protease inhibitors (Protease Inhibitor Cocktail I, Calbiochem,

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There is an Inside *Blood* commentary on this article in this issue.

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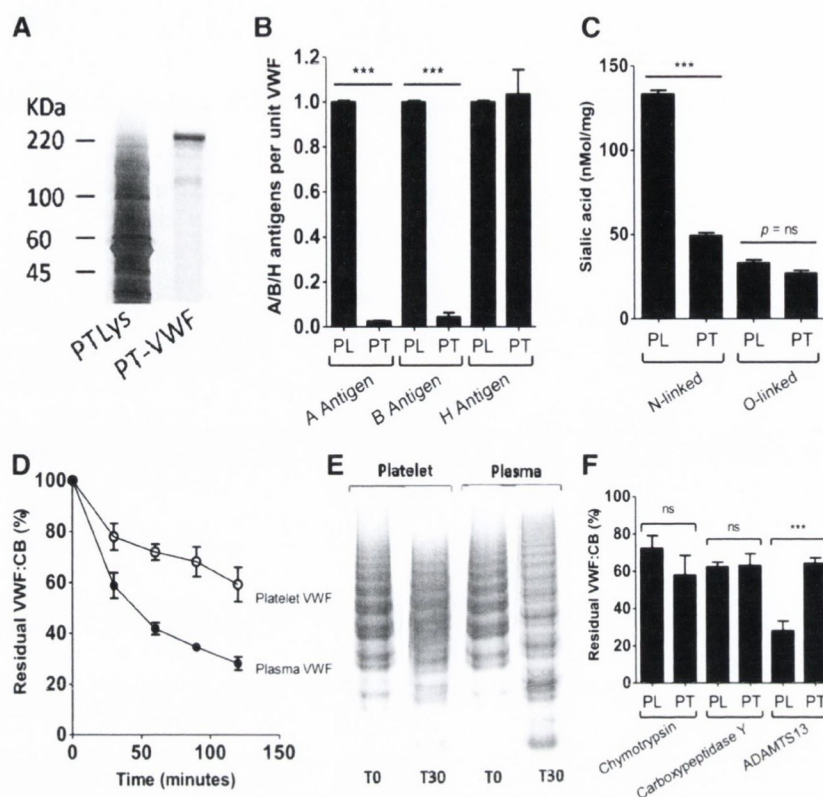


Figure 1. Glycosylation of platelet-VWF and susceptibility to ADAMTS13 proteolysis. (A) Platelet (PT)-VWF was purified from lysed (Lys) human platelets by immunoaffinity chromatography and analyzed by SDS-polyacrylamide gel electrophoresis and subsequent silver staining. (B) ABO(H) blood group antigen expression on plasma (PL)-VWF and PT-VWF were quantified using lectin plate-binding assays. All experiments were performed in triplicate, and the results shown represent the mean $\pm$ SEM ($***P < .001$). (C) Reverse-phase HPLC analysis was used to quantify sialic acid expression on purified PL-VWF and PT-VWF. Total sialic acid expression on PT-VWF was significantly reduced compared with PL-VWF (76 vs 167 nmol/mg; $***P < .001$). To determine relative quantitative sialic acid expression levels on the *N*-linked glycans of VWF, HPLC analysis of residual VWF-bound sialic acid was performed following digestion with *O*-glycosidase. To determine quantitative sialic acid expression on the *O*-linked glycans of VWF, HPLC analysis of residual VWF-bound sialic acid was performed following digestion with PNGase F. (ns, *P* value is nonsignificant). (D) To investigate whether altered glycosylation on platelet-VWF influences susceptibility to ADAMTS13 proteolysis, PT-VWF and PL-VWF were incubated with 3 nM recombinant human ADAMTS13 in the presence of 1.5 M urea. Rate of cleavage was assessed by determining the reduction in VWF:CB over time. Results (mean of 5 experiments $\pm$ SEM) are expressed as the percentage residual collagen-binding activity. In some cases, SEM cannot be seen due to its small size. (E) The susceptibility of PT-VWF and PL-VWF multimers to digestion with recombinant human ADAMTS13 in the presence of 1.5 M urea was further assessed by performing standard nonreducing SDS agarose gel electrophoresis at baseline (T0) and following a 30-minute incubation (T30). (F) To establish whether the altered glycosylation profile of PT-VWF influenced susceptibility to other nonspecific proteases, PL-VWF and PT-VWF were also treated with chymotrypsin (30 U/mg VWF) and carboxypeptidase Y (19 U/mg VWF) at 37°C for 90 minutes. Results are shown as residual VWF:CB at 90 minutes $\pm$ SEM.

Merck, UK) followed by centrifugation at 10 000g to clear cellular debris. Finally, platelet-VWF was purified by immunoaffinity chromatography employing the monoclonal antibody CLB-RAG20 as previously described in detail.²⁰ Plasma-derived VWF was purified by cryoprecipitation and 2BCL gel filtration as before,¹⁸ followed by CLB-RAG20 immunoaffinity chromatography.²⁰

ADAMTS13 expression and proteolysis of VWF

Recombinant human ADAMTS13 was purified and quantified following stable expression in HEK293 cells as previously described.¹⁶ ADAMTS13-VWF cleavage assays were then performed. In brief, 10 μ g/mL of VWF was incubated with 3 nM ADAMTS13 in the presence of 1.5 M urea and 10 mM BaCl₂. At specific time points (0, 30, 60, 90, and 120 minutes), subsamples were removed and 10 mM EDTA was added to stop the cleavage reaction. VWF proteolysis was analyzed using VWF collagen-binding assay (VWF:CB) and sodium dodecyl sulfate (SDS) agarose gel electrophoresis.¹⁶ In preliminary experiments, we established that plasma- and platelet-VWF bound to type III collagen with similar affinities (data not shown). Chymotrypsin (30 U/mg VWF) and carboxypeptidase Y (19 U/mg VWF) digestion of VWF were performed at 37°C for 90 minutes and the extent of cleavage was assessed using a VWF:CB as before.

Glycan analysis and glycosidase treatment of VWF

Lectin plate-binding assays were used to analyze quantitative ABO(H) expression on plasma- and platelet-VWF.²¹ In addition, terminal *N*- and

O-linked sialic acid expression on VWF was quantified using high-performance liquid chromatography (HPLC) analysis as before.¹⁸ To modify endogenous VWF glycan structures, purified plasma- or platelet-VWF (final concentration, 65 μ g/mL) were incubated for 2 hours at 37°C with specific exoglycosidases including α 2-3,6,8,9 neuraminidase (2500 U/mg; Calbiochem, Merck Chemicals Ltd, UK); PNGase F (1000 U/mg; New England BioLabs, UK); α 2-3 neuraminidase (Sigma, Ireland), and *O*-glycosidase (5 U/mg; Sigma, Ireland). This methodology has been described in detail elsewhere.¹⁸

Data were analyzed using the GraphPad Prism program (GraphPad Prism version 5.0 for Windows; GraphPad Software, San Diego, CA). Experiments were performed in triplicate. All data are expressed as mean $\pm$ standard error of the mean (SEM). To assess statistical differences, the data were analyzed using unpaired 2-tailed Student *t* test. $P < .05$ was considered significant.

Results and discussion

Upon purification, platelet-VWF was visible as a ~220-kDa band when analyzed by SDS-polyacrylamide gel electrophoresis analysis, similar to that previously observed for plasma-VWF (Figure 1A).^{14,16} Low-resolution SDS agarose gel electrophoresis confirmed that

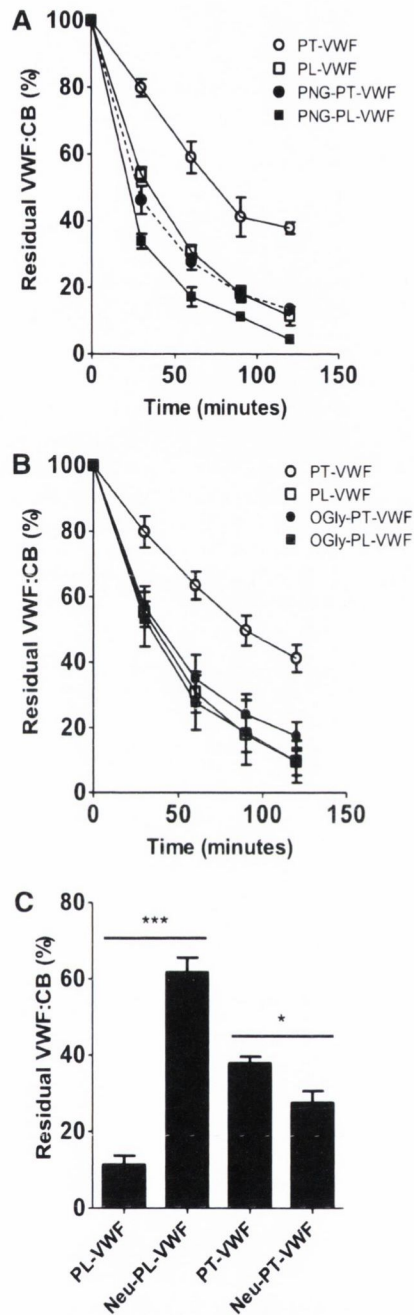


Figure 2. Role of glycans expression in regulating the ADAMTS13-resistant phenotype of PL-VWF. (A) PL-VWF and PT-VWF were incubated with PNGase F to remove *N*-linked glycans. Subsequently, the effects of *N*-linked deglycosylation in regulating susceptibility to ADAMTS13 proteolysis were investigated. Removal of *N*-linked glycans significantly increased PL-VWF cleavage by ADAMTS13 (PL-VWF [□] vs PNG-PL-VWF [■]; $P < .001$ at all time points). Treatment of platelet-VWF with PNGase F was also associated with significantly enhanced proteolysis by ADAMTS13 (PT-VWF [□] vs PNG-PT-VWF [●]; $P < .001$). Nevertheless, PNG-treated PT-VWF remained more resistant to ADAMTS13 proteolysis compared with PNG-treated PL-VWF. All experiments were performed a minimum of 3 times and results are expressed as mean $\pm$ SEM at 120 minutes. (B) PL-VWF and PT-VWF were incubated with *O*-glycosidase (OGly) to remove *O*-linked glycans. Subsequently, the effects of *O*-linked deglycosylation in regulating susceptibility to ADAMTS13 proteolysis were investigated. Removal of *O*-linked glycans from PL-VWF had no significant effect on cleavage by ADAMTS13 (PL-VWF [□] vs OGly-PL-VWF [■]). In contrast, treatment of PL-VWF with OGly was associated with significantly enhanced proteolysis by ADAMTS13 (PT-VWF [□] vs PNG-PT-VWF [●]; $P < .01$). (C) PL-VWF and PT-VWF were incubated with α 2-3,6,8,9 neuraminidase to remove terminal sialic acid. Subsequently, the effects of desialylation

platelet-VWF was enriched in HMWM compared with plasma-VWF (data not shown). In keeping with previous reports, A and B blood group antigens were strongly expressed on plasma-VWF in a blood group-specific manner (Figure 1B).^{16,22} In contrast however, A and B antigens were not expressed on platelet-VWF. This finding is interesting given that ABO(H) blood group determinants are abundantly expressed on several different platelet membrane glycoproteins including GpIa, Ib, IIa, and IV.⁴ H antigen (Fuc α 1 $\rightarrow$ 2 Gal β 1 $\rightarrow$ 4 GlcNAc β 1 $\rightarrow$) is the essential carbohydrate acceptor for either α -1,3-*N*-acetylgalactosaminyltransferase (A transferase) or α -1,3-galactosyltransferase (B transferase).¹⁶ Individuals with the rare Bombay phenotype fail to express H antigen and therefore cannot synthesize A or B antigenic structures regardless of their ABO blood group genotype. In spite of the absence of A and B determinant expression on platelet-VWF, we observed strong expression of H antigen on platelet-VWF (Figure 1B). Indeed, quantitative H antigen expression levels on platelet-VWF were strikingly similar to that observed on plasma-VWF.

Plasma-VWF is heavily sialylated.^{12,13} Moreover, *N*- and *O*-linked sialic acid expression plays a critical role in modulating VWF function, proteolysis, and clearance.²³⁻²⁵ Reverse-phase HPLC analysis demonstrated that total sialic acid expression on platelet-VWF was significantly reduced compared with plasma-VWF (76 nmol/mg vs 167 nmol/mg; $P < .001$). Importantly, this reduction in total sialylation was almost entirely attributable to a specific reduction in sialic acid expression on the *N*-glycans of platelet-VWF (49.2 nmol/mg vs 134.9 nmol/mg; $P < .001$) (Figure 1C). In contrast, quantitative *O*-linked sialic acid expression on platelet-VWF (26.9 nmol/mg) and plasma-VWF (30.2 nmol/mg) were similar.

Terminal sialic acid and ABO(H) determinants expressed on plasma-VWF critically regulate susceptibility to ADAMTS13 proteolysis.^{15,16,18} Given the marked differences in terminal *N*-linked glycan expression on platelet-VWF, we investigated platelet-VWF proteolysis by ADAMTS13. Interestingly, ADAMTS13 proteolysis of platelet-VWF was significantly attenuated compared with plasma-VWF (Figure 1D-E). At all time points after 30 minutes, platelet-VWF showed significant increased resistance to ADAMTS13 ($P < .05$). For example, after incubation with ADAMTS13 (3 nM for 90 minutes) platelet VWF:CB was reduced to 68.2% compared with 34.5% for plasma-VWF ($P < .01$). In contrast to its ADAMTS13-resistant phenotype, platelet-VWF displayed similar susceptibility to cleavage by other serine and cysteine proteases as plasma-VWF (Figure 1F).

To elucidate the molecular basis underlying this specific ADAMTS13-resistant phenotype, plasma- and platelet-VWF were treated with specific exoglycosidases. In keeping with previous findings, removal of *N*-linked glycans from plasma-VWF (PNG-PL-VWF) enhanced the rate of proteolysis by ADAMTS13 (Figure 2A).^{17,18} Similarly, removal of *N*-linked glycans from platelet-VWF (PNG-PT-VWF) also significantly increased the rate of ADAMTS13 cleavage ($P < .05$). Nevertheless, PNG-PT-VWF still cleaved significantly more slowly than PNG-PL-VWF,

Figure 2 (continued) in regulating susceptibility to ADAMTS13 proteolysis were investigated as previously described. Removal of sialic acid from PL-VWF (neuraminidase [Neu]-PL-VWF) significantly attenuated proteolysis by ADAMTS13 ($***P < .001$). In keeping with its markedly reduced sialic acid expression levels, Neu digestion of PT-VWF demonstrated only a small effect on susceptibility to ADAMTS13 cleavage.

demonstrating that *O*-linked glycans on platelet-VWF also play a role in modulating ADAMTS13 resistance. This hypothesis was supported by the observation that removal of *O*-linked glycans from platelet-VWF also enhanced the rate of proteolysis by ADAMTS13 (Figure 2B).

Recent work from our laboratory demonstrated that expression of α 2-6-linked sialic acid on plasma-VWF promotes cleavage by ADAMTS13. Consequently, neuraminidase treatment of plasma-VWF significantly attenuates ADAMTS13 proteolysis ($P < .01$; Figure 2C). In contrast, in keeping with its markedly reduced *N*-linked sialic acid expression levels, neuraminidase digestion of platelet-VWF had a negligible effect upon susceptibility to ADAMTS13 cleavage (Figure 2C). Given the reduced sialic acid present on platelet-VWF, we hypothesize that the wild-type platelet-VWF glycoform is similar to neuraminidase-treated plasma-VWF, such that further removal of sialic acid from platelet-VWF does not promote resistance to ADAMTS13. However, definitive glycome mapping studies will be required to determine how sialic acid expression varies across the 12 *N*-linked glycan sites of platelet-VWF.

In conclusion, these novel data demonstrate that platelet-VWF exists as a distinct natural glycoform, with a particularly marked reduction in *N*-linked sialylation. As a result of this differential glycan profile, platelet-VWF exhibits resistance to ADAMTS13 proteolysis. Thus, at sites of vascular injury, not only will high local concentrations of HMW platelet-VWF be released, but this VWF will be partially resistant to ADAMTS13 further enhancing platelet plug formation.

Further in vivo studies will be needed to elucidate the physiological significance of the ADAMTS13-resistant phenotype of platelet-VWF.

Acknowledgments

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Authorship

Contribution: R.T.M., M.v.d.B., B.B., J.M.O., and O.R. performed experiments; R.T.M., M.v.d.B., B.B., O.R., R.O., J.V., R.J.S.P., and J.S.O. designed the research and analyzed the data; and all authors were involved in writing and reviewing the paper.

Conflict-of-interest disclosure: J.S.O. has served on the speaker's bureau for Baxter, Bayer, Novo Nordisk, Leo Pharma, and Octapharma; has served on the advisory boards of Baxter, Bayer, Octapharma, and Pfizer; and has received research grant funding awards from Baxter, Bayer, and Novo Nordisk.

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References

- Howard MA, Montgomery DC, Hardisty RM. Factor-VIII-related antigen in platelets. *Thromb Res*. 1974;4(5):617-624.
- Nachman RL, Jaffe EA. Subcellular platelet factor VIII antigen and von Willebrand factor. *J Exp Med*. 1975;141(5):1101-1113.
- Mannucci PM. Platelet von Willebrand factor in inherited and acquired bleeding disorders. *Proc Natl Acad Sci USA*. 1995;92(7):2428-2432.
- McGrath RT, McRae E, Smith OP, O'Donnell JS. Platelet von Willebrand factor—structure, function and biological importance. *Br J Haematol*. 2010; 148(6):834-843.
- Sultan Y, Jeanneau C, Lamaziere J, Maisonneuve P, Caen JP. Platelet factor VIII-related antigen: studies in vivo after transfusion in patients with von Willebrand disease. *Blood*. 1978;51(4): 751-761.
- Ruggeri ZM, Zimmerman TS. Variant von Willebrand's disease: characterization of two subtypes by analysis of multimeric composition of factor VIII/von Willebrand factor in plasma and platelets. *J Clin Invest*. 1980;65(6):1318-1325.
- Fressinaud E, Baruch D, Rothschild C, Baumgartner HR, Meyer D. Platelet von Willebrand factor: evidence for its involvement in platelet adhesion to collagen. *Blood*. 1987;70(4): 1214-1217.
- Bowie EJ, Solberg LA Jr, Fass DN, et al. Transplantation of normal bone marrow into a pig with severe von Willebrand's disease. *J Clin Invest*. 1986;78(1):26-30.
- Nichols TC, Bellinger DA, Reddick RL, et al. The roles of von Willebrand factor and factor VIII in arterial thrombosis: studies in canine von Willebrand disease and hemophilia A. *Blood*. 1993;81(10):2644-2651.
- Kanaji S, Fahs SA, Shi Q, Haberichter SL, Montgomery RR. Contribution of platelet vs. endothelial VWF to platelet adhesion and hemostasis. *J Thromb Haemost*. 2012;10(8): 1646-1652.
- Williams SB, McKeown LP, Krutzsch H, Hansmann K, Gralnick HR. Purification and characterization of human platelet von Willebrand factor. *Br J Haematol*. 1994;88(3):582-591.
- Canis K, McKinnon TAJ, Nowak A, et al. The plasma von Willebrand factor O-glycome comprises a surprising variety of structures including ABH antigens and disialosyl motifs. *J Thromb Haemost*. 2010;8(1):137-145.
- Canis K, McKinnon TA, Nowak A, et al. Mapping the N-glycome of human von Willebrand factor. *Biochem J*. 2012;447(2):217-228.
- Kagami K, Williams S, Horne M, Gralnick H. A preliminary analysis of platelet von Willebrand factor oligosaccharides. *Nagoya J Med Sci*. 2000; 63(1-2):51-56.
- Bowen DJ. An influence of ABO blood group on the rate of proteolysis of von Willebrand factor by ADAMTS13. *J Thromb Haemost*. 2003;1(1): 33-40.
- O'Donnell JS, McKinnon TA, Crawley JT, Lane DA, Laffan MA. Bombay phenotype is associated with reduced plasma-VWF levels and an increased susceptibility to ADAMTS13 proteolysis. *Blood*. 2005;106(6):1988-1991.
- McKinnon TAJ, Chion ACK, Millington AJ, Lane DA, Laffan MA. N-linked glycosylation of VWF modulates its interaction with ADAMTS13. *Blood*. 2008;111(6):3042-3049.
- McGrath RT, McKinnon TA, Byrne B, et al. Expression of terminal alpha2-6-linked sialic acid on von Willebrand factor specifically enhances proteolysis by ADAMTS13. *Blood*. 2010;115(13): 2666-2673.
- Coppinger JA, Cagney G, Toomey S, et al. Characterization of the proteins released from activated platelets leads to localization of novel platelet proteins in human atherosclerotic lesions. *Blood*. 2004;103(6):2096-2104.
- van den Biggelaar M, Meijer AB, Voorberg J, Mertens K. Intracellular cotrafficking of factor VIII and von Willebrand factor type 2N variants to storage organelles. *Blood*. 2009;113(13): 3102-3109.
- O'Donnell J, Boulton FE, Manning RA, Laffan MA. Amount of H antigen expressed on circulating von Willebrand factor is modified by ABO blood group genotype and is a major determinant of plasma von Willebrand factor antigen levels. *Arterioscler Thromb Vasc Biol*. 2002;22(2):335-341.
- Brown SA, Collins PW, Bowen DJ. Heterogeneous detection of A-antigen on von Willebrand factor derived from platelets, endothelial cells and plasma. *Thromb Haemost*. 2002;87(6):990-996.
- Ellies LG, Ditto D, Levy GG, et al. Sialyltransferase ST3Gal-IV operates as a dominant modifier of hemostasis by concealing asialoglycoprotein receptor ligands. *Proc Natl Acad Sci USA*. 2002;99(15):10042-10047.
- van Schooten CJ, Denis CV, Lisman T, et al. Variations in glycosylation of von Willebrand factor with O-linked sialylated T antigen are associated with its plasma levels. *Blood*. 2007; 109(6):2430-2437.
- Grewal PK, Uchiyama S, Ditto D, et al. The Ashwell receptor mitigates the lethal coagulopathy of sepsis. *Nat Med*. 2008;14(6): 648-655.



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

Rachel T. McGrath, Maartje van den Biggelaar, Barry Byrne, Jamie M. O'Sullivan, Orla Rawley, Richard O'Kennedy, Jan Voorberg, Roger J. S. Preston and James S. O'Donnell

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BRIEF REPORT

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

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Summary. *Background:* Enhanced von Willebrand factor (VWF) clearance is important in the etiology of type 1 and type 2 von Willebrand disease (VWD). More than 20 different VWF point mutations have already been reported in patients with enhanced clearance. These include the VWD-Vicenza variant, which is characterized by an Arg1205His substitution in the VWF D3 domain. Critically, however, the molecular mechanisms through which single amino acid substitutions in VWF result in enhanced clearance of this complex multimeric glycoprotein have not been defined. *Objectives:* In this study, we have investigated the biological basis underlying the enhanced clearance of the VWF-R1205H variant. *Methods:* Using VWF^{-/-} mice, *in vivo* clearance rates were determined for a series of full-length and truncated recombinant VWF variants. In addition, the role of macrophages in modulating enhanced VWD-Vicenza clearance was investigated using clodronate liposome administration. *Results:* Our findings demonstrate that substitutions of R1205 with histidine, cysteine or serine all result in markedly reduced survival of full-length recombinant VWF. Importantly, D'A3 fragments containing these same R1205 substitutions also demonstrated significantly enhanced clearance. In contrast to the reduced *in vivo* survival observed with R1205H, clearance of R1204H was not enhanced. Recent studies have dem-

onstrated that hepatic and splenic macrophages play key roles in regulating VWF clearance. Importantly, macrophage-depletion also served to markedly attenuate the enhanced clearance phenotypes associated with VWF-R1205H, VWF-R1205S and VWF-R1205C. *Conclusions:* Collectively, these novel findings demonstrate a specific and critical role for the R1205 residue in modulating macrophage-mediated clearance of VWF *in vivo*.

Keywords: glycosylation; metabolic clearance rate; von Willebrand disease, Type 1; von Willebrand disease, Type 2; von Willebrand factor.

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 to VWF antigen (VWF : Ag) ratio is markedly elevated. Following DDAVP administration, plasma VWF : Ag levels increase significantly in VWD-Vicenza individuals [11,12]. However, the half-life of the secreted VWF-R1205H is markedly reduced compared with that of wild-type (WT) VWF [10–12].

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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 with that of human WT-VWF in VWF [13]. Subsequently, Pruss *et al.* showed that the half-life of murine VWF-R1205H was also significantly reduced following intravenous injection [14]. In addition, enhanced clearance of R1205H-VWF was also observed following hydrodynamic expression in these VWF-deficient mice [14]. 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, and have described a number of putative macrophage receptors [15,16]. Furthermore, variation in VWF glycans including terminal sialic acid and ABO(H) blood group antigen expression have also been shown to regulate VWF clearance [17,18]. Finally, an important role for the hepatic asialoglycoprotein receptor (ASGPR) in modulating VWF half-life has also been demonstrated [19]. 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 has been described previously [20]. The Kapa HiFi HotStart PCR Kit (Anachem, Bedfordshire, UK) was used to generate point mutations (R1204H, R1205C, R1205H, and R1205S) in both full-length VWF and D'A3-VWF constructs. The presence of all mutations was verified by Sanger sequencing. 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 h and concentrated via anion exchange chromatography as before [21]. Full-length VWF variants were further concentrated using 100-kDa cut-off spin filters (Millipore, Cork, Ireland). D'A3-VWF variants were further purified via nickel affinity chromatography. VWF concentration was subsequently determined by ELISA as previously described [22].

VWF clearance studies in VWF^{-/-} mice

VWF^{-/-} mice were obtained from the Jackson Laboratory (Sacramento, CA, USA) on a C57Bl/6 background.

All animal experiments were performed in compliance with the Irish Medicines Board regulations, on mice between 6 and 8 weeks of age. Briefly, VWF^{-/-} mice were intravenously injected with 300 nM VWF or fragments thereof, diluted in 100 µL sterile-filtered PBS. Blood from a subclavicle incision was sampled into heparin-coated microcontainers (BD Unitech, Dublin, Ireland) at 3 min post-injection and then at 5-min intervals up to 30 min. Plasma VWF : Ag levels were then 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 where residual VWF represents the amount of VWF present in plasma at given time-points relative to the initial amount injected. For macrophage depletion, mice were intravenously injected with clodronate liposomes (100 µL per 10 g body weight) 24 h prior to injection of VWF as previously described [23]. In preliminary studies, and in keeping with previous studies, we determined that pretreatment with clodronate liposomes resulted in a 75% reduction of F4/80⁺/CD11b⁺ macrophages compared with PBS-liposome treated controls (data not shown).

Statistical analysis

Data were analyzed using GraphPad Prism software (GraphPad Prism version 5.0 for Windows; GraphPad Software, San Diego, CA, USA). 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 (Fig. 1A). In keeping with a previous study that investigated clearance of recombinant murine R1205H expressed in HEK293-T cells [13], clearance of recombinant human VWF-R1205H was approximately 1.5-fold faster than that of human WT-VWF. Two other substitutions of arginine 1205 (with cysteine and serine, respectively) have also been reported in individual patients with significantly reduced plasma VWF levels [24]. Both patients had reduced plasma VWF : Ag levels, and a reduced VWF half-life following DDAVP administration. To determine whether these substitutions also directly influenced VWF survival *in vivo*, we further characterized 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 with WT-VWF (Fig. 1B). Indeed, VWF-R1205C and VWF-R1205S were actually cleared more rapidly than VWF-R1205H.

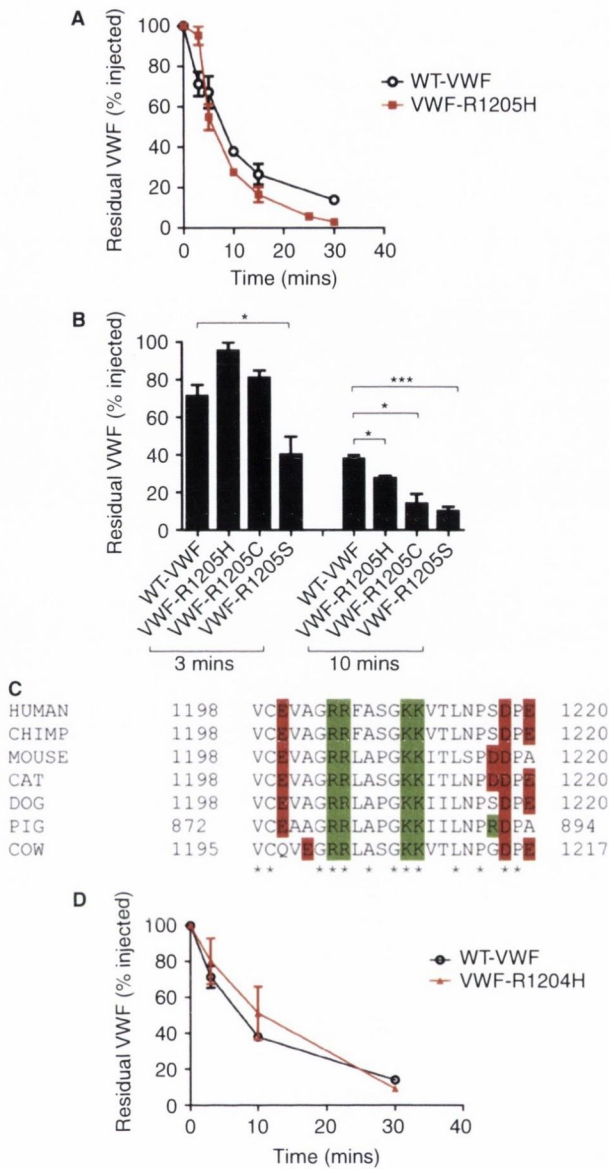


Fig. 1. Arginine 1205 in the D3 domain plays a critical role in regulating von Willebrand factor clearance *in vivo*. (A) Recombinant wild-type (WT) and R1205H-human VWF were expressed in HEK293T cells. To study *in vivo* survival, VWF^{-/-} mice were intravenously injected with purified VWF and blood 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 min following intravenous administration and clearance compared with 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 that 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. In contrast to the enhanced clearance phenotypes associated with the R1205H, R1205S and R1205C substitutions, VWF-R1204H clearance did not differ significantly from that of WT-VWF. In some cases, SEM cannot be seen due to its small size.

The arginine 1205 residue in human VWF is highly conserved across other species. Of note, another conserved arginine is located at residue 1204 (Fig. 1C). 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

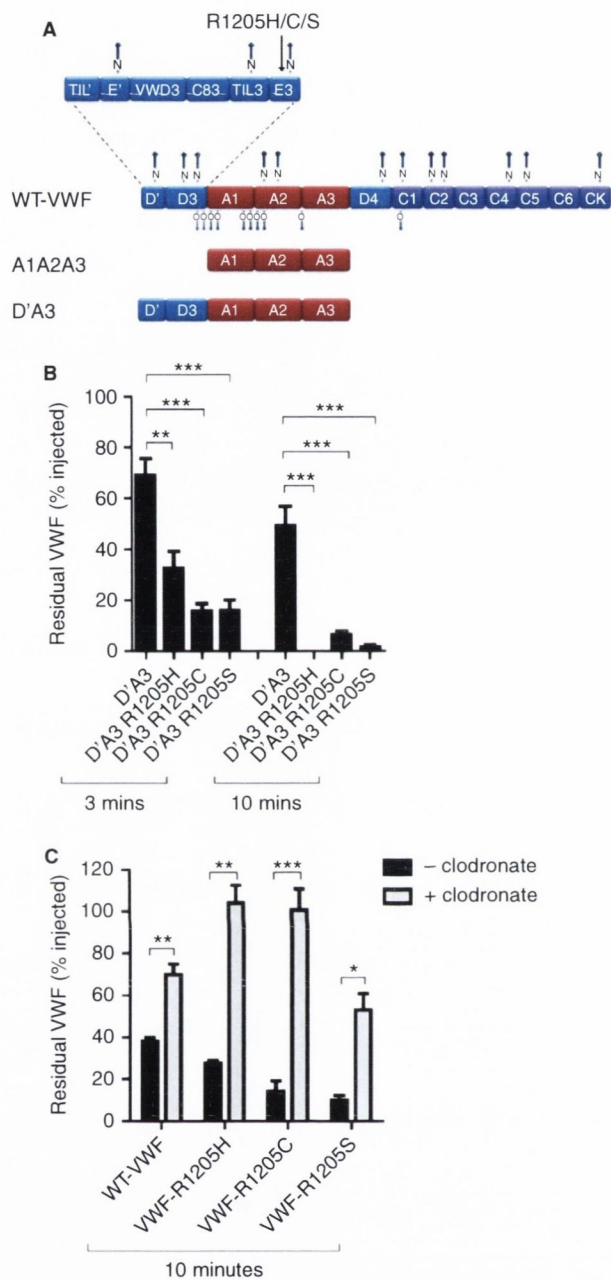


Fig. 2. Macrophages play a major role in modulating the enhanced clearance associated with VWF R1205 substitutions. (A) Diagram illustrates the domain structure of full-length VWF. In addition, the domains present in the truncated VWF variants expressed in this study are also shown. In the amplified 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 with wild-type D'A3. Plasma samples were collected at 3 and 10 min following intravenous administration. Ten minutes following infusion, residual plasma levels of the D'A3 truncations containing R1205H, R1205S and R1205C were significantly reduced compared with wild-type D'A3 ($^{**}P < 0.001$ and $^{***}P < 0.0001$, respectively). (C) 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 h following clodronate administration. 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 at 10 min post-VWF infusion ($^{*}P < 0.05$, $^{**}P < 0.001$ and $^{***}P < 0.0001$, respectively).

phenotype observed with the R1205H substitution, clearance of VWF-R1204H was not significantly different from that of WT-VWF (Fig. 1D). 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 (Fig. 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 [13]. 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 [13]. To investigate the molecular mechanism(s) responsible for the reduced plasma survival associated with R1205 substitution, we further characterized the effects of introducing the R1205H, R1205S and R1205C substitutions into the D3 domain of the D'A3 fragment. Interestingly, in keeping with their involvement in reducing the survival of full-length VWF, each of these R1205 substitutions resulted in significantly enhanced clearance of the D'A3 fragment (Fig. 2B). Moreover, the effect of these mutations in causing enhanced clearance was more pronounced in the D'A3 fragment compared with full-length VWF. 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 [25]. Alternatively, R1205 substitutions may serve to enhance VWF clearance by creating a novel receptor-binding site within the D'D3 region.

Emerging data suggest that hepatic and splenic macrophages can also bind and endocytose VWF, and thus play a role in regulating VWF clearance [15,16]. In keeping with these previous reports, we confirmed that macrophage depletion with clodronate significantly attenuated *in vivo* clearance of WT-VWF in VWF^{-/-} mice (Fig. 2C). Importantly, macrophage depletion also significantly prolonged the survival of VWF-R1205H, R1205S and R1205C. Interestingly, macrophage depletion inhibited VWF-R1205H clearance to a greater degree than that observed with WT-VWF, suggesting that macrophage-mediated clearance is the predominant pathway through which R1205H is cleared.

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. Furthermore, this enhanced clearance of VWF was mediated through a

macrophage-dependent mechanism. Further studies will be necessary to define the specific macrophage receptors involved in the etiology of this clinically important enhanced VWF clearance phenotype. Moreover, it remains unclear whether additional distinct VWF clearance pathways may also be important in causing the accelerated clearance associated with other VWF amino acid substitutions, particularly those located in different VWF domains.

Addendum

O. Rawley, J. M. O'Sullivan, A. Chion, S. Keyes, R. J. S. Preston and T. M. Brophy performed experiments; O. Rawley, J. M. O'Sullivan, A. Chion, S. Keyes, M. Lavin, N. van Rooijen, P. Fallon, T. M. Brophy and J. S. O'Donnell designed the research and analyzed the data. All authors were involved in writing and reviewing the paper.

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Disclosure of Conflict of Interests

J. S. O'Donnell 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'Donnell has also received research grant funding awards from Baxter, Bayer and Novo Nordisk.

References

- 1 Casari C, Lenting PJ, Wohner N, Christophe OD, Denis CV. Clearance of von Willebrand factor. *J Thromb Haemost* 2013; **11** (Suppl 1): 202–11.
- 2 Castaman G, Tosi A, Rodeghiero F. Reduced von Willebrand factor survival in von Willebrand disease: pathophysiologic and clinical relevance. *J Thromb Haemost* 2009; **7** (Suppl 1): 71–4.
- 3 Brown SA, Eldridge A, Collins PW, Bowen DJ. Increased clearance of von Willebrand factor antigen post-DDAVP in Type 1 von Willebrand disease: is it a potential pathogenic process? *J Thromb Haemost* 2003; **1**: 1714–7.
- 4 Denis CV, Christophe OD, Oortwijn BD, Lenting PJ. Clearance of von Willebrand factor. *Thromb Haemost* 2008; **99**: 271–8.
- 5 Schooten CJ, Tjernberg P, Westein E, Terraube V, Castaman G, Mourik JA, Hollestelle MJ, Vos HL, Bertina RM, Berg HM, Eikenboom JC, Lenting PJ, Denis CV. Cysteine-mutations in von Willebrand factor associated with increased clearance. *J Thromb Haemost* 2005; **3**: 2228–37.
- 6 Habichter SL, Balistreri M, Christopherson P, Morateck P, Gavazova S, Bellissimo DB, Manco-Johnson MJ, Gill JC, Montgomery RR. Assay of the von Willebrand factor (VWF) propeptide to identify patients with type 1 von Willebrand disease with decreased VWF survival. *Blood* 2006; **108**: 3344–51.
- 7 Habichter SL, Castaman G, Budde U, Peake I, Goodeve A, Rodeghiero F, Federici AB, Batlle J, Meyer D, Mazurier C, Goudemand J, Eikenboom J, Schneppenheim R, Ingerslev J,

- Vorlova Z, Habart D, Holmberg L, Lethagen S, Pasi J, Hill FG, et al. Identification of type 1 von Willebrand disease patients with reduced von Willebrand factor survival by assay of the VWF propeptide in the European study: molecular and clinical markers for the diagnosis and management of type 1 VWD (MCMDM-1VWD). *Blood* 2008; **111**: 4979–85.
- 8 Casonato A, Gallinaro L, Cattini MG, Pontara E, Padrini R, Bertomoro A, Daidone V, Pagnan A. Reduced survival of type 2B von Willebrand factor, irrespective of large multimer representation or thrombocytopenia. *Haematologica* 2010; **95**: 1366–72.
 - 9 Eikenboom J, Federici AB, Dirven RJ, Castaman G, Rodeghiero F, Budde U, Schneppenheim R, Batlle J, Canciani MT, Goude-mand J, Peake I, Goodeve A, Group M-VS. VWF propeptide and ratios between VWF, VWF propeptide, and FVIII in the characterization of type 1 von Willebrand disease. *Blood* 2013; **121**: 2336–9.
 - 10 Casonato A, Pontara E, Sartorello F, Cattini MG, Sartori MT, Padrini R, Girolami A. Reduced von Willebrand factor survival in type Vicenza von Willebrand disease. *Blood* 2002; **99**: 180–4.
 - 11 Mannucci PM, Lombardi R, Castaman G, Dent JA, Lattuada A, Rodeghiero F, Zimmerman TS. von Willebrand disease “Vicenza” with larger-than-normal (supranormal) von Willebrand factor multimers. *Blood* 1988; **71**: 65–70.
 - 12 Gezsi A, Budde U, Deak I, Nagy E, Mohl A, Schlammadinger A, Boda Z, Masszi T, Sadler JE, Bodo I. Accelerated clearance alone explains ultra-large multimers in von Willebrand disease Vicenza. *J Thromb Haemost* 2010; **8**: 1273–80.
 - 13 Lenting PJ, Westein E, Terraube V, Ribba AS, Huizinga EG, Meyer D, de Groot PG, Denis CV. An experimental model to study the in vivo survival of von Willebrand factor. Basic aspects and application to the R1205H mutation. *J Biol Chem* 2004; **279**: 12102–9.
 - 14 Pruss CM, Golder M, Bryant A, Hegadorn CA, Burnett E, Laverty K, Sponagle K, Dhala A, Notley C, Haberichter S, Lillicrap D. Pathologic mechanisms of type 1 VWD mutations R1205H and Y1584C through in vitro and in vivo mouse models. *Blood* 2011; **117**: 4358–66.
 - 15 van Schooten CJ, Shahbazi S, Groot E, Oortwijn BD, van den Berg HM, Denis CV, Lenting PJ. Macrophages contribute to the cellular uptake of von Willebrand factor and factor VIII in vivo. *Blood* 2008; **112**: 1704–12.
 - 16 Rastegarlar G, Pegon JN, Casari C, Odouard S, Navarrete AM, Saint-Lu N, van Vlijmen BJ, Legendre P, Christophe OD, Denis CV, Lenting PJ. Macrophage LRP1 contributes to the clearance of von Willebrand factor. *Blood* 2012; **119**: 2126–34.
 - 17 Ellies LG, Ditto D, Levy GG, Wahrenbrock M, Ginsburg D, Varki A, Le DT, Marth JD. Sialyltransferase ST3Gal-IV operates as a dominant modifier of hemostasis by concealing asialoglycoprotein receptor ligands. *Proc Natl Acad Sci U S A* 2002; **99**: 10042–7.
 - 18 Gallinaro L, Cattini MG, Sztukowska M, Padrini R, Sartorello F, Pontara E, Bertomoro A, Daidone V, Pagnan A, Casonato A. A shorter von Willebrand factor survival in O blood group subjects explains how ABO determinants influence plasma von Willebrand factor. *Blood* 2008; **111**: 3540–5.
 - 19 Grewal PK, Uchiyama S, Ditto D, Varki N, Le DT, Nizet V, Marth JD. The Ashwell receptor mitigates the lethal coagulopathy of sepsis. *Nat Med* 2008; **14**: 648–55.
 - 20 McKinnon TA, Chion AC, Millington AJ, Lane DA, Laffan MA. N-linked glycosylation of VWF modulates its interaction with ADAMTS13. *Blood* 2008; **111**: 3042–9.
 - 21 Nowak AA, Canis K, Riddell A, Laffan MA, McKinnon TA. O-linked glycosylation of von Willebrand factor modulates the interaction with platelet receptor glycoprotein Ib under static and shear stress conditions. *Blood* 2012; **120**: 214–22.
 - 22 McGrath RT, McKinnon TA, Byrne B, O’Kennedy R, Terraube V, McRae E, Preston RJ, Laffan MA, O’Donnell JS. Expression of terminal alpha2-6-linked sialic acid on von Willebrand factor specifically enhances proteolysis by ADAMTS13. *Blood* 2010; **115**: 2666–73.
 - 23 van Rooijen N, Kors N, Kraal G. Macrophage subset repopulation in the spleen: differential kinetics after liposome-mediated elimination. *J Leukoc Biol* 1989; **45**: 97–104.
 - 24 Millar CM, Riddell AF, Brown SA, Starke R, Mackie I, Bowen DJ, Jenkins PV, van Mourik JA. Survival of von Willebrand factor released following DDAVP in a type 1 von Willebrand disease cohort: influence of glycosylation, proteolysis and gene mutations. *Thromb Haemost* 2008; **99**: 916–24.
 - 25 Ulrichs H, Udvardy M, Lenting PJ, Pareyn I, Vandeputte N, Vanhoorelbeke K, Deckmyn H. Shielding of the A1 domain by the D’D3 domains of von Willebrand factor modulates its interaction with platelet glycoprotein Ib-IX-V. *J Biol Chem* 2006; **281**: 4699–707.