

**Defining the mechanisms through which von Willebrand Factor
sialic acid expression regulates *in vivo* clearance**



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Declaration

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Signed,

Soracha E. Ward

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- **von Willebrand factor clearance – biological mechanisms and clinical significance.**

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Summary

Von Willebrand Factor (VWF) is a complex plasma sialoglycoprotein which plays a key role in maintaining normal haemostasis. While the biosynthesis, structure, and functions of VWF are well characterised, the molecular mechanisms regarding VWF clearance remain poorly understood. Nonetheless, enhanced VWF clearance is of major importance in the pathophysiology underlying von Willebrand disease (VWD). The programme of work detailed in this thesis examined the role of VWF glycosylation and lectin receptors in modulating circulatory clearance.

Previous studies have shown that loss of terminal sialic acid causes enhanced VWF clearance through the Asialoglycoprotein receptor (ASGPR). Importantly however a number of other lectin clearance receptors have also been implicated in regulating the clearance of hyposialylated glycoproteins. In initial studies, the specific importance of N- vs O-linked sialic acid in protecting against VWF clearance was investigated. Subsequently, a potential role for contributions by additional lectin receptors in modulating the reduced half-life of hyposialylated VWF was also studied.

α 2-3-linked sialic acid accounts for less than 20% of the total sialic acid expressed on VWF and is predominantly present on O-linked glycans. Nevertheless, specific digestion with α 2-3 neuraminidase (α 2-3Neu-VWF) was sufficient to cause markedly enhanced VWF clearance. Interestingly, *in vivo* clearance experiments in dual *VWF^{-/-}/Asgr1^{-/-}* mice demonstrated enhanced clearance of α 2-3Neu-VWF even in the absence of the ASGPR. Collectively these findings suggest that O-linked glycan structures on VWF play an important role in protecting against premature clearance, and that other lectin receptors besides ASGPR are important in regulating this clearance.

The macrophage galactose-type lectin (MGL) is a C-type lectin that binds to glycoproteins expressing terminal N-acetylgalactosamine or galactose residues. Importantly, the markedly enhanced clearance of hyposialylated VWF in *VWF^{-/-}/Asgr1^{-/-}* mice was significantly attenuated in the presence of an anti-MGL inhibitory antibody. Furthermore, dose-dependent binding of human VWF to purified recombinant human MGL was confirmed using surface plasmon resonance. Additionally, plasma VWF:Ag levels were significantly elevated in *MGL1^{-/-}* mice compared with controls. Together, these findings identify MGL as a novel macrophage lectin receptor involved in VWF clearance *in vivo*. Importantly, these studies also demonstrate that MGL is important in regulating the clearance of both wild-type and hyposialylated VWF.

Previous studies have reported that as glycoproteins age in plasma, there is progressive loss of terminal sialylation from N-linked glycans. Interestingly, in further studies we have shown that VWF ageing is also associated with hyposialylation. This loss of terminal sialic acid residues is modulated through neuraminidase enzymes present in normal plasma. Resulting in exposure of sub-terminal galactose, driving clearance through the ASGPR and MGL receptors. Interestingly, inhibition of plasma neuraminidases was associated with a significant prolongation in VWF half-life and a secondary increase in endogenous murine plasma VWF levels. These exciting data suggest that neuraminidase inhibition may offer a novel mechanism through which to increase plasma VWF levels in patients with VWD.

Abbreviations

AA	Amino acid
ADAMTS13	A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13
AIC	Akaike Information Criterion
AMR	Ashwell Morell receptor
ASF	Asialofetuin
ASGPR	Asialoglycoprotein receptor
ASGPR1	Asialoglycoprotein receptor subunit 1
ASGPR2	Asialoglycoprotein receptor subunit 2
ASOR	Asialoorsomuroid
BCA	Bicinchoninic acid
bPEI	Branched polyethylenimine
BSA	Bovine serum albumin
BFA	Brefeldin A
C8	Cysteine 8
cAMP	Cyclic adenosine monophosphate
CHO	Chinese hamster ovary cells
CLEC4M	C-type lectin domain family 4-member M
CRD	Carbohydrate recognition domain
CTCK	C-terminal cysteine knot
DC	Dendritic cell
DDAVP	1-desamino-8-D arginine vasopressin
EC	Endothelial cell
ECA	<i>Erythrina cristagalli</i> lectin
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
ER	Endoplasmic reticulum
FPLC	Fast protein liquid chromatography
Fuc	Fucose
FVIII	Factor VIII
Gal	Galactose
Gal-1	Galectin 1
Gal-3	Galectin 3
Galgt2	N-acetylgalactosaminyltransferase
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
HEK	Human embryonic kidney
HIS	Histidine
HMWM	High molecular weight multimers
HPLC	High performance liquid chromatography
HPRA	Health product regulatory authority

HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cells
IP	Intraperitoneal
IV	Intravenous
LMWM	Low molecular weight multimers
LoVIC	Low von Willebrand in Ireland cohort
LRP1	Low density lipoprotein receptor-related protein 1
LSEC	Liver sinusoidal endothelial cell
Man	Mannose
MAL II	<i>Maackia amurensis</i> lectin II
MGL	Macrophage galactose lectin Macrophage mannose receptor
MMR	receptor
MOPS	3-(N-morpholino)propanesulfonic acid
MRT	Mean residence time
MS	Mass spectrometry
NeuAc	Neuraminic acid
Neu-VWF	Neuraminidase treated VWF
NHS-biotin	N-hydroxysuccinimidobiotin
OC	Oseltamivir carboxylate
OP	Oseltamivir phosphate
PACE	Paired dibasic amino-acid cleaving enzyme
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pd-VWF	Plasma derived von Willebrand factor
PLA	Proximity ligation assay
PNA	Peanut Agglutinin
PNG	Peptide N-glycosidase
RAP	Receptor associated protein
RCA	<i>Ricinus communis</i> agglutinin I
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SE	Size exclusion
SEM	Standard error of the mean
Siglec5	Sialic acid-binding Ig-like lectin 5
SNA	<i>Sambucus Nigra</i>
SPR	Surface plasmon resonance
SR-A1	Scavenger receptor class A member 1
SRCL	Scavenger receptor C-type lectin
STAB2	Stabilin 2
ST3Gal-IV	Gal-Beta-1,4-GalNAc-Alpha-2,3-Sialyltransferase
T _{1/2}	Half life
T antigen	Thomas Friedenreich antigen
TAE	Tris-acetate-EDTA buffer

TBE	Tribromoethanol
TC	Tris citrate
TIL	Trypsin-inhibitor-like
TTP	Thrombotic thrombocytopenic purpura
UL-VWF	Ultra large von Willebrand factor
WiN	Willebrand in the Netherlands
VWD	von Willebrand disease
VWF	von Willebrand factor
VWF:Ag	von Willebrand factor antigen
VWFpp	von Willebrand factor propeptide
WGA	Wheat germ agglutinin
WP	Weibel Palade

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1. Introduction

The program of work in this thesis is focused on examining the role of sialylation in regulating VWF clearance. Consequently, Chapter 1 provides background information relating to three discrete sections; (i) VWF structure function, (ii) VWF sialylation and (iii) VWF clearance.

von Willebrand disease (VWD) was first described by Erik von Willebrand in the Aland Islands in 1926 (Willebrand, 1926). von Willebrand described a significant bleeding disorder in a young girl who exhibited profound menstrual bleeding and died upon her fourth period (Nilsson, 1999). The disease could be differentiated from Haemophilia A by the autosomal pattern of inheritance and the type of bleeding, with mucocutaneous bleeding being a hallmark of the condition.

Von Willebrand factor (VWF) is a large multimeric plasma glycoprotein which plays an integral role in regulating haemostasis. VWF acts as the protective carrier molecule for pro-coagulant Factor VIII (FVIII), preventing premature proteolytic degradation and rapid removal from circulation (Weiss *et al*, 1977; Lollar *et al*, 1988). VWF also plays a role in mediating primary haemostasis whereby VWF binds exposed subendothelial collagen at sites of vascular injury. Subsequently, VWF binds and sequesters platelets, enabling formation of the primary platelet plug (Savage *et al*, 1996, 1998). VWF circulates in normal plasma at a concentration of approximately 100 IU/dL. However a wide normal plasma range is observed from 50-200 IU/dL (Favaloro *et al*, 1993). Quantitative or qualitative VWF deficiency results in VWD, the commonest inherited bleeding disorder in man (Rodeghiero *et al*, 1987). VWD is classified into three major

groups (Table 1.1). Type 1 VWD involves a partial quantitative deficiency in VWF antigen (VWF:Ag). Patients who exhibit intermediate plasma VWF:Ag levels (30-50 IU/dL) are considered in a distinct category labelled "Low VWF". Type 2 VWD is characterised by a qualitative defect in VWF function. Finally, Type 3 VWD results from a complete absence of VWF from circulation (Sadler *et al*, 2006). Conversely, elevated plasma levels of the VWF are associated with an increased risk of myocardial infarction, ischaemic stroke and venous thromboembolism (Nossent *et al*, 2006; Atiq *et al*, 2018; Conlan *et al*, 1993; Wieberdink *et al*, 2010; Apostolova *et al*, 2018; Bongers *et al*, 2006; Crawley *et al*, 2008).

VWD Type	Description
<u>Low VWF</u>	Partial quantitative VWF deficiency Plasma VWF:Ag 30-50 IU/dL
<u>Type 1</u>	Partial quantitative VWF deficiency Plasma VWF:Ag <30 IU/dL
<u>Type 2</u>	Qualitative VWF deficiency
2A	Decreased VWF-dependant platelet adhesion and loss of HMWM
2B	Increased affinity for platelet GpIb α
2M	Decreased VWF dependent platelet adhesion with no loss of HMWM
2N	Decreased binding affinity of VWF for FVIII
<u>Type 3</u>	Virtual complete deficiency of VWF

Table 1.1: Classification of VWD

1.1. VWF gene

The *VWF* gene is positioned on chromosome 12p13.3. The cDNA sequence of human *VWF* was elucidated by a number of groups in the late 1980's (Ginsburg *et al*, 1985; Sadler *et al*, 1985; Verweij *et al*, 1986; Bonthron *et al*, 1986). The *VWF* gene is composed of 52 exons and is 178kb in size. The first 17 exons encode the signal peptide and the *VWF* propeptide (*VWF:pp*), with the remaining exons encoding the mature *VWF* subunit. In addition, chromosome 22 (22q11) contains a non-processed *VWF* pseudogene which consists of exons 23-34, with 97% sequence homology with the *VWF* gene (Mancuso *et al*, 1991).

1.2. VWF biosynthesis

1.2.1. VWF tissue expression

Biosynthesis of *VWF* is limited to vascular endothelial cells (EC) and megakaryocytes (Sporn *et al*, 1985; Wagner & Marder, 1983). In adult C57Bl/6 mice, *VWF* gene expression levels have been shown to vary in different vascular beds, such that *VWF* mRNA concentrations were highest in the lung, followed by the spleen, aorta, brain, kidney, skeletal muscle, gut and liver (Yamamoto *et al*, 1998). Furthermore, immunohistochemical analysis has demonstrated that *VWF* expression is higher in venous vascular beds rather than arterial (Rand *et al*, 1987). Additionally, *VWF* expression can be modulated in diseased states such as cancer, chronic hypertension and cirrhosis (O'Sullivan *et al*, 2018a; Atiq *et al*, 2018; Lisman *et al*, 2006).

1.2.2. *VWF* gene regulation

Early investigations into cell specific expression of *VWF* *in vitro* demonstrated a complex mechanism with both positive and negative regulatory factors (Jahroudi & Lynch, 1994). A 734-bp cell specific promoter was identified -487 to +247-bp relative to the *VWF* transcription start site. A negative regulatory region is present in most cell types. ECs overcome this inhibition via a positive regulatory motif, consisting of SP1, GATA and octamer binding sites resulting in endothelial specific gene expression (Jahroudi & Lynch, 1994). Further studies have identified transcription factors GATA, Ets, H1, NFAT5 and E4BP4 as activators of *VWF* transcription (Liu *et al*, 2009; Schwachtgen *et al*, 1997; Wang *et al*, 2004; Dmitrieva & Burg, 2014; Hough *et al*, 2005), while transcription factor Oct-1 is a repressor of transcription (Schwachtgen *et al*, 1998). Recently, epigenetic regulation of *VWF* gene expression has been implicated in modulating VWD severity, with higher promoter methylation being associated with lower *VWF* levels (Kuldanek *et al*, 2014).

1.3. VWF domain structure

The *VWF* gene translational product consists of a 22 amino acid signal peptide, a 741 amino acid propeptide and the mature 2050 amino acid VWF subunit (Wagner, 1990).

The historical annotation of the VWF domain structure described the VWF propeptide as domains D1-D2, with the mature VWF subunit consisting of D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK (Sadler, 1998). With advancements in electron microscopy and *in silico* analysis, the structure has recently been re-annotated D1-D2-D'D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CTCK wherein each D domain has been subdivided into VWD-C8-TIL-E motifs (Zhou *et al*, 2012) (Figure 1.1).

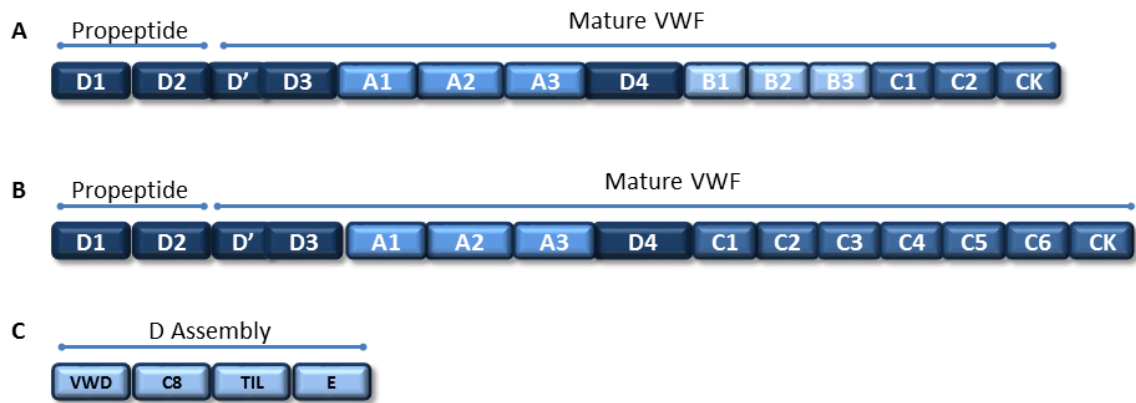


Figure 1.1: VWF Domain Structure

A) Historical annotation of VWF domains

B) Re-annotated VWF domain structure

C) Components of D assembly

1.3.1. VWF D domains

VWF propeptide consists of the D1 and D2 domains only, while the mature VWF monomer begins with a D'D3 domain. The VWF D1, D2 and D'D3 domains are essential for the formation of Weibel-Palade bodies within ECs (Journet *et al*, 1993; Huang *et al*, 2008). Electron microscopy (EM) illustrates the D domains as composed of smaller lobules which self-associate (Zhou *et al*, 2012). VWF D domains are subdivided into; VWD, cysteine 8 (C8), trypsin-inhibitor-like (TIL) and an E module, with D' containing only TIL and E sub-domains, and D4 also containing a unique motif between A3 and D4 called D4N (Zhou *et al*, 2012).

1.3.2. VWF A domains

VWF A domains, are large globular structures with hydrophobic cores (Zhou *et al*, 2012). Unlike other VWF domains which are cysteine rich, VWF A domains together contain only 6 cysteines (Sadler, 1998). The VWF A1 domain contains the principal binding site for platelet glycoprotein GpIb. Determination of the crystal structure of A1 indicates GpIb binding site is likely at the front and upper surfaces of the domain. Interestingly, VWD mutations that disrupt VWF platelet binding have been mapped to the lower surface of the A1 domain, indicating indirect disruption of platelet binding (Emsley *et al*, 1998). The VWF cleaving protein ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) cleaves VWF within the A2 domain at Tyr1065 – Met1606. Critical for this interaction is the unfolding of A2 to expose the ADAMTS13 scissile bond, buried within A2 (Zhang *et al*, 2009; Xu & Springer, 2012) .

Crystal structure of the VWF A3 domain demonstrates that collagen binding occurs via interactions through negatively charged residues within the A3 domain and positively charged residues within collagen (Huizinga *et al*, 1997).

1.3.3. VWF C domains

VWF C domains share sequence homology with thrombospondin and procollagen types I and III (Hunt & Barker, 1987). Structurally homologous VWF C domains contain two subdomains linked in tandem by disulphide bonds, with nuclear magnetic resonance spectroscopy showing flexibility between the 2 tandem motifs (O'Leary *et al*, 2004). EM has demonstrated the VWF C domain region is stem-like (Zhou *et al*, 2011), depicting 6 paired motifs lying beside/atop one another (Zhou *et al*, 2012). VWF C4 contains an Arg-Gly-Asp-Ser (RGDS) domain which is integral to VWF binding to platelet GpIIb-IIIa (Beacham *et al*, 1992; Zhou *et al*, 2012).

1.3.4. VWF CTCK domain

The C-terminal cystine knot (CTCK) domain is a motif found in many proteins. In this motif, a ring structure is formed by two disulphide bridges, with a third disulphide passing through the centre of the ring (Meitinger *et al*, 1993). VWF CTCK domain mediates dimerisation of proVWF via disulphide linkage and also associates with VWF C domains, in a perpendicular fashion, forming the base for the dimeric bouquet observed in EM (Zhou *et al*, 2012).

1.4. VWF biosynthesis and post-translational modification

Under normal physiological conditions, *in vivo* biosynthesis of VWF is restricted to vascular ECs and megakaryocytes (Wagner & Marder, 1983; Sporn *et al*, 1985). Within both EC and megakaryocytes VWF biosynthesis involves a number of sequential processes including proteolytic processing of the signal peptide, dimerisation through C-terminal disulphide bond formation, sulfation and significant glycosylation. Ultimately, this results in secretion of both VWF-propeptide (VWF:pp) and mature VWF multimers (Vischer & Wagner, 1994).

After translation, pre-pro-VWF moves to the ER, where proteolytic removal of the signal peptide, addition of precursor glycans and dimerisation all occurs. Further processing continues in the Golgi, including N-glycan modification, addition of O-linked glycans, multimerisation and propeptide cleavage. VWF is then secreted into plasma (EC only) or stored in WP bodies or platelet α -granules (Figure 1.2).

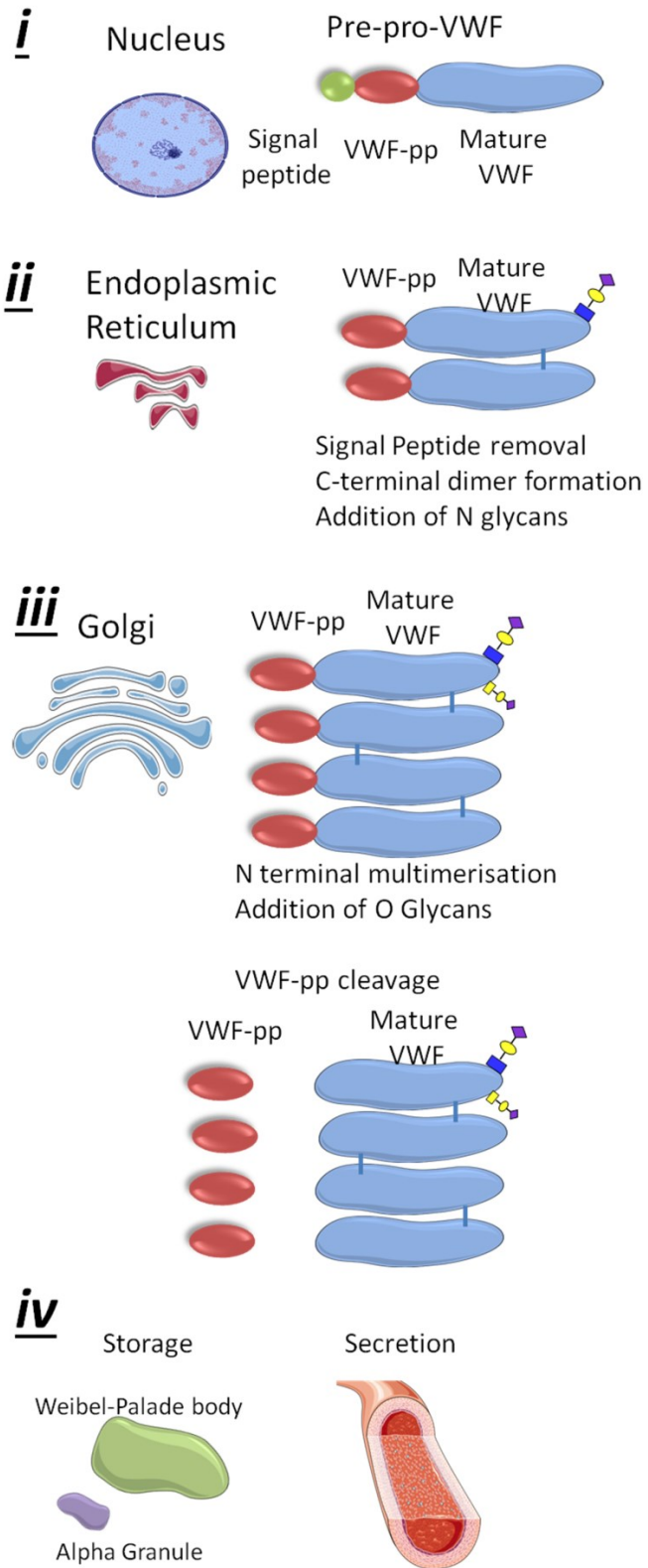


Figure 1.2: VWF Synthesis and Secretion Pathway

VWF-pp; VWF propeptide.

1.4.1. Dimerisation and multimerisation

Following N-linked glycosylation, VWF forms C-terminal end-to-end dimers through disulphide bonds within the CTCK domains (Marti *et al*, 1987; Voorberg *et al*, 1991; Fretto *et al*, 1986). The conserved residue Cys2010 has been identified to play a key role in pro-VWF dimerisation (Schneppenheim *et al*, 1996).

Following dimerisation in the ER, VWF moves to the Golgi where multimerisation occurs. Multimerisation within the Golgi is pH dependent, with an optimum pH of approximately 5.8 (Wagner *et al*, 1986). Multimerisation is also dependent upon the high concentration of calcium within the Golgi as EDTA chelation can inhibit multimerisation (Vischer & Wagner, 1994).

Following multimerisation, the VWF propeptide is cleaved by the calcium-dependant PACE (paired dibasic amino-acid cleaving enzyme) at Ser-763 adjacent to the dibasic amino acid pair Lys761-Arg762 (Rehemtulla & Kaufman, 1992). Studies have demonstrated that propeptide cleavage is essential for effective FVIII binding, with propeptide cleavage mutants (R760K, K762D and R763K) exhibiting defective VWF-FVIII interaction (Bendetowicz *et al*, 1998).

Mature 250kDa VWF subunits are subsequently secreted as a series of heterogeneous oligomers with the largest multimers; termed high molecular weight multimers (HMWM) having molecular weights in excess of 20,000kDa (Wagner & Marder, 1983; Sadler, 1998).

1.4.2. VWF storage

VWF synthesised within EC can be secreted into the plasma or stored within Weibel Palade (WP) bodies (Wagner, 1982). In contrast, VWF synthesised within megakaryocytes is stored within α -granules (Cramer *et al*, 1985). Consequently, under steady state conditions, plasma VWF is predominantly derived from EC.

Approximately 4 μ M in length and 0.1 μ M in diameter, WP bodies are membrane bound organelles unique to EC (Weibel & Palade, 1964). VWF and VWFpp are stored within WP bodies in a 1:1 ratio. Furthermore, VWF stored within WP bodies is composed of the highest molecular weight multimers (Ribes *et al*, 1987).

Platelet α -granules contain a variety of proteins in addition to VWF, including immunoglobulins, growth factors, fibrinogen, thrombospondin, Factor V, platelet factor 4, albumin and neuraminidases (Harrison & Cramer, 1993). Unlike EC VWF there is no constitutive secretion of VWF from platelets (Harrison & Cramer, 1993; Sporn *et al*, 1985).

1.4.3. VWF secretion

VWF secretion occurs by a number of mechanisms including constitutive, basal and regulated pathways. Regulated VWF secretion occurs upon acute EC activation by different secretagogues such as thrombin, fibrin and histamine. These secretagogues act by increasing intracellular calcium concentration, resulting in the release of VWF from WP bodies (de Groot *et al*, 1984; Ribes *et al*, 1987; Erent *et al*, 2007). VWF stored within WP bodies can also be secreted in the absence of stimulation (basal secretion) (Giblin *et*

al, 2008). Regulated secretion of VWF is utilised therapeutically to stimulate release of stored VWF in VWD patients. A synthetic derivative of vasopressin, 1-desamino-8-D arginine vasopressin (DDAVP) acts upon V2 receptors on ECs to increase intracellular cyclic adenosine monophosphate (cAMP) activation. cAMP in turn results in secretion of VWF from peripherally located WP bodies (Kaufmann *et al*, 2000).

A recent study demonstrated that inhibition of constitutive secretion (using brefeldin A (BFA); an inhibitor of anterograde carriers in the Golgi network) resulted in a reduction of VWF predominantly secreted to the basolateral surface of HUVECs (Lopes da Silva & Cutler, 2016). This suggests that constitutive VWF secretion localises to the basolateral surface of ECs rather than into plasma. Constitutive VWF secretion therefore likely provides a proportion of collagen-bound subendothelial VWF. This differs from early studies which demonstrated that 95% of VWF synthesised is constitutively secreted from EC directly into plasma (Sporn *et al*, 1986). The relative contribution of this pathway was assessed using BFA to block constitutive VWF secretion, in the absence of regulatory pathway stimulation. Lopes *et al* observed secretion of ultra large (UL-VWF) and HMW rich VWF from WPB into the vessel lumen in the absence of stimulation. In contrast, the constitutive pathway leads to LMW VWF, from the trans Golgi network moving to the EC basolateral surface for secretion into the sub-endothelial matrix (Lopes da Silva & Cutler, 2016).

1.5. VWF glycosylation

VWF is heavily glycosylated, with 13 asparagine (N-) linked and 10 serine/threonine (O-) linked glycans being expressed on mature VWF monomer (Canis *et al*, 2012). VWF glycans comprise 20% of the total monomeric mass of VWF and are important determinants of VWF functional activity and plasma antigen levels (Ellies *et al*, 2002; Mohlke *et al*, 1999; McGrath *et al*, 2010a, 2013; O'Sullivan *et al*, 2016a).

1.5.1. N-linked glycosylation

Subsequent to synthesis of the 2,813 amino acid (aa) pre-pro-VWF translational product, VWF is transferred to the ER where the signal peptide is removed to form pro-VWF (Wagner, 1990). VWF N-linked glycans are initially coupled to asparagine sites within the rough ER as high-mannose containing carbohydrate structures. N-linked high-mannose oligosaccharides are trimmed, and galactose (Gal) and neuraminic acid/sialic acid (NeuAc) residues are added to produce complex type glycans through the action of a series of glycosidases and glycosyltransferases. Addition of N-linked glycosylation is essential for dimerisation VWF, such that inhibition of N-linked glycan synthesis *in vitro* results in accumulation of pro-VWF monomers within the ER of human umbilical vein endothelial cells (HUVECs) (Wagner & Marder, 1984; Matsui *et al*, 1992).

Analysis of the VWF amino acid sequence identified 13 potential N-glycosylation consensus sequons (N857, N1147, N1231, N1515, N1574, N2223, N2290, N2357, N2400, N2546, N2585, N2635, N2790) (Figure 1.3 A). Subsequent studies reported all that all sites with the exception of N2635 were occupied (Matsui *et al*, 1992; Titani *et al*, 1986). However, recent mass spectrometry (MS) studies on human plasma-derived (pd-) VWF

have confirmed occupancy of N2635 and N1147 (Canis *et al*, 2012; Gashash *et al*, 2017). Importantly, these studies also highlighted that the glycans expressed at individual positions across the VWF monomer differed significantly. For example, smaller N-glycan structures were observed at sequons N857 and N1147, whereas larger more complicated sugar structures were present at N1515 and N1574 respectively (Canis *et al*, 2012). Overall, complex-type carbohydrates predominated, such that less than 1% of VWF glycan chains were high-mannose in type. The most common structures observed were monosialylated and disialylated biantennary chains (Figure 1.3 B) (Canis *et al*, 2012; Gashash *et al*, 2017).

1.5.2. O-Linked glycosylation

In addition to its extensive N-glycosylation, each VWF monomer also contains ten O-glycosylation sites. Unlike the N-glycans which are distributed across multiple different domains of VWF, eight of the ten O-linked glycans (Figure 1.3 A) (T1248, T1255, T1256, S1263, T1468, T1477, S1486, T1487) are clustered in two groups of four at either side of the A1 domain (Samor *et al*, 1989; Canis *et al*, 2010). The other O-glycan chains are located within the A3 and C1 domains of VWF (T1679 and T2298 respectively). In contrast to the complexity of its N-glycans, the O-glycans of VWF predominantly exist as short mucin-type carbohydrates (Samor *et al*, 1989; Canis *et al*, 2010; Solecka *et al*, 2016). Recent MS analyses have improved our understanding of the composition of these O-glycan structures (Solecka *et al*, 2016; Gashash *et al*, 2017). The predominant structure seen was a disialylated core 1 tetrasaccharide (commonly referred to as the Thomas Friedenreich (T) antigen) accounting for approximately 70% of the total O-glycan population (Canis *et al*, 2010) (Figure 1.3 C). Core 2 structures have also been

identified. Interestingly core 2 structures are not evenly distributed among all ten glycosylation sites. Moreover, analysis of the glycan clusters which flank the A1 domain revealed that more core 2 type structures were present on the C-terminal Cluster 2 compared to N-terminal Cluster 1 (Solecka *et al*, 2016).

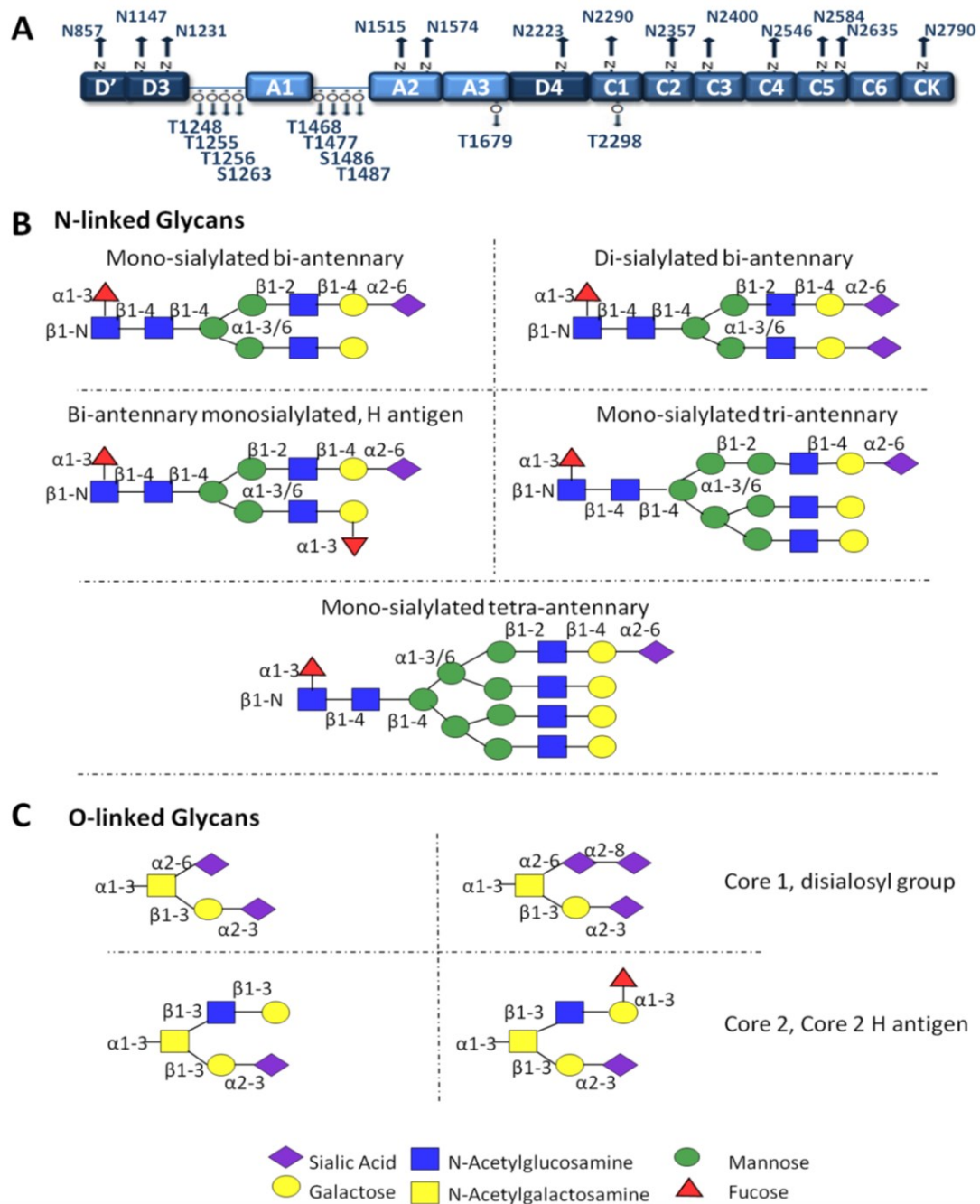


Figure 1.3: VWF glycan structures

A) VWF N and O linked glycan positions. B) Commonest N glycan structures are illustrated. C) Commonest O glycan structures are illustrated. Adapted from Ward *et al*, JTH 2019.

1.5.3. ABO (H) blood group determinants

VWF is unusual amongst circulating glycoproteins in that it expresses ABO(H) blood group determinants on its glycans (Matsui *et al*, 1992). The H antigen is generated via the action of fucosyltransferase and is a precursor for the addition of A (N-acetylgalactosamine –GalNAc) and/or B (Gal) antigens. The presence of ABO(H) blood group determinants on VWF is of direct clinical importance, as ABO is a critical determinant of plasma VWF:Ag levels (Orstavik *et al*, 1985). Blood group O individuals have reduced VWF:Ag levels that are 20% lower than non-O individuals. Moreover, individuals exhibiting the rare Bombay blood group (lacking expression of H antigen and thus A/B antigens) have been shown to have even lower VWF levels (O'Donnell *et al*, 2005). Terminal ABO (H) blood group determinants have been identified on approximately 15% of the N-linked carbohydrates of human plasma-derived pd-VWF (pd-VWF). These ABO(H) structures were not localized to specific N-glycan sites, but rather were disseminated across all of the occupied sequons (Matsui *et al*, 1992; Canis *et al*, 2012; Gashash *et al*, 2017). ABO (H) moieties have also been detected on approximately 1% VWF O linked glycans.

1.6. VWF Sialylation

The vast majority of the N- and O-glycans on human VWF are capped by terminal negatively-charged sialic acid residues (Figure 1.3). Approximately 80% of the total sialic acid on VWF is expressed on its N-linked glycans where is present in an α 2-6 linkage (Canis *et al*, 2012; McGrath *et al*, 2010a) (Figure 1.3 B). O-linked sialic acid expression accounts for <20% of the total sialylation present on human pd-VWF, and is either α 2-3, or α 2-6 linked (McGrath *et al*, 2010a; Solecka *et al*, 2016). Of note, MS analysis has also identified rare disialosyl groups on VWF O-linked glycans which consist of two sialic acids linked together in an α 2-8 linkage (Canis *et al*, 2010) (Figure 1.3 C).

1.6.1. Sialylation and VWF ageing in plasma

Like other plasma glycoproteins, emerging evidence suggests that VWF glycosylation may vary with protein ageing. Interestingly, Yang *et al* recently reported that ageing of secreted plasma glycoproteins is not only associated with loss of sialic acid, but also progressive stepwise loss of terminal sugar residues from N-glycan chains (Yang *et al*, 2015). Loss of terminal α 2-6 linked sialic acid (catalysed by plasma neuraminidases) constitutes the first step of this process. Subsequently, through the actions of other plasma glycosidase enzymes, further progressive N-glycan trimming ensues (Yang *et al*, 2015) (Figure 1.4). In keeping with these data, lectin studies performed on plasma samples collected pre- and post-DDAVP stimulated VWF release, have demonstrated significant differences in VWF glycan expression. For example, Aguila *et al* showed that *Sambucus nigra agglutinin* (SNA) (a plant lectin which specifically binds α 2-6 linked sialic acid) binding to VWF secreted following DDAVP was significantly elevated compared to

circulating steady-state plasma VWF, suggesting that HMWM VWF stored within WP bodies are more highly sialylated compared with VWF in circulation (Aguila *et al*, 2019). In addition, decreased *Arachis hypogaea* (Peanut agglutinin (PNA)) (a plant lectin with affinity for the T antigen structure) binding and increased blood group antigen expression on plasma VWF has also been described in post-DDAVP samples (Brown *et al*, 2002; van Schooten *et al*, 2007). Thus, although the majority of the N- and O-linked glycans of VWF initially secreted from EC are capped with terminal sialic acid residues, the level of plasma VWF sialylation is dynamic in nature and likely to be regulated by multiple inherited and environmental influences.

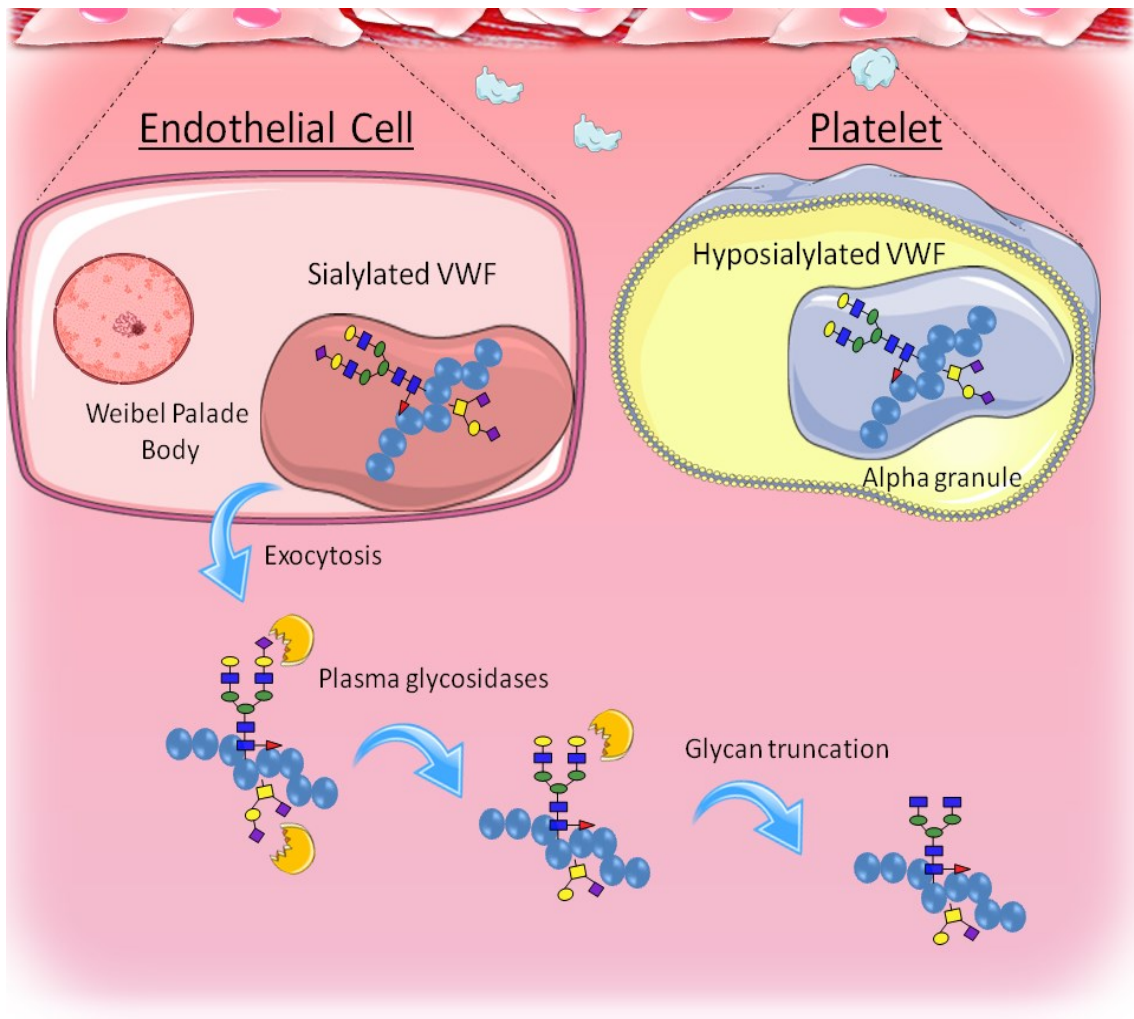


Figure 1.4: Physiological changes in VWF sialylation

The sialylation status of VWF is heterogeneous. Once secreted, VWF is exposed to the action of plasma glycosidases. This image was used as the front cover of the Journal of Thrombosis and Haemostasis, July 2019.

1.6.2. Sialylation of human platelet-VWF

Platelet α -granules contain an estimated 10-20% of the total VWF present in platelet rich plasma (Howard *et al*, 1974; Nachman & Jaffe, 1975). This pool of platelet-VWF is discrete from pd-VWF, with no interchange between the two compartments. Previous studies have demonstrated that sialic acid expression on platelet-VWF is markedly reduced compared to pd-VWF (Williams *et al*, 1994; Kagami *et al*, 2000) (Figure 1.4). Using HPLC (high performance liquid chromatography) analysis, McGrath *et al* showed that this reduced sialic acid expression was mainly attributable to a specific decrease (>50%) in N-linked sialylation on platelet-VWF (McGrath *et al*, 2013). In contrast, O-linked sialylation on platelet-VWF and pd-VWF were similar. Again, in contrast to pd-VWF, studies have also consistently shown that A and B blood group determinants are not present on platelet-VWF. These glycosylation differences presumably reflect differences in the post-translational modification of platelet-VWF (synthesised within megakaryocytes) compared to pd-VWF (synthesised within EC) (Brown *et al*, 2002; Matsui *et al*, 1999; McGrath *et al*, 2013).

1.6.3. VWF sialylation influences functional activity

Previous studies conducted under both static and shear-based conditions have demonstrated that VWF glycan structures significantly influence its functional properties (Federici *et al*, 1988; Nowak *et al*, 2012; Fallah *et al*, 2013). A specific role for sialylation in regulating aspects of VWF activity has also been defined. Federici *et al* showed that neuraminidase treatment of VWF removed >95% of total sialic acid from VWF (Neu-VWF) (Federici *et al*, 1988). In the presence of protease inhibitors, desialylation had no direct effect on the multimer pattern on VWF (Berkowitz & Federici, 1988). However, Neu-VWF was also shown to induce spontaneous platelet aggregation in platelet-rich plasma, and also to be more effective in modulating platelet adhesion to a collagen surface under shear. Desialylation of VWF is associated with exposure of penultimate Gal residues. Importantly, removal of these Gal moieties significantly attenuated the increased adhesion and aggregation properties of Neu-VWF (Federici *et al*, 1988).

As previously discussed, N-linked sialylation on platelet-VWF is markedly reduced when compared to pd-VWF. Given this reduction in sialic acid expression, it is of interest that current evidence suggests important functional differences between platelet-VWF and pd-VWF (McGrath *et al*, 2010b). For example, although platelet-VWF is enriched in HMWM, it binds GP1b α with significantly reduced affinity compared to pd-VWF (Williams *et al*, 1994). In contrast, platelet-VWF displays significantly enhanced binding to both GpIIb/IIIa and heparin (Williams *et al*, 1994). Further studies will be necessary to elucidate the molecular mechanisms underlying these differences in specific activity,

and in particular to determine the contribution of differences in both N- and O-linked VWF glycans.

1.6.4. VWF sialylation regulates susceptibility to proteolysis

VWF multimer distribution is a key determinant of its functional activity (Sadler, 1998). HMWMs bind collagen and GpIb α with increased affinity compared to low molecular weight LMWMs and are thus more effective in facilitating platelet plug formation. In normal plasma, VWF multimer distribution is regulated by ADAMTS13, which cleaves VWF at a specific Tyr1605-Met1606 bond within the A2 domain (Sadler, 2008; Zheng, 2015). Recent studies have suggested that other proteases including plasmin may also play roles in regulating VWF multimer conditions under specific circumstances (Tersteeg *et al*, 2014; Brophy *et al*, 2017). The clinical importance of regulating VWF multimer distribution is illustrated by the fact that ADAMTS13 deficiency in patients with thrombotic thrombocytopenic purpura (TTP) results in accumulation of ultra-large (UL-) VWF multimers and life-threatening thrombotic microvascular occlusion (Zheng, 2015). Conversely, loss of HMWM due to enhanced proteolysis in patients with type 2A VWD is associated with significant bleeding (Lillicrap, 2013). In this context, understanding the biological mechanisms through which VWF proteolysis is regulated is of direct clinical importance. It is well recognised that terminal sialic acid expression on glycoproteins plays a key role in protecting against premature proteolytic destruction. In keeping with this concept, previous studies demonstrated that VWF desialylation resulted in significantly enhanced proteolysis by a number of proteases including cathepsin B, trypsin and chymotrypsin (McGrath *et al*, 2010a; Berkowitz & Federici, 1988). Paradoxically however, more recent studies have shown that removal of sialic acid from

VWF causes it to be significantly more resistant to cleavage by ADAMTS13 (McGrath *et al*, 2010a). In particular, enzymatic desialylation of pd-VWF with α 2-3,6,8,9 neuraminidase markedly impaired ADAMTS13-mediated VWF proteolysis in a dose-dependent manner (McGrath *et al*, 2010a). Interestingly, treatment with α 2-3 neuraminidase to specifically digest α 2-3 linked sialylation from the O-glycans of pdVWF had no significant effect on ADAMTS13 proteolysis (McGrath *et al*, 2010a). Together, these data suggest that α 2-6 linked sialylation on pd-VWF may play a critical role in enhancing the susceptibility of VWF to proteolysis by ADAMTS13. Although the molecular mechanism underpinning this sialylation effect remains unclear, site-directed mutagenesis studies have suggested an important role for the N-linked glycans at Asn1574 in the VWF A2 domain in regulating ADAMTS13 proteolysis (McKinnon *et al*, 2008).

As previously discussed, levels of N-linked sialylation are significantly reduced on platelet-VWF compared to pd-VWF (Kagami *et al*, 2000; Williams *et al*, 1994). Presumably, as a result of this quantitative change in terminal α 2-6 linked sialic acid expression, platelet-VWF has been shown to exhibit specific resistance to ADAMTS13-mediated proteolysis compared to pd-VWF (McGrath *et al*, 2013). Hence, not only are high local concentrations of platelet-VWF released at sites of vascular injury, but due to the fact that it has undergone different post-translational modification within megakaryocytes, this released platelet-VWF exists as a discrete natural glycoform that is at least partially resistant to ADAMTS13 proteolysis.

Recent studies have suggested that plasmin-induced cleavage of VWF may also be of both physiological and potential pharmacological significance (Tersteeg *et al*, 2014;

Herbig & Diamond, 2015). For example, significant activation of plasminogen to plasmin has been reported in patients during acute TTP (Tersteeg *et al*, 2014). Furthermore, a role for plasmin in preventing accumulation of pathological UL-VWF multimers in the absence of ADAMTS13 regulation has also been proposed (Herbig & Diamond, 2015). Interestingly, VWF glycans also play a role in regulating susceptibility to plasmin-mediated proteolysis (Berkowitz & Federici, 1988; Brophy *et al*, 2017). However, in contrast to ADAMTS13 proteolysis, ABO blood group does not influence VWF cleavage by plasmin (Brophy *et al*, 2017) and Berkowitz *et al* previously showed that desialylation of VWF is associated with enhanced plasmin-mediated proteolysis (Berkowitz & Federici, 1988).

In addition to influencing the susceptibility of VWF to proteolysis, sialic acid expression has been shown to play a critical role in modulating VWF clearance (O'Sullivan *et al*, 2018b). Current state of the art understanding of VWF clearance and the particular role of VWF sialylation in regulating this process will be considered in the final section of this Chapter.

1.6.5. VWF sialylation in healthy normal individuals

In light of the important effects of sialic acid expression in regulating VWF biology, it is interesting that variation in VWF sialylation has been reported between normal individuals. In a study of 68 healthy blood donors, Aguila *et al* observed significant inter-individual variation in VWF-binding for three lectins that recognize terminal sialic acid residues SNA, *Maackia amurensis lectin II* (MAL-II) and *Triticum vulgare* (Wheat germ agglutinin (WGA)) (Aguila *et al*, 2019). In keeping with these findings, exposure of sub-

terminal β -Gal also varied significantly between different normal individuals (Aguila *et al*, 2019). Collectively, these data suggest that, even amongst normal individuals, there may be marked inter-individual heterogeneity in quantitative N- and O-linked sialic acid expression on pd-VWF. Further studies will be required to determine the biological mechanisms underpinning this inter-individual heterogeneity in VWF sialylation, and to define whether the changes in sialic acid expression may contribute to quantitative and qualitative variations in plasma VWF levels in the normal population.

1.6.6. VWF sialylation in patients with VWD

Perhaps unsurprisingly given the key roles for sialylation in regulating VWF biology, several groups have investigated VWF glycosylation in patients with VWD. Two independent studies reported significantly increased binding of the lectin RCA-I to pd-VWF (suggestive of increased Gal or GalNAc exposure) in patients with VWD compared to controls (Ellies *et al*, 2002; Millar *et al*, 2008) (Figure 1.5 A). Importantly, these studies both included patients with different types of VWD, although the total numbers enrolled were limited (n=19 and n=26 respectively). Furthermore, significantly enhanced binding of the lectin PNA (suggesting increased expression of O-linked desialylated T antigen) has also been observed in patients with type 1 VWD compared to healthy controls (van Schooten *et al*, 2007). Collectively, these data raise the intriguing question as to whether alteration in VWF sialylation could be important in the pathogenesis of VWD, particularly in patients with Low VWF levels in whom the disease is not linked to the *VWF* gene locus (Lavin *et al*, 2017).

To further address this question, Aguila *et al* developed a novel panel of lectin assays to assess VWF sialylation in 110 patients with Low VWF (plasma VWF levels in the range 30-50IU/dL) compared to ABO-matched healthy controls (Aguila *et al*, 2019). For each patient and control, pd-VWF sialylation was assessed using the lectins SNA, MAL-II and WGA. Significant inter-individual variation in VWF sialylation was observed amongst the cohort of Low VWF patients. Importantly however, SNA lectin binding to VWF was significantly reduced in the Low VWF cohort compared to controls, suggesting a specific reduction in terminal α 2-6 linked sialic acid expression in these patients (Aguila *et al*, 2019). In keeping with this reduction in terminal α 2-6 linked sialylation, a significant

increase in RCA-I binding (consistent with increased Gal exposure) was also seen in the Low VWF cohort compared to healthy controls. Interestingly, highest levels of RCA-I binding were seen in Low VWF patients who did not have any *VWF* gene mutations to explain their reduced plasma VWF levels. Finally, an inverse correlation was observed between enhanced RCA-I binding and estimated VWF half-life was observed in Low VWF patients. Together, these findings support that loss of terminal sialylation may contribute to the underlying pathophysiology in at least a sub-group of Low VWF patients by promoting enhanced clearance (Aguila *et al*, 2019) (Figure 1.5 A).

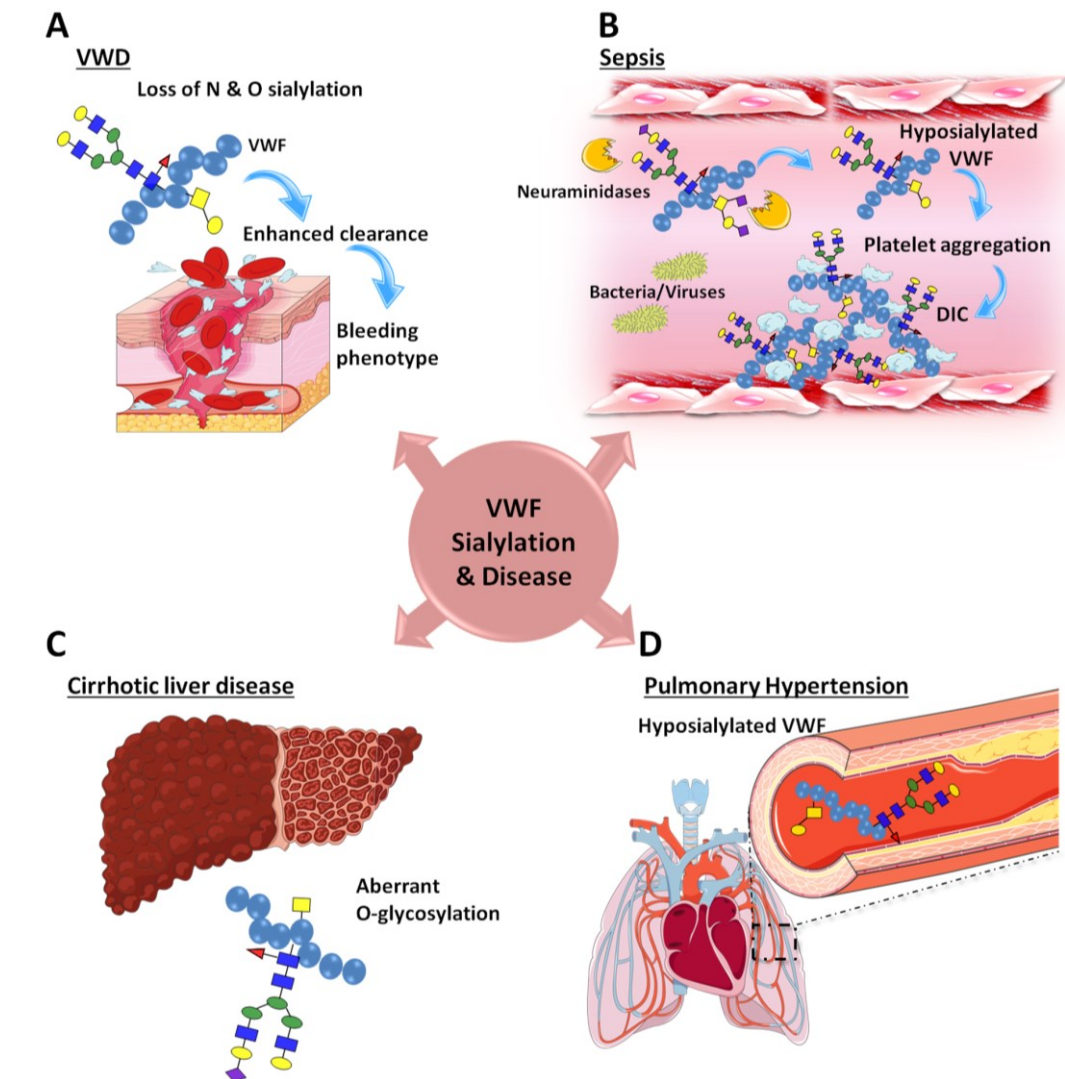


Figure 1.5: VWF Sialylation and Disease

(A) Galactose exposure is correlated with an increase in VWF clearance *in vivo* and decreased VWF:Ag levels, contributing to the pathophysiology of VWD. (B) Viral and bacterial neuraminidases can mediate the de-sialylation of circulating VWF and platelets during sepsis. De-sialylated VWF has been shown to trigger platelet aggregation and thus contribute to the coagulopathy of sepsis. (C) PNA lectin binding has been shown to inversely correlate with VWF:Ag in cirrhotic patients. (D) Pulmonary hypertension is associated with aberrantly sialylated VWF. Adapted from Ward *et al*, JTH 2019.

1.6.7. Altered VWF sialylation in miscellaneous physiological and pathological conditions

In addition to the evidence that VWF sialylation may be abnormal in some patients with VWD, previous studies have suggested that a number of other conditions may also be associated with alterations in sialic acid expression on plasma VWF. Several pathogens including *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Pseudomonas aeruginosa* have been shown to express neuraminidase enzymes that can target host sialoglycoproteins (Roggentin *et al*, 1993; Soong, 2006). Furthermore, platelet desialylation by bacterial NanA sialidase has been implicated in causing enhanced platelet clearance and thrombocytopenia in patients with *S. pneumoniae* infection (Grewal *et al*, 2008). Desialylation of VWF resulting in enhanced clearance and reduced plasma VWF levels has also been observed in mice infected with *S. pneumoniae* (Grewal *et al*, 2008) (Figure 1.5 B).

Several studies have shown that plasma VWF levels are markedly elevated in patients with liver cirrhosis (van Schooten *et al*, 2007; Lisman *et al*, 2006). Furthermore, an inverse correlation between PNA lectin binding to VWF and plasma VWF levels has also been described in patients with cirrhosis (Figure 1.5 C). In addition, a correlation between severity of cirrhosis and relative PNA binding was also observed (van Schooten *et al*, 2007). Since PNA is known to preferentially bind to the desialylated O-linked T antigen carbohydrate structures on VWF, these data suggest that abnormal VWF glycosylation, and in particular sialylation, may be common in cirrhotic patients. Reduced VWF sialylation has also been reported in patients with pulmonary hypertension (Lopes *et al*, 2000). In a study of 16 patients with moderate to severe

pulmonary hypertension, Lopes *et al* observed reduced binding of WGA lectin to patient-VWF compared to VWF from healthy controls (Lopes *et al*, 2000) (Figure 1.5 D). Subsequent studies demonstrated that this decrease in WGA binding was attributable to a significant reduction in VWF sialic acid expression. Overall, the authors estimated that VWF sialylation in patients with pulmonary hypertension was reduced in the order of 20-25% (Lopes *et al*, 2000). However, in some of the patients studied, VWF sialic acid expression was reduced up to 70%. These findings are of clinical relevance as pulmonary hypertension has also been associated with a significant reduction in HMWM VWF. It seems likely that other diseases (e.g. cancer) may also be associated with quantitative and/or qualitative alterations in VWF sialylation. Further studies will be required to define the nature of these glycan changes and determine how they may impact upon (i) VWF structure and function, and (ii) disease pathogenesis respectively.

In addition to these pathological conditions, current evidence suggests that VWF glycosylation and sialylation may also undergo physiological variations for example during pregnancy or with increasing age. It is well established that plasma VWF levels increase progressively during the course of normal pregnancy. For example, Drury-Stewart *et al* recently showed that mean plasma VWF:Ag levels were 120.3 IU/dL in the first trimester, 139.9 IU/dL in the second trimester and 191.3 IU/dL in the third trimester (Drury-Stewart *et al*, 2014). This increase in levels appears to relate in part to a reduction in VWF clearance. Alterations in plasma VWF multimer distribution (including a reduction in HMWM and altered triplet pattern) have also been associated with pregnancy (Drury-Stewart *et al*, 2014). Although the biology underpinning these changes in VWF clearance and multimer distribution remains unclear, changes in

isoelectric focussing studies raise the possibility that VWF glycan expression may also differ during pregnancy (Drury-Stewart *et al*, 2014).

1.6.8. VWF sialylation and therapeutic development

Recent studies have investigated the glycosylation and sialylation of VWF in a number of different therapeutic concentrates (Riddell *et al*, 2019). Since all of these concentrates were plasma-derived, it is perhaps unsurprising that Riddell *et al* observed no significant differences in SNA (which prefers α 2-6 linked over α 2-3 linked sialic acid) or RCA-1 (terminal Gal residues) binding between ten commercially available VWF-containing concentrates. In addition to the pd-VWF concentrates available for clinical use, a first recombinant VWF (rVWF) concentrate has recently been licensed (VONVENDI®; Takeda) (Lavin & O'Donnell, 2016). This rVWF is expressed in Chinese hamster ovary (CHO) cells and consequently expresses differences in glycosylation and sialylation compared to pd-VWF. In particular, ABO(H) blood group antigens are not expressed on the rVWF product and sialylation levels are increased (Turecek *et al*, 2009). Given these differences in glycosylation, it is interesting that data from the phase 1 and phase 3 studies suggest that the half-life of infused rVWF (19.6 hours) is considerably longer than that of pd-VWF (range 12.8-15.8 hours) (Mannucci *et al*, 2013; Gill *et al*, 2015). Moreover, the data further suggest that rVWF may be more effective in stabilizing endogenous FVIII compared to pd-VWF. Additional studies will be required to confirm these initial observations and to define the biological mechanisms underlying these findings.

1.7. VWF Clearance

1.7.1. Cellular basis of VWF clearance

Despite significant insights into the biology underlying VWF biosynthesis, glycosylation and function, our understanding of the mechanisms involved in regulating VWF clearance remains limited (Pipe *et al*, 2016). Initial clearance studies in a variety of different animal models (including New Zealand White rabbits, Sprague Dewley rats and C57BL/6J VWF-deficient mice) consistently demonstrated biphasic clearance of VWF, with an initial rapid distribution phase followed by a second slower elimination phase (Sodetz *et al*, 1977; Stoddart *et al*, 1996; Lenting *et al*, 2004). The biological mechanism(s) underpinning this biphasic clearance pattern have not been defined. Nevertheless, following intravenous injection of radiolabelled VWF into VWF-deficient mice, Lenting *et al* showed that the liver and spleen were both efficient in modulating VWF uptake (Figure 1.6). However, given its relative size and blood flow, current data suggest that the majority of intravenously-injected VWF is primarily targeted to the liver (Lenting *et al*, 2004). In contrast, other organs, such as the spleen and kidneys, took up relatively small amounts of VWF (Van Schooten *et al*, 2008). Subsequent immunohistochemical studies demonstrated that VWF specifically co-localised with CD68+ Kupffer macrophage cells within the murine liver (Van Schooten *et al*, 2008). Moreover, macrophage depletion with either gadolinium or clodronate was associated with significantly prolonged survival of VWF in this VWF-deficient mouse model (Van Schooten *et al*, 2008; Rawley *et al*, 2015). Interestingly, similar clearance rates were observed for HMWM, suggesting that VWF clearance is independent of its multimer size (Lenting *et al*, 2004). This finding is in keeping with data from other studies that found

no relationship between VWF proteolysis by ADAMTS13 and rate of VWF clearance (Badirou *et al*, 2012).

In keeping with these *in vivo* data, *in vitro* studies have shown that primary human macrophages bind VWF in a dose-dependent and saturable manner (Van Schooten *et al*, 2008; Castro-Núñez *et al*, 2012; Chion *et al*, 2016). Furthermore, this macrophage binding was followed by VWF uptake and degradation (Van Schooten *et al*, 2008). Several groups have reported that VWF binding to macrophages is significantly enhanced in the presence of shear stress or ristocetin, suggesting that VWF conformation plays a critical role in regulating macrophage-mediated clearance (Castro-Núñez *et al*, 2012; Chion *et al*, 2016). Together, these data demonstrate that macrophages play a prominent role in regulating VWF clearance and thereby modulating plasma levels of the VWF-FVIII complex. Nevertheless, it remains possible that other cells may also contribute to physiological and/or pathological VWF clearance. For example, Sorvillo *et al* recently reported that immature monocyte-derived dendritic cells (DCs) bind to VWF. In their capacity as key antigen-presenting cells, DCs are known to have a wide variety of endocytic mechanisms. Interestingly however, despite binding to the cell surface, VWF was not efficiently internalised by DCs (Sorvillo *et al*, 2016). Neutrophil binding to VWF under both static and shear conditions has been demonstrated, but there is no evidence to date that neutrophils contribute to VWF clearance (Pendur *et al*, 2006).

Lenting *et al* demonstrated that the liver is the primary destination of radiolabelled-VWF following intravenous infusion. In addition to resident macrophages/Kupffer cells both hepatocytes and liver sinusoidal endothelial cells (LSEC) have been demonstrated to mediate VWF clearance (Figure 1.6). The endocytic capacity of LSECs is one of the highest of any cell type in the body (Poisson *et al*, 2017). In keeping with this, genetic variation in LSEC endocytic receptors *C-type lectin domain family 4 member M* (CLEC4M) and *Stabilin 2* (STAB2) are associated with VWF:Ag levels (Smith *et al*, 2010). Moreover, cyclophosphamide induced disruption of the sinusoidal endothelium results in a significant increase in VWFpp:Ag ratio, a surrogate marker for VWF clearance (Swystun *et al*, 2018). Furthermore, hepatocytes have been described to mediate the clearance of hyposialylated VWF (Grewal *et al*, 2008).

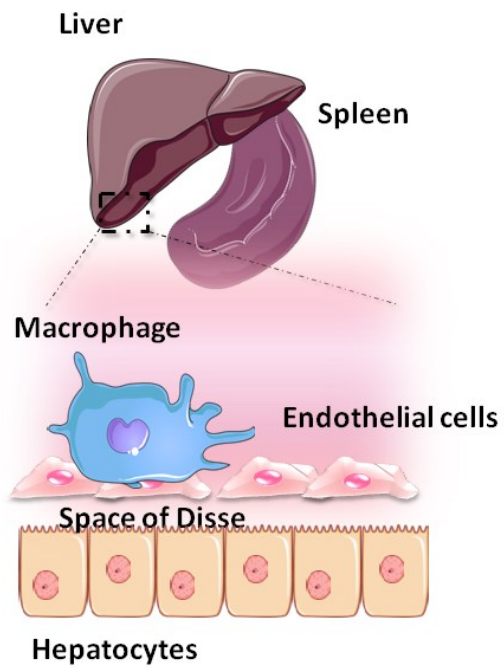


Figure 1.6: Cellular basis of VWF clearance

VWF clearance is predominantly localised to the hepatic sinusoids where hepatocytes, resident kupffer cells and LSECs mediate VWF endocytosis.

1.7.2. Lectin receptors and VWF clearance

1.7.2.1. The Asialoglycoprotein receptor (ASGPR)

The first VWF clearance receptor identified was the asialoglycoprotein receptor (ASGPR; also termed Ashwell-Morell receptor, AMR) (Grewal *et al*, 2008) (Figure 1.7 A). This receptor is a member of the calcium-dependent (C-type) lectin family of receptors (Ashwell & Harford, 1982; Stockert, 1995). Importantly, although the ASGPR is expressed abundantly in the liver on hepatocytes, it is not expressed on macrophages. The ASGPR is composed of two trans-membrane polypeptides (ASGPR1 and ASGPR2) that assemble into hetero-oligomers on the cell surface (Stockert, 1995). The C-terminal extracellular domains of ASGPR1 and ASGPR2 form a carbohydrate recognition domain (CRD) that selectively binds glycoproteins expressing either β -D-galactose (Gal) or N-acetyl-D-galactosamine (GalNAc) determinants (Spiess, 1990). These Gal and GalNAc residues are more typically expressed on plasma glycoproteins as sub-terminal moieties on oligosaccharide chains capped by sialic acid. As glycoproteins age in plasma, terminal sialic acid residues are lost so that the ASGPR can then bind to the exposed Gal or GalNAc and mediate endocytosis (Yang *et al*, 2015). In keeping with other plasma glycoproteins, enzymatic removal of terminal sialylation from VWF has been associated with a markedly reduced plasma half-life (Sodetz *et al*, 1977; O'Sullivan *et al*, 2016a). Grewal *et al* first reported that enhanced clearance of hyposialylated VWF occurs via the ASGPR. Thus, plasma VWF levels are significantly elevated due to attenuated clearance in *Asgr1* knockout mice (Grewal *et al*, 2008).

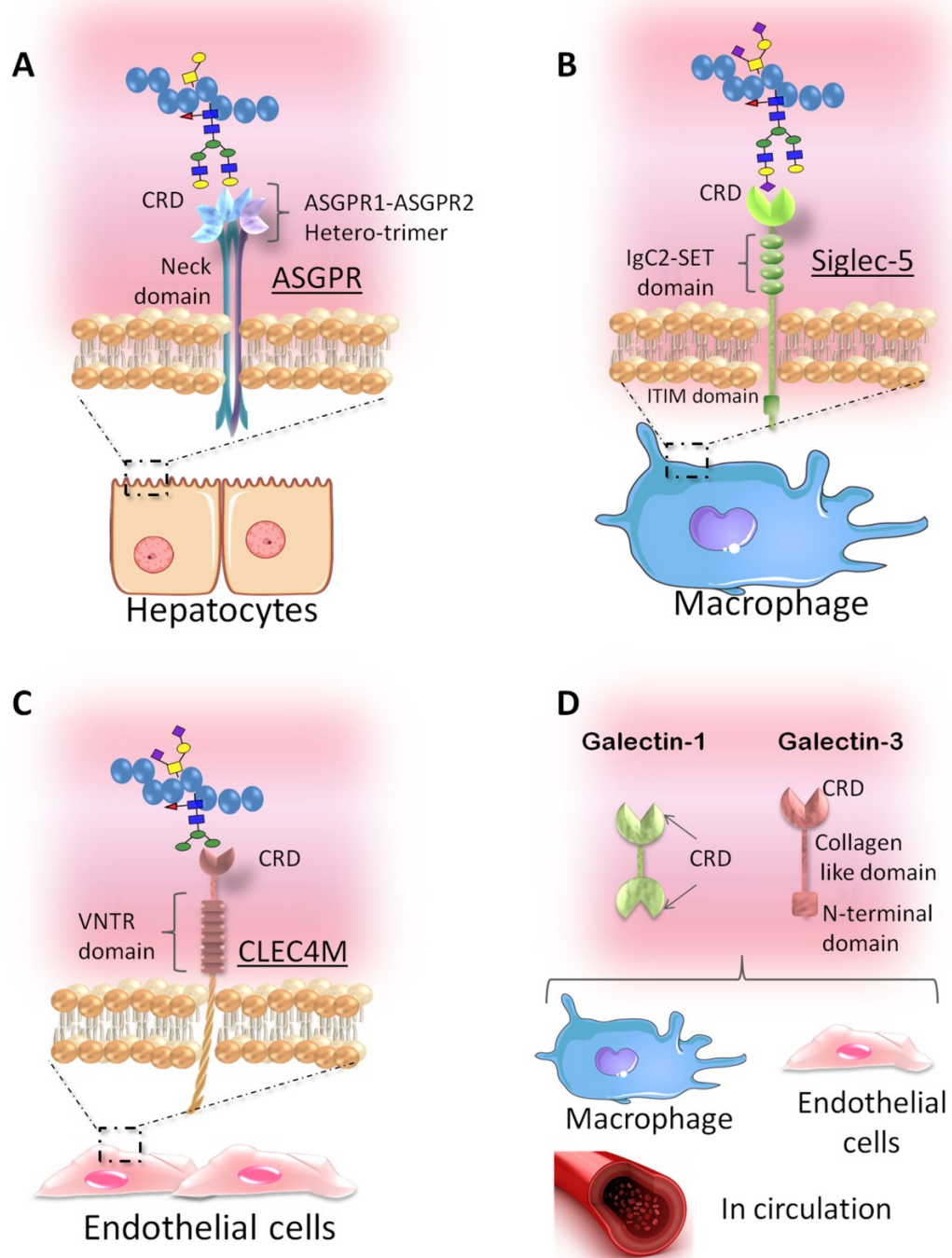


Figure 1.7 Lectin Receptors in VWF clearance

(A) ASGPR expressed on hepatocytes as a hetero-trimer is composed of major (ASGPR1) and minor (ASGPR2) subunits. (B) Siglec-5 binds sialylated glycan structures. (C) CLEC4M expressed on LSECs binds mannose structures. (D) Gal-1 and Gal-3 are present in normal plasma and expressed on EC and macrophages and bind Gal moieties.

1.7.2.2. Sialic-acid binding immunoglobulin-like lectin (Siglec 5)

Unlike ASGPR which binds with enhanced affinity to hyposialylated VWF, Siglec-5 is a member of the sialic-acid binding immunoglobulin-like lectin (Siglec) family (Lock *et al*, 2004) (Figure 1.7 B). Siglec-5 is expressed on monocytes/macrophages, neutrophils and B lymphocytes (Jandus *et al*, 2011). Pegon *et al* demonstrated dose dependent binding of Siglec-5 to human VWF in a sialic-acid dependent manner. Also, Siglec-5 expressed on HEK293 cells was able to bind VWF and regulate endocytosis into early endosomes (Pegon *et al*, 2012). Although there is no Siglec-5 homolog in mice, hydrodynamic expression of human Siglec-5 in murine hepatocytes resulted in a marked reduction in murine plasma VWF levels (Pegon *et al*, 2012).

1.7.2.3. C-type lectin domain family 4-member M

CLEC4M has affinity for mannose and is specifically expressed on EC in liver sinusoids and lymph nodes (Khoo *et al*, 2008) (Figure 1.7 C). Rydz *et al* reported that CLEC4M binds to human VWF, and that CLEC4M expressed on HEK293 cells can bind VWF on the cell surface, facilitate internalization, and subsequently target VWF to early endosomes (Rydz *et al*, 2013). Similar to Siglec-5, mice do not express CLEC4M. However hydrodynamic expression of human CLEC4M in murine hepatocytes resulted in a 46% decrease in murine plasma VWF levels (Rydz *et al*, 2013). In addition, significant correlations between CLEC4M polymorphisms and human plasma VWF:Ag levels have been reported (Smith *et al*, 2010; Antoni *et al*, 2011; Sanders *et al*, 2015a; Manderstedt *et al*, 2018).

1.7.2.4. Galectins

Galectin-1 (Gal-1) and galectin-3 (Gal-3) are lectins with specific affinity for β -galactose structures (Vasta, 2012). These galectins are expressed in many different cell types (including EC and macrophages) and are also present in normal plasma (Thijssen *et al*, 2008) (Figure 1.7 D). Saint-Lu *et al* observed that Gal-1 and Gal-3 could bind to VWF within EC and remained bound to circulating VWF in the plasma (Saint-Lu *et al*, 2012). Plasma VWF:Ag levels were normal in Gal-1/Gal-3 double-deficient mice, suggesting that although galectins can bind to VWF and may influence its functional properties, they may not play a direct role in modulating its clearance (Saint-Lu *et al*, 2012). In this context, it is interesting that Gal-1 and Gal-3 have also both recently been shown to bind to FVIII in a glycan dependent manner (O'Sullivan *et al*, 2016b).

1.7.3. Scavenger receptors and VWF clearance

1.7.3.1. Low-density lipoprotein receptor related protein-1

LRP1 is a large endocytic scavenger receptor that is highly expressed on a number of tissues including macrophages (Strickland *et al*, 2014)(Figure 1.9 A). The extracellular domain of LRP1 is composed of a modular structure consisting including four clusters (I, II, III and IV) of LDL receptor type A repeats that regulate ligand binding. *In vitro* studies have demonstrated that cluster IV of LRP1 can bind to VWF and that LRP1 modulates VWF endocytosis into early endosomes (Rastegarlarlari *et al*, 2012; Wohner *et al*, 2015). Interestingly however, the binding of wild type VWF to LRP1 occurs only in the presence of shear stress or ristocetin, suggesting that VWF needs to be at least partially unfolded in order to interact with LRP1 (Rastegarlarlari *et al*, 2012; Wohner *et al*, 2015). LRP1 binding can also be accelerated by truncation of the N-linked glycans of VWF (O'Sullivan *et al*, 2016a). In keeping with the importance of shear, the A1 domain of VWF has been shown to be important in modulating interaction with LRP1 (Rastegarlarlari *et al*, 2012; Chion *et al*, 2016). *In vitro* studies further suggest that additional domains of VWF (including D'D3 and D4) may also contribute to LRP1 binding (Wohner *et al*, 2015). Although the molecular mechanisms through which multimeric VWF interacts with the large LRP1 receptor remain poorly understood, plasma VWF levels were significantly increased (1.6-fold) in mice with macrophage-specific deficiency of LRP1 compared to controls (Rastegarlarlari *et al*, 2012). Of note, several human studies have also reported that polymorphisms in the LRP1 gene are associated with variations in plasma VWF:Ag levels (Cunningham *et al*, 2005; Morange *et al*, 2005).

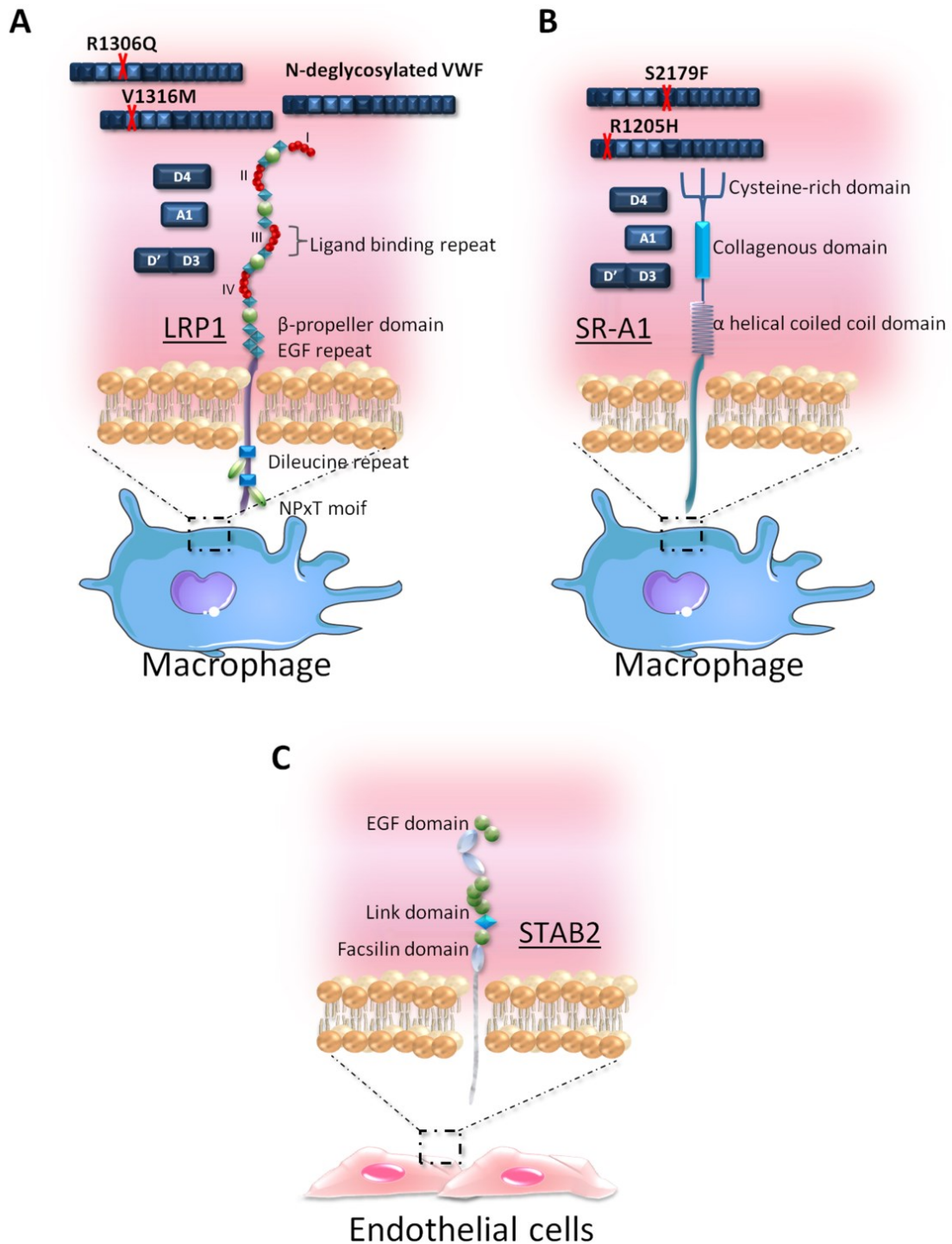


Figure 1.8 Scavenger Receptors in VWF clearance

(A) LRP1 is a large endocytic receptor consisting of four ligand binding repeats termed cluster I-IV. (B) SR-A1 is a homotrimer with a cysteine-rich N-terminal domain and collagen-like repeats. (C) STAB2 is expressed on LSECs is composed of multiple epidermal growth factor (EGF)-like repeats and a fascilin domain.

1.7.3.2. Scavenger receptor class A member

The scavenger receptor class A member I (SR-A1; also known as SCARA1 or CD204) is another scavenger receptor expressed in macrophages and DCs (Zani *et al*, 2015) (Figure 1.8 B). Recent *in vitro* studies have demonstrated that VWF binds to purified SR-A1 in a dose-dependent and saturable manner (Wohner *et al*, 2018). Similar to LRP1, multiple different domains of VWF have been implicated in regulating SR-A1 binding (including the D'D3 region, the A1 domain and the D4 domain) (Wohner *et al*, 2018). However, in marked contrast to LRP1, binding of VWF to SR-A1 can occur under static conditions, without the need for either shear stress or ristocetin.

1.7.3.3. Stabilin 2

Stabilin 2 (STAB2) is a class H scavenger receptor expressed on LSECs (Figure 1.8 C). Genome-wide association studies identified that genetic variations in *STAB2* were associated with VWF:Ag (Antoni *et al*, 2011). Swystun *et al* demonstrated that STAB2 expressing cells bind and internalise human VWF and FVIII (Swystun *et al*, 2018). Moreover, the circulatory half-life of infused pd-VWF is significantly prolonged in *VWF/STAB2* double knockout mice (Swystun *et al*, 2018). However, the molecular mechanisms underpinning STAB2-VWF interactions remain to be elucidated.

1.7.3.4. Scavenger receptor class A member 5

In addition to LRP1, SR-A1, and STAB 2 genome-wide association studies have also reported significant associations between VWF-FVIII plasma levels and another scavenger receptor; scavenger receptor class A member 5 (SCARA5) (Souto et al, 2003; Smith et al, 2010; Antoni et al, 2011). In a recent study Swystun et al demonstrated that VWF binds and is internalised by to SCARA5 expressing cells *in vitro*. Moreover, murine studies demonstrated that the half-life of human VWF is weakly extended in *SCARA5*^{-/-} mice. The molecular mechanisms regulating VWF binding to SCARA5 remain to be fully elucidated (Swystun *et al*, 2019).

1.7.4. Enhanced VWF clearance in the pathogenesis of type 1 VWD

Understanding the biological basis involved in regulating VWF clearance has direct clinical significance. In particular, a series of studies have reported that that enhanced VWF clearance constitutes an important pathogenic mechanism in patients with type 1 VWD (Casonato *et al*, 2002, 2010; Federici *et al*, 2004; Schooten *et al*, 2005; Haberichter *et al*, 2006; Castaman *et al*, 2008; Millar *et al*, 2008; Flood *et al*, 2011; Eikenboom *et al*, 2013; Sanders *et al*, 2015b; Haberichter *et al*, 1992). The observation that VWF circulatory half-life was significantly reduced in selected patients with VWD originated from fall-off studies performed following DDAVP administration. Subsequently, it was demonstrated that enhanced clearance in VWD patients could also be identified by measuring steady state plasma VWFpp and VWF:Ag levels (Haberichter *et al*, 2006, 1992; Haberichter, 2015). On the basis of DDAVP and/or VWFpp:Ag ratio studies, several large cohort studies have demonstrated that enhanced VWF clearance is prevalent in patients with type 1 VWD. For example, the US Zimmerman Program included 140

patients with type 1 VWD (plasma VWF levels < 30 IU/dL) (Flood *et al*, 2011). Importantly, 57 (41%) of these patients had significantly reduced plasma VWF levels (typically <15 IU/dL) together with increased plasma VWFpp/VWF:Ag ratios (>3.0) consistent with enhanced VWF clearance. Similarly, the Willebrand in The Netherlands (WiN) study investigated 380 type 1 VWD patients, all with plasma VWF levels \leq 30 IU/dL (Sanders *et al*, 2015b). In keeping with the US results, an increased VWFpp/VWF: Ag ratio (>2.2) consistent with enhanced clearance was identified in 46% of individuals. Other studies, including the MCMDM-1VWD European study, have also reported that reduced VWF half-life is a common finding among individuals with type 1 VWD (Castaman *et al*, 2008; Eikenboom *et al*, 2013). Furthermore, the Low VWF Ireland Cohort (LoVIC) study recently reported evidence that subtle enhanced VWF clearance is also common in patients with Low VWF levels (range 30–50 IU/dL) (Lavin *et al*, 2017). Taken together, these findings define pathologically-enhanced clearance as an important mechanism in the pathogenesis of type 1 VWD and have led to the suggestion that affected patients should be considered as a distinct type 1C (1-Clearance) subgroup (Haberichter *et al*, 2006). Given the fact that many patients with type 1 VWD and Low VWF do not have *VWF* gene coding mutations, nor indeed demonstrate linkage to the *VWF* gene, further studies into the biological mechanisms underpinning enhanced clearance may provide exciting insights into VWD pathogenesis. Importantly, accumulating data have reported associations between polymorphisms and rare mutations of several clearance receptors (including LRP1, CLEC4M and STAB 2) in patients with VWD (O’Sullivan *et al*, 2018).

1.7.5. Enhanced VWF clearance in types 2 and 3 VWD

Besides the importance of increased VWF clearance in type 1 VWD pathogenesis, emerging data suggest that significant reductions in VWF half-life are also present in many patients with type 2 VWD. For example, the WiN study also studied VWFpp:Ag and FVIII:C/VWF:Ag ratios in 240 patients with type 2 VWD (including 158 with type 2A, 42 with type 2B and 27 with type 2M) (Sanders *et al*, 2015b). Interestingly 59% of type 2A patients, 95% of 2B patients and 48% of 2M patients demonstrated increased VWFpp/VWF:Ag ratios but normal FVIII:C/VWF:Ag ratios. Overall, Sanders *et al* observed that the VWFpp/VWF:Ag ratio was actually higher in patients with type 2 VWD compared to those with type 1 VWD, suggesting that the pathogenic importance of enhanced clearance is even more important in type 2 VWD (Sanders *et al*, 2015b). This hypothesis is supported by data derived from other studies confirming enhanced VWF clearance in patients with type 2 VWD (Haberichter *et al*, 2006; Casari *et al*, 2013). According to the International Society on Thrombosis and Haemostasis and United Kingdom Haemophilia Centre Doctors' Organisation diagnostic guidelines, type 3 VWD is characterised by plasma VWF levels <5% (Nichols *et al*, 2009; Laffan *et al*, 2014). The WiN study included 37 patients with type 3 VWD (Sanders *et al*, 2015b). In 59% of these patients, plasma VWFpp levels were undetectable, consistent with a complete absence of VWF synthesis. Surprisingly however, high levels of VWFpp were identified in 41% of type 3 VWD patients, suggesting that markedly enhanced clearance may contribute to the very low plasma VWF levels seen in some type 3 VWD patients (Sanders *et al*, 2015b).

1.7.6. VWF mutations and enhanced clearance

Despite recent advances in our understanding of the biology underpinning VWF clearance, and the evidence that enhanced clearance is important in VWD pathogenesis, the molecular mechanisms through which individual VWF mutations serve to trigger enhanced VWF clearance remain poorly understood. Nevertheless, more than 30 different VWF point mutations have already been reported in patients with increased VWF clearance (Castaman *et al*, 2008; Casari *et al*, 2013). The first mutation characterized was the VWD Vicenza variant which involves a single amino acid substitution R1205H in the D3 domain of VWF (Casonato *et al*, 2002). Patients with VWD-Vicenza typically have reduced plasma VWF:Ag levels (<10 IU/dL) coupled with significantly elevated VWFpp/VWF:Ag ratios (Casonato *et al*, 2002; Haberichter *et al*, 1992). Following DDAVP, the half-life of the secreted VWF-R1205H is also markedly reduced compared to wild-type VWF (Castaman *et al*, 2008). Two other substitutions of arginine 1205 (with cysteine and serine respectively) have also been reported in individuals with significantly reduced plasma VWF levels and reduced half-lives following DDAVP (Millar *et al*, 2008). *In vivo* clearance studies demonstrated that substitutions of R1205 with histidine, cysteine or serine all resulted in markedly reduced survival of recombinant VWF in VWF-deficient mice (Rawley *et al*, 2015). The enhanced clearance of these R1205 variants *in vivo* was significantly attenuated following clodronate-induced macrophage depletion. Subsequent *in vitro* studies have confirmed enhanced binding of VWF-R1205H to differentiated THP1 macrophages (Wohner *et al*, 2018). Although the precise mechanisms remain unclear, recent data suggest that the SR-A1 scavenger receptor may be important in regulating macrophage-mediated clearance of

VWF-R1205H (Wohner *et al*, 2018). Further studies will be required to investigate whether additional macrophage receptors are also involved.

Besides the R1205 substitutions, a number of other point mutations have also been described in patients with type 1C VWD (Casari *et al*, 2013). Amongst these other mutations in the D3 domain (including C1130F, W1144G and C1149R) have been reported. An increasing number of different point mutations in other VWF domains have also been implicated (e.g. I1416N in A1, S2179F in the D4 domain and C2671Y in the CK domain) (Schooten *et al*, 2005; Haberichter *et al*, 2006, 1992; Castaman *et al*, 2008; Casari *et al*, 2013). In addition, Wohner *et al* recently demonstrated that specific type 2B VWD variants also result in enhanced macrophage-mediated clearance. In particular, the R1306Q and V1316M substitutions within the A1 domain were shown to significantly enhance VWF clearance via macrophages through an LRP1-dependent mechanism (Wohner *et al*, 2015). Further studies will be required to define the molecular mechanisms through which all of these different mutations all lead to increased VWF clearance.

1.8. VWF glycosylation and sialylation and enhanced clearance

In keeping with the role for lectin receptors (including ASGPR, CLEC4M and Siglec-5) in regulating VWF clearance, several lines of evidence have shown that VWF glycosylation is a critical determinant of its half-life. For example, plasma VWF levels are 20–30% lower in blood group O compared to non- O individuals (Jenkins & O'Donnell, 2006). Gallinaro *et al* reported that this effect of ABO blood group on plasma VWF levels was modulated through altered clearance, with significantly increased clearance in group O individuals (Gallinaro *et al*, 2008). More recent studies have proposed alternative mechanisms may also contribute to this ABO effect (Groeneveld *et al*, 2015). Interestingly, plasma VWF levels are further reduced in persons with the rare Bombay phenotype who lack expression of ABO(H) antigens (O'Donnell *et al*, 2005).

1.8.1. Specific glycosites within VWF monomer regulate clearance

Similar to the role of specific VWF glycan sites in modulating susceptibility to ADAMTS13 proteolysis, the individual glycans have also been shown to play a key role in protecting VWF against *in vivo* clearance. Chion *et al* demonstrated that ablation of N glycans at N1515 and N1574 resulted in a marked reduction of VWF half-life (Chion *et al*, 2016). Clodronate induced macrophage depletion, and co-injection of LRP1 antagonist RAP (receptor associated protein) resulted in restoration of VWF half-life. Chion *et al* hypothesised that large, complex N-linked glycan structures within the A2 domain may shield cryptic LRP1 binding sites; such that loss of these glycans results in conformation changes in A2 structure and enhanced LRP1 mediated clearance. Moreover, utilising *in vivo* transfection methods, Badirou *et al* demonstrated that mutation of the O-glycan

doublet T1255/T1256 or loss of all O-glycosylation sites resulted in significantly enhanced VWF clearance compared to wild-Type VWF (Badirou *et al*, 2012).

1.8.2. The relationship between VWF sialylation and clearance

A critical role of VWF sialylation in regulating circulatory half-life was first deduced by Sodetz *et al*. This observation has subsequently been validated by several independent groups, all demonstrating that enzymatic removal of sialic acid residues from VWF markedly reduces plasma half-life (Sodetz *et al*, 1977; Stoddart *et al*, 1996; O'Sullivan *et al*, 2016a). Moreover, genetic inactivation of a specific sialyltransferase (ST3Gal-IV), which catalyses the addition of α 2-3 linked sialic acid to glycoproteins, in mice causes reduced plasma VWF levels due to significantly enhanced clearance (Ellies *et al*, 2002). Genetic ablation of this enzyme results in a significant bleeding phenotype with a significant reduction in plasma VWF levels. The circulatory half-life of endogenous VWF in *ST3Gal-IV*^{-/-} mice was also significantly reduced when compared to wild type (1.9 and 4.5 hours respectively). Furthermore, the inbred RIIS/J mouse strain exhibits aberrant VWF glycosylation profile caused by a change in the expression of N-acetylgalactosaminyltransferase (*Galgt2*) (Mohlke *et al*, 1999). Interestingly, this altered VWF glycosylation results in a significant reduction in plasma VWF levels in these mice which are reduced up to 20-fold. Normal expression of *Galgt2* is limited to gastrointestinal epithelial cells. However, in the RIIS/J inbred strain, expression of *Galgt2* is observed in vascular ECs. Thus, VWF synthesised undergoes aberrant glycosylation with abnormal GalNAc residue expression and subsequently VWF circulatory clearance is enhanced.

Despite the evidence that glycosylation and in particular sialylation influence VWF clearance, the biological mechanism(s) through which these glycans modulate their effects via specific lectin receptors remains poorly defined. This important question will be addressed in the studies presented in this thesis.

1.9. Purpose of Investigation

The aim of this research program was to elucidate the biological mechanisms through which glycosylation, and in particular sialylation, function to regulate VWF circulatory half-life. In addition, the relative importance of VWF N-linked and O-linked sialic acid was investigated. Finally, studies were performed to address the hypothesis that additional lectin receptors beyond the ASGPR may be important in regulating the physiological and pathological clearance of VWF.

2. Materials and methods

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

2.1.1. Purification of VWF from Fandhi®

A commercial VWF concentrate licensed for VWD patient treatment was used as the source of plasma derived (pd-) VWF for this study. Fandhi® (Grifols, Spain) contains high concentrations of VWF, FVIII, albumin and excipients. Size exclusion chromatography using gel filtration was used to further purify pd-VWF from Fandhi® concentrate.

2.1.2. Gel filtration

A Sepharose 2B-CL gel filtration column (600mm x 26mm x 320ml volume; Amersham Pharmacia, UK) stored in 20% ethanol was first washed with filtered deionised H₂O and was subsequently equilibrated with two column volumes of Tris-Citrate (TC) buffer (20mM Tris, 10mM sodium citrate, pH 7.4) at a flow rate of 0.5ml/min. Three vials of Fandhi were reconstituted in 20mL of sterile H₂O and applied to the column at a flow rate of 1ml/min. The 280nm UV read out was monitored to identify when protein elution was occurring and 5mL pd-VWF fractions were collected. After elution, the column was cleaned with two column volumes of 0.1M NaOH and finally washed with 20% ethanol for storage. VWF concentration and multimeric composition was determined as described in Section 2.4.

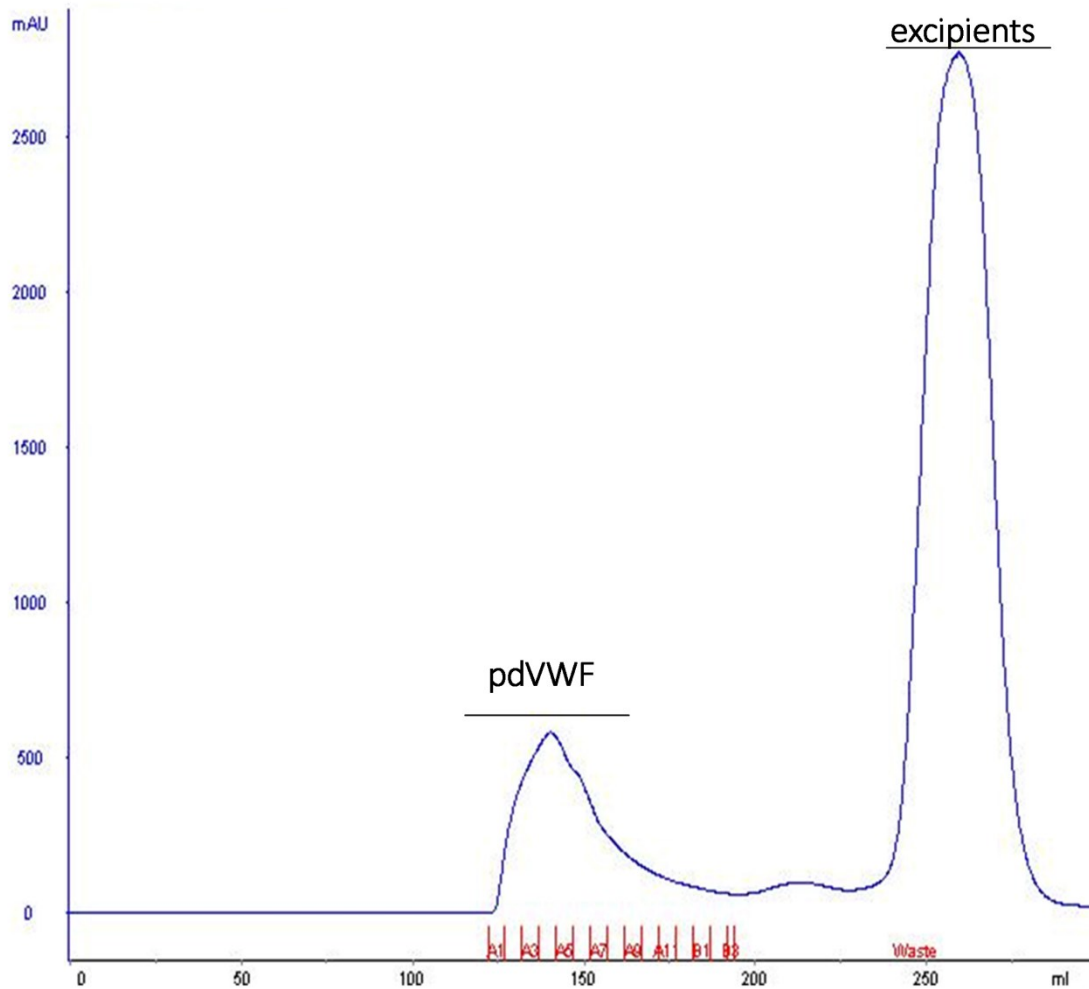


Figure 2.1: FPLC of Fandhi® pd-VWF

UV 280nm indicates elution of protein. The first UV peak represents the elution of pd-VWF multimers according to size, HMWM first followed by LMWM containing fractions. The second peak indicated elution of excipients within the Fandhi® preparation.

2.2. Generation of pd-VWF glycoforms

N- and O-linked glycan structures of pd-VWF were modified to generate a series of pd-VWF glycoforms. pd-VWF was treated with various exoglycosidases overnight at 37°C (Table 2.1), under non-denaturing conditions. Subsequently, quantitative lectin analysis (Section 2.4.3) was performed to confirm efficacy of each digestion.

Enzyme	Substrate	Source	Optimised use	Supplier
α2,3,6,8,9 Neuraminidase	α 2-3,6,8,9 linked sialic acid	<i>Arthrobacter ureafaciens</i>	1 μ l/10 μ g VWF	New England Biolabs, UK
α2-3 Neuraminidase	α 2-3 linked sialic acid	<i>Streptococcus pneumonia</i>	1 μ l/20 μ g VWF	New England Biolabs, UK
Peptide N Glycosidase	N-linked glycan	<i>Flavobacterium meningosepticum</i>	1 μ l/10 μ g VWF	New England Biolabs, UK
O Glycosidase	O-linked glycan	<i>Enterococcus faecalis</i>	10 μ l/5 μ g VWF	New England Biolabs, UK

Table 2.1: Optimised concentration of exoglycosidases for digestion of VWF

2.3. Production of recombinant VWF

The VWF expression vector used in this study was a kind gift from Dr McKinnon (Imperial College London, UK). This vector encodes an ampicillin resistance gene to allow for bacterial selection in *Escherichia coli*. This expression vector also contains a cytomegalovirus promoter for mammalian cell expression.

A number of full-length and truncated VWF variants (Figure 2.2) used in this study have previously been developed by Dr. Alain Chion (Haemostasis Research Group, TCD) and collaborator Dr. Thomas McKinnon (Imperial College London, UK). All recombinant VWF variants were expressed in Human Embryonic Kidney (HEK-293T) cells (ATCC, LGC Standards UK). T-175 (Nunc, UK) tissue culture flasks were seeded with HEK-293T cells and grown in Dulbecco's modified eagles medium (Sigma Aldrich, Ireland), supplemented with 10% foetal bovine serum (FBS) (Gibco, UK) and 1U/mL penicillin, 0.1mg/mL streptomycin (Sigma Aldrich, Ireland). Cells were routinely cultured in T-175 tissue culture flasks (Nunc, UK) and passaged at 75-85% confluence.

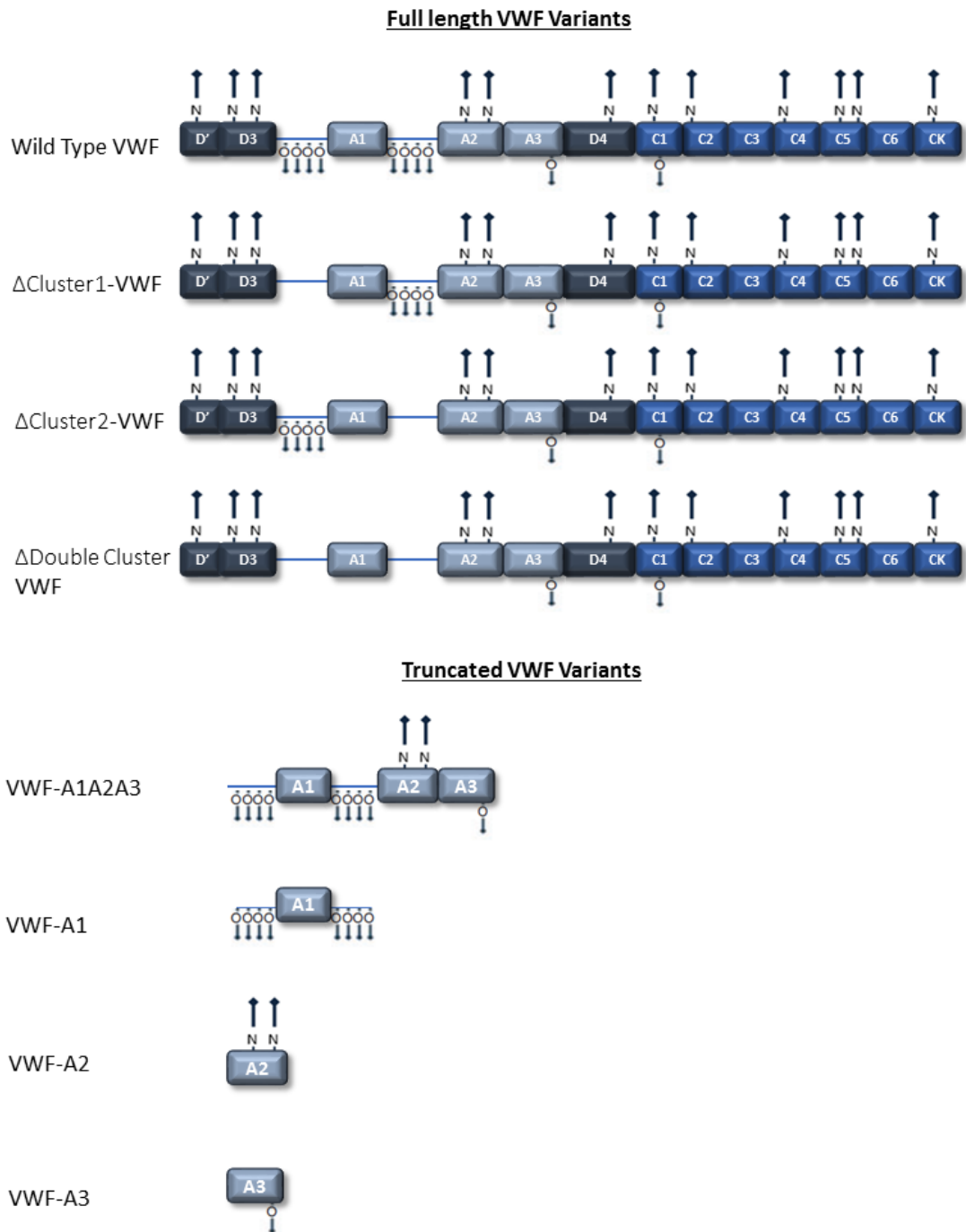


Figure 2.2: VWF variants used in this study

2.3.1. Transient transfection of HEK-293T cells

HEK-293T cells were seeded in T-175 cell culture flasks and grown to 90% confluency. Cells were washed with 10mL of sterile PBS (Sigma Aldrich, Ireland) after which 18mL of serum free Opti-MEM (Gibco, UK) was added to each flask. Plasmid DNA was diluted to a final concentration of 2µg/mL in 150mM NaCl. The transfection reagent bPEI (branched polyethylenimine; Sigma Aldrich, Ireland) was also diluted in sterile 150mM NaCl to a final concentration of 1µg/mL. Subsequently, bPEI was added to diluted plasmid DNA (2:1 ratio) in a drop-wise manner and incubated at room temperature for 20 minutes to allow complex formation. Following incubation, 2mL of the bPEI:DNA complex was added to each T-175 flask also in a drop-wise manner. Serum free conditioned media was harvested 72 hours after transfection. Media was centrifuged at 4,000rpm for 30minutes to pellet any cellular debris. Supernatant was collected and stored at -80°C until purification.

2.3.2. Concentration of recombinant VWF

Conditioned medium containing full length recombinant VWF was concentrated using anion-exchange chromatography (Figure 2.4). Conditioned media was thawed and filtered before being loaded onto a 5mL HiTrap Q HP column (Q Sepharose High Performance; GE Healthcare, UK). The column was equilibrated with 20mM Tris, pH 7.4 and VWF containing media was loaded at 1mL/minute. Column was washed with low salt buffer (20mM Tris, 100mM NaCl, pH 7.4) at 2mL/min. Elution was performed with high salt buffer (20mM Tris, 500mM NaCl, pH 7.4) at a flow rate of 0.5mL/min. Eluted 2mL VWF containing fraction was dialyzed in 2L of 20mM Tris, pH 7.4 at 4°C overnight.

After dialysis, full-length VWF was further concentrated to a final volume of 0.5mL using Amicon Ultra-15 100KDA molecular weight cut off filter (Merck Millipore, Ireland). Finally, VWF concentration was assessed using ELISA (Section 2.4.1) and rVWF was analysed by western blot (Section 2.4.5).

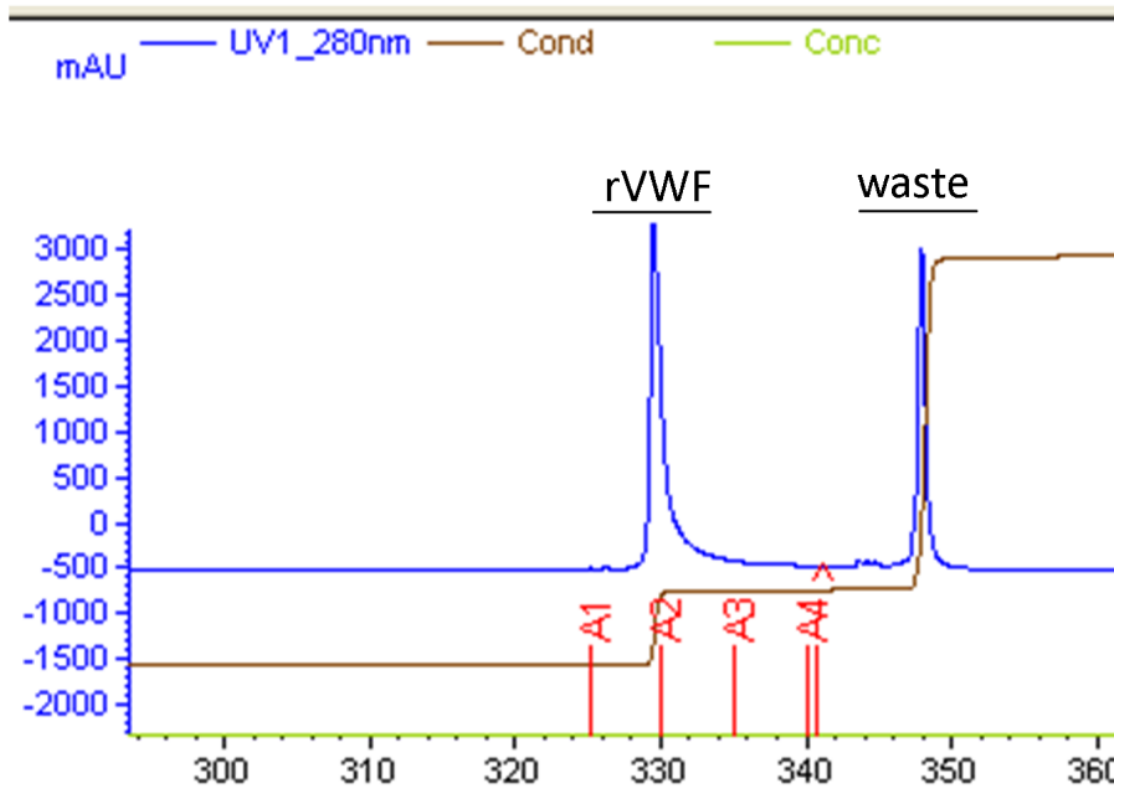


Figure 2.3: Anion exchange chromatography elution peak for recombinant VWF.

2.3.3. Purification of VWF using metal affinity chromatography.

Truncated VWF constructs (VWF-A1A2A3, VWF-A1, VWF-A2, and VWF-A3) were expressed with polyhistidine tag to allow for purification using metal ion affinity chromatography. A 1mL HiTrap Chelating Column (GE Healthcare, UK) was used as per manufacturers guidelines. Briefly, the column was first charged with 1mL of 0.1M NiCl₂. VWF fragment containing conditioned media was loaded onto the column at a flow rate of 0.5mL/min in 20mM Tris, 150mM NaCl 5mM Imidazole pH7.4. The column was washed with the same buffer at a flow rate of 1mL/min. VWF fragments were eluted from the column using 20mM Tris, 150mMNaCl, 500mM Imidazole, pH 7.4. Eluted VWF fragments were dialysed in 20mM Tris, pH 7.4 at 4°C overnight. Eluted VWF fragments were finally assessed for purity and concentration.

2.4. Analysis of VWF

2.4.1. VWF:Ag ELISA

VWF antigen (VWF:Ag) was measured by a sandwich enzyme-linked immunosorbant assay (ELISA). Briefly, maxisorp plates (Thermo Scientific™ Pierce™, UK) were coated with polyclonal anti-VWF antibody (Dako, Denmark) diluted 1:1000 in carbonate buffer (50mM Na₂CO₃, pH 9.6) overnight at 4°C. Wells were blocked for 1 hour at 37°C with 3% bovine serum albumin (BSA) in PBS-T (PBS containing 0.1% Tween-20 (Sigma Aldrich, Ireland)). VWF containing samples were diluted in PSB-T were added to wells. Reference plasma (Siemens, Germany) was diluted 1:80-1:2560 in PBS-T and used as a standard. Test samples and standard were incubated for 2 hours at 37°C, subsequently polyclonal anti-VWF-HRP (Dako, Denmark) diluted 1:1000 in PBS-T was added to the wells and incubated for 1 hour at 37°C. After washing 100µl of Horseradish Peroxidase (HRP) specific substrate 3,3',5,5'-Tetramethylbenzidine (TMB) (Substrate Reagent Pack, R&D Systems, UK) was added to wells. The reaction was subsequently stopped with 50µL 1M H₂SO₄. Optical density was measured at 450nm using a VERSAmax microplate reader (Molecular Devices, UK). 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 r² values were calculated using Microsoft Excel. All ELISAs were performed in triplicate and dilutions were measured per duplicate.

2.4.2. BCA Protein assay

For purified VWF fragments, total protein was quantified following purification using a commercial BCA (bicinchoninic acid) total protein assay kit (Thermo Scientific™ Pierce™, UK). BCA is a colourimetric assay which measures total protein concentration based on the reduction of Cu^{2+} ions to Cu^+ , resulting in a purple coloured product which absorbs at 562 nm

A standard curve was created using bovine serum albumin (BSA) which was diluted over a range of 2000-20 $\mu\text{g}/\text{mL}$, VWF test samples were serially diluted and 25 μl of each standard/sample was added to a 96 well plate in duplicate. BCA working reagent was prepared according to manufacturer's guidelines (50:1, Reagent A:B) and 200 μl was added to each well. The plate was incubated for 30minutes at 37°C and optical density was measured at 562nm. A standard curve was generated, and sample concentrations were extrapolated from the curve using linear regression analysis. GraphPad Prism software was used for data analysis (GraphPad Prism version 5.0 for windows; GraphPad Software, CA, USA).

2.4.3. Lectin ELISA

Polyclonal rabbit anti-VWF antibody (Dako, Denmark) was N-deglycosylated by overnight digestion with Peptide N Glycosidase (New England Biolabs, UK). Subsequently, Polysorp (Thermo Scientific™ Pierce™, UK) 96 well plates were coated overnight with deglycosylated polyclonal rabbit anti-VWF diluted 1:250 in carbonate buffer pH9.6. Non-specific binding was blocked with protein free blocking solution (Thermo Scientific™ Pierce™, UK) for 1 hour at 37°C. Samples containing VWF diluted to

(1 – 0.125µg/mL) in PBS-T were added and incubated for 2 hours at 37°C. Biotinylated plant lectins (Table 2.2) were also diluted in PBS-T and incubated for 1 hour at 37°C. For detection, high sensitivity streptavidin-HRP (Thermo Scientific™ Pierce™, UK) diluted 1:1000 was added to wells and incubated for 1 hour at 37°C. Finally, 100µl of TMB (Substrate Reagent Pack, R&D Systems, UK) was added to wells. The reaction was subsequently stopped with 50µL 1M H₂SO₄. Optical density was measured at 450nm. A standard curve was generated by plotting VWF concentration (µg/mL) on the x-axis against optical density (absorbance @ 450nm) on the y-axis. Lectin binding was quantified by calculating the relative slopes of the of the curves. Reference plasma was used as a standard, the slope for which was referred to as 100%.

Plant Lectin	Concentration	Glycan Specificity
Elderberry bark lectin/ <i>Sambucus Nigra</i> (SNA)	0.1µg/mL	α2-6 linked sialic acid
<i>Maackia amurensis</i> lectin II (MAL II)	2.5µg/mL	α2-3 linked sialic acid
Wheat germ agglutinin (WGA)	1µg/mL	Polysialic acid & N-acetylgalactosamine
Peanut Agglutinin (PNA)	1µg/mL	Thomas Friedenreich antigen (Gal-β1-3-GalNAc-Ser/Thr)
<i>Ricinus communis</i> agglutinin I (RCA I)	0.5µg/mL	Galactose and N-acetylgalactosamine
<i>Erythrina cristagalli</i> lectin (ECA)	1µg/mL	Galactose, N-acetylgalactosamine and H antigen

Table 2.2: Biotinylated Plant lectin specificity

2.4.4. VWF multimer gel

VWF multimer analysis was performed using a Bio-Rad mini gel casting system (Fannin LTD, UK). Glass plates (1.5mm depth) were cleaned with methanol and placed in a 37°C incubator for warming. A 1.8% agarose gel was 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 ~75°C before pouring 11mLs per/gel into the pre-heated plates. A 10 well comb (1.5mm depth) was inserted immediately before incubating the gels at 4°C to allow them to set.

VWF samples (containing 5ng VWF) were mixed with NuPAGE LDS sample buffer (4X; Life Technologies, UK) and heated at 70°C. Gels were electrophorised for 180 minutes at 15mAmp/gel in MOPS buffer (0.02M 3-(N-morpholino)propnesulfonic acid, pH7.4). Subsequently, protein was transferred to PVDF membrane (Immobilon-FL, Millipore, USA) using an electroblot system (Trans-Blot Electrophoretic Transfer Cell, Bio-Rad, Fannin LTD, UK). The membrane was blocked for 1 hour at room temperature with PBS-T containing 5% BSA. Membranes were incubated with anti-VWF-HRP (Dako, Denmark) diluted 1:1000 in PBS-T for 1 hour at room temperature. Finally, after extensive washing (10 minutes washes in triplicate) with PBS-T, bound antibody was detected using SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Scientific™ Pierce™, UK). HRP signal was subsequently visualised using an automated imager (Amersham™ Imager 600, GE Healthcare, UK) (Figure 2.4).

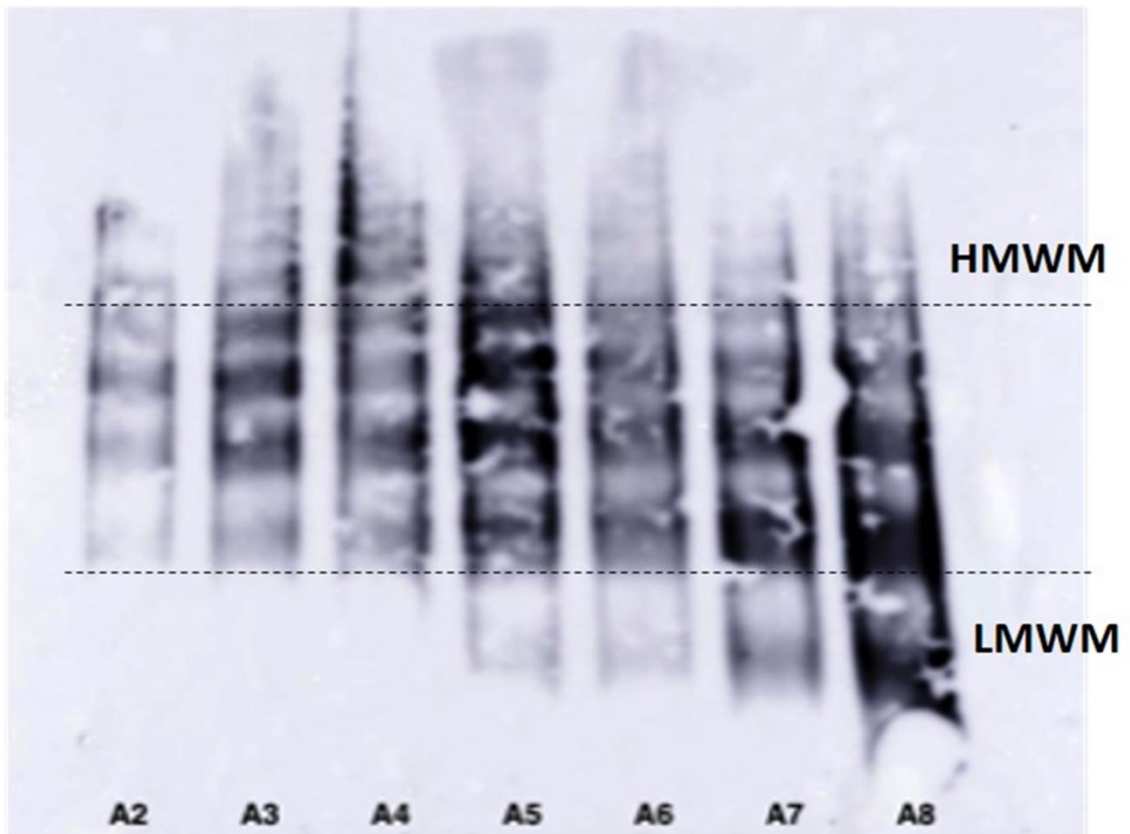


Figure 2.4: Multimer gel of pd-VWF containing fractions post FPLC purification of Fandhi.

2.4.5. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Commercial gradient gels were used for analysis of VWF (4-12% NuPAGE Bis-Tris, Life Technologies, UK). VWF samples were mixed with NuPAGE LDS sample buffer (4X; Life Technologies, UK) and heated at 70°C for 10 minutes (for reducing conditions samples were incubated with 1M 1,4-dithiothreitol). A pre-stained molecular weight marker (PageRuler Prestained Protein Ladder; Thermo Scientific™ Pierce™, UK) was loaded in parallel with VWF samples. Electrophoresis was carried out at 200V for 50 minutes in MOPS buffer. Protein detection was performed as described below.

2.4.5.1. Coomassie staining

For staining of total protein, GelCode™ Blue Stain (Thermo Scientific™ Pierce™, UK) was used according to manufacturer's guidelines. Following SDS-PAGE gels were rinsed with dH₂O and incubated with GelCode™ blue stain for 30 minutes. Finally, gels were de-stained with dH₂O for 1 hour with gentle agitation. GelCode™ is reported to be sensitive to protein detection in the range of 5-25ng.

2.4.5.2. Western blotting

Proteins were transferred from the gel to PVDF membrane using an electroblot system (Trans-Blot Electrophoretic Transfer Cell, Bio-Rad, Fannin LTD, UK) with commercial transfer buffer (Thermo Scientific™ Pierce™, UK). Following protein transfer, the membrane was blocked using PBS-T containing 5% BSA at room temperature for 1 hour. The membrane was then washed with PBS-T and incubated with polyclonal anti-human VWF-HRP (Dako, Denmark) diluted 1:1000 in PBS-t for 1 hour at room temperature.

After incubation the membrane was again washed with PBS-T and bound antibody was detected using SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Scientific™ Pierce™, UK). HRP signal was subsequently visualised using an automated imager (Amersham™ Imager 600, GE Healthcare, UK) (Figure 2.5).

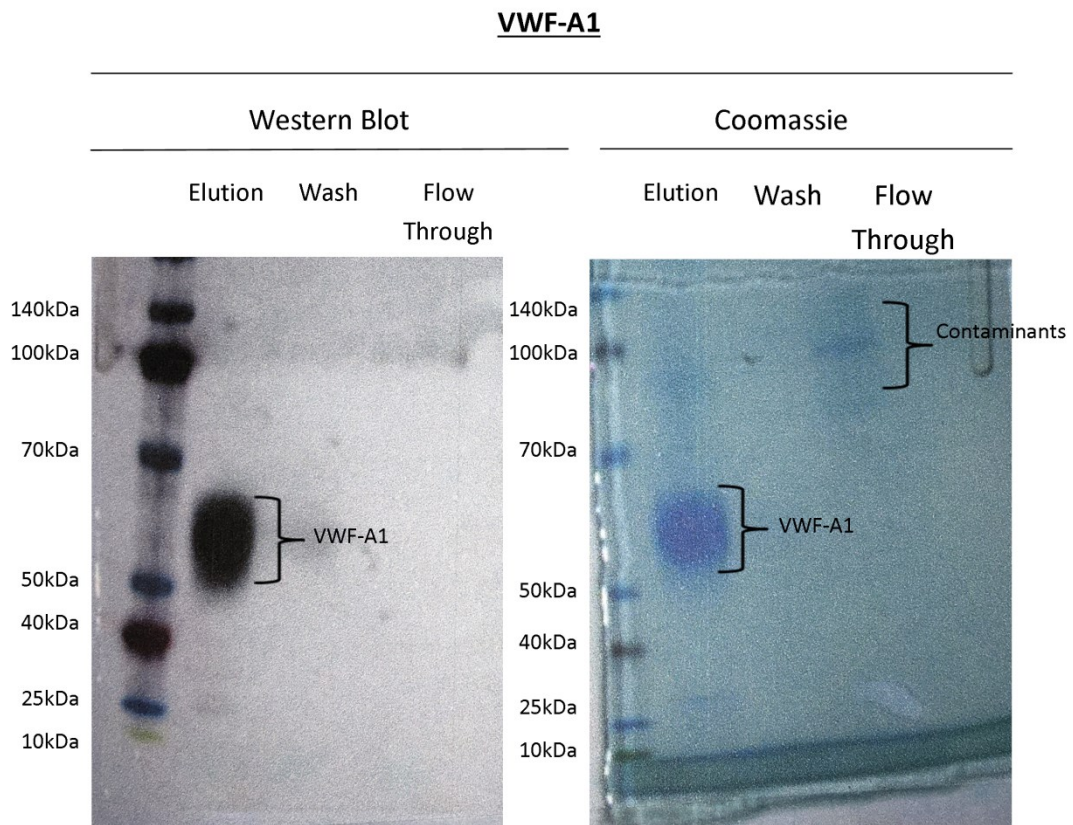


Figure 2.5: Western blot and Coomassie stain; VWF-A1 protein

2.5. Solid phase immunosorbant lectin receptor binding studies

2.5.1. *In vitro* Macrophage Galactose Lectin binding

Recombinant human MGL (Stratech, UK) was immobilised at 5µg/mL on a polysorp 96 well plate (Thermo Scientific™ Pierce™, UK) in 50mM carbonate buffer pH 9.6 for 1 hour at 37 °C. Wells were blocked with 5% BSA in PBS-T for 1 hour at 37 °C. Pd-VWF or glycoforms thereof diluted in PBS-T supplemented with 2.5mM CaCl₂, 1mg/mL ristocetin (MP Biomedicals, UK) were added to wells and incubated at 37°C for 1 hour. For detection of full length VWF, HRP conjugated polyclonal anti-VWF (Dako, Denmark) diluted 1:1000 was added to wells for 1 hour 37°C. Conversely for His-tagged truncated VWF fragments anti-His-HRP antibody (Qiagen, UK), diluted 1:2500 in 5% BSA in PBS-T, was incubated for 1 hour at 37°C. Finally, bound VWF was detected with HRP substrate TMB (Substrate Reagent Pack, R&D Systems, UK). The reaction was subsequently stopped with 50µL 1M H₂SO₄. Optical density was measured at 450nm using a VERSAmax microplate reader (Molecular Devices, UK).

2.5.2. *In vitro* Asialoglycoprotein receptor 1 binding

Recombinant human Asialoglycoprotein receptor 1 (ASGPR1) (Strattech, UK) was immobilised on a Polysorp 96 well plate at 5µg/mL (Thermo Scientific™ Pierce™, UK) in 50mM carbonate buffer pH 9.6 for 1 hour at 37°C. Wells were blocked with 5% BSA in PBS-T for 1 hour at 37°C. Pd-VWF or glycoforms thereof diluted in PBS-T supplemented with 2.5mM CaCl₂ and 1mg/mL ristocetin (MP Biomedicals, UK) were added to wells and incubated at 37°C for 1 hour. Subsequently, HRP conjugated polyclonal anti-VWF antibody (Dako, Denmark) diluted 1:1000 was added to wells for 1 hour 37°C. Finally, bound VWF was detected with HRP substrate TMB (Substrate Reagent Pack, R&D Systems, UK). The reaction was subsequently stopped with 50µL 1M H₂SO₄. Optical density was measured at 450nm using a VERSAmax microplate reader (Molecular Devices, UK).

2.5.3. *In vitro* Macrophage Mannose Receptor binding

Maxisorb 96 well plates (Thermo Scientific™ Pierce™, UK) were coated with pd-VWF 20µg/ml in 50mM carbonate buffer pH 9.6 supplemented with 1mg/mL ristocetin (MP Biomedicals, UK) for 1 hour at 37°C. Wells were blocked with 5% BSA in PBS-T for 1 hour at 37 °C. Histidine tagged recombinant human macrophage mannose receptor (MMR) (R&D Systems, UK) 10µg/ml in PBS-t 0.1% Tween-20 were added to wells and incubated at 37 °C for 1 hour. HRP conjugated anti-His antibody (Qiagen, UK) diluted 1:2500 in 5% BSA in PBS-T for 1 hour at 37°C. Bound MMR was detected with HRP substrate TMB (Substrate Reagent Pack, R&D Systems, UK). The reaction was subsequently stopped

with 50 μ L 1M H₂SO₄. Optical density was measured at 450nm using a VERSAmax microplate reader (Molecular Devices, UK).

2.6. Surface plasmon resonance binding analysis

Surface plasmon resonance (SPR) was used to evaluate MGL binding to VWF. Highly purified pd-VWF (Cambridge Biosciences, UK) was covalently immobilized on a CM5 chip at a surface density of 1500RU using an Amine Coupling Kit (GE Healthcare, UK) in 10mM sodium acetate buffer pH 5.0. Interaction experiments were performed using recombinant human MGL (50 - 1.56 μ g/mL) (R&D Systems, UK) in 10mM HEPES, 150mM NaCl, 5mM CaCl₂, 0.05% v/v Tween-20 buffer. Binding was assessed over a contact time of 120 sec and a dissociation time of 180 sec.

2.7. THP1 macrophage binding studies

To investigate the role of macrophages in modulating the enhanced clearance of hyposialylated VWF, *in vitro* binding studies were performed using THP1 cells (ATCC, LGC Standards UK). These are a human monocytic suspension cell line derived from a patient with acute monocytic leukaemia which can be differentiated to a macrophage phenotype for experiments. Cells were maintained in RPMI media (containing 2mM glutamine) (Gibco, UK) supplemented with 10% FBS (Sigma Aldrich, Ireland).

To assess binding of VWF variants to differentiated THP1 macrophages, cells were seeded onto 96 well plates at a density of 5 x 10⁶ cells/mL and differentiated with 100nM PMA for 72 hours, binding was performed after a 4-day resting period. VWF was diluted (10 and 5 μ g/mL) in ice-cold serum free growth medium and incubated with

differentiated THP1 cells for 1 hour at 4°C to prevent endocytosis. Non-adherent cells were removed by gentle washing with PBS and the wells were fixed with 4% paraformaldehyde (Sigma-Aldrich, Ireland). VWF binding to differentiated THP1 macrophages was assessed using flow cytometry. Fc receptors were blocked using a Fc-gamma receptor inhibitor (Thermofisher, UK). Bound VWF was detected using polyclonal rabbit anti-human VWF (Dako, Denmark) for 30minutes followed by anti-rabbit Alexa-488 (Thermofisher, UK) for 30minutes. Cells were acquired by BD FACSCanto™II flow cytometer (Becton Dickinson, USA) and live, single cells were gated. Data was analysed using FlowJo version 10 software (FlowJo, USA).

To examine whether VWF is able to bind to MGL expressed natively on macrophages, Proximity Ligation Assay (DuoLink-PLA, Sigma-Aldrich, Ireland) was performed on differentiated THP1 macrophages on glass coverslips (Nunc, UK). Cells were incubated with 5µg/mL VWF or PBS control in RPMI supplemented with 1M CaCl₂ for 30 minutes at room temperature. Following binding and washing, primary antibody rabbit anti-VWF (Dako, Denmark) and mouse anti-MGL (R&D Systems, UK) were incubated with THP1 macrophages in 1.5% BSA/PBS for 30 mins at 4°C. Species-specific secondary antibodies conjugated with a short DNA probe were subsequently incubated with the THP1 macrophages according to manufacturer's guidelines. When the DNA probes are in close proximity (~40 nm), they hybridise and undergo amplification to reveal discrete fluorescent red dots. Co-localisation was analysed using the fluorescence microscopy IN Cell Analyzer 63 (GE Healthcare, UK). Eight fields of view were imaged at a magnification of ×20. Data are presented as images of representative cells, quantification of co-localisation was not performed.

2.8. Animal Husbandry

For all *in vivo* experiments male and female wild type (WT), *VWF*^{-/-}, *Asgr1*^{-/-}, *VWF*^{-/-}/*Asgr1*^{-/-}, *MGL1*^{-/-} and *MMR*^{-/-} (Table 2.3) were used between 20-25 grams body weight/ at 6-10 weeks of age. Mice were housed in individually ventilated cages under positive air flow pressure (Techniplast, UK) and food and water were supplied *ad libitum*. All murine experiments were reviewed by the ethical committee in Trinity College Dublin and approved by the Health Product Regulatory Authority (HPRA) in Ireland.

Abbreviation	Strain identification	Background
WT	C57 BL/6J	C57BL/6J
<i>VWF</i> ^{-/-}	B6.129S2- <i>Vwf</i> ^{tm1Wgr} /J	C57BL/6J
<i>Asgr1</i> ^{-/-}	B6.129S4- <i>Asgr1</i> ^{tm1Sau} /SaubJxmJ	C57BL/6J
<i>MGL1</i> ^{-/-}	B6.129- <i>Clec10a</i> ^{tm1Hed} /J	C57BL/6J
<i>MMR</i> ^{-/-}	B6.129P2- <i>Mrc1</i> ^{tm1Mnz} /J	C57BL/6J

Table 2.3: Mouse strains

2.8.1. Generation of $VWF^{-/-}Asgr1^{-/-}$ double knockout mice

To generate $VWF^{-/-}Asgr1^{-/-}$ mice, $VWF^{-/-}$ (B6.129S2- Vwf^{tm1Wgr}/J) and $Asgr1^{-/-}$ (B6.129S4- $Asgr1^{tm1Sau}/SaubJxmJ$) mice were cross bred (Figure 2.6). First generation mice were heterozygous for all traits ($VWF^{+/-}Asgr1^{+/-}$). These mice were then bred with $VWF^{-/-}$ to fix the $VWF^{-/-}$ mutation and subsequent progeny were genotyped. Once progeny was confirmed $VWF^{-/-}Asgr1^{+/-}$, these mice were bred together until the desired double mutation ($VWF^{-/-}Asgr1^{-/-}$) was observed. $VWF^{-/-}Asgr1^{-/-}$ mice are viable and do not exhibit a pathological phenotype.

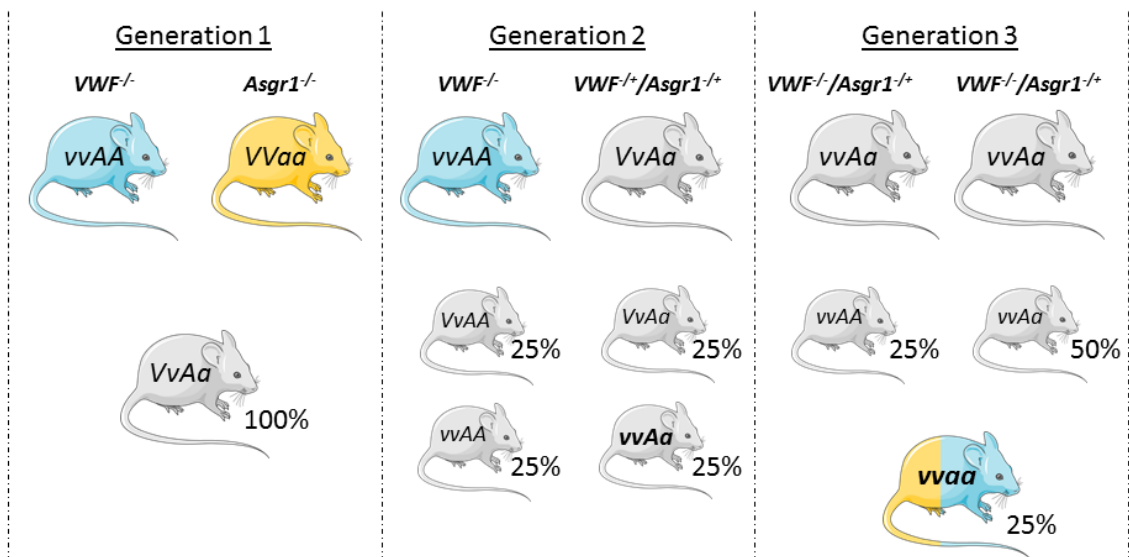


Figure 2.6: Breeding protocol schematic

2.8.2. Genotyping protocols

Ear clippings were taken after weaning each litter (3 weeks of age), anaesthetic was not used for this minimally invasive procedure. Ear samples were digested in 75µl of 25mM NaOH, 0.2mM EDTA at 98°C for 2 hours, vortexing samples at 15-minute intervals. Samples were neutralised by the addition of 75µl of 40mM Tris-HCL pH 5.5 and centrifuged at 4,000rpm for 3 minutes. Polymerase chain reaction (PCR) was carried out on 2µl of the DNA lysis above using PrimeSTAR™ HS DNA Polymerase (Takara, Clontech, USA) according to manufacturer's guidelines with appropriate primers for each mouse strain as listed below. PCR amplicons were run on a 1% agarose (w/v) gels dissolved in Tris Borate EDTA buffer (90mM Tris, 90mM Boric Acid, 2mM EDTA, pH 8.3). SYBRsafe (Invitrogen, UK), a non-hazardous alternative for ethidium bromide was added 10% (v/v) and gels were poured in a horizontal electrophoresis tank. Samples were prepared with 10x loading buffer and run in parallel with a 1kb DNA base pair ladder and subsequently visualised with ultraviolet light.

Primer Name	5'-3' Sequence	Band size
VWF/wild type	CCC AAC TTT TGC CAA CAA ATA	965bp
VWF/mutant	CCT TCT ATC GCC TTC TTG ACG	670bp
VWF/common	AGT GAG ACC TTT GGC TTT GC	

Table 2.4: *VWF* genotyping primer sequences.

Genotype	Multiplex reaction result
VWF ^{+/+}	A single band at 965bp
VWF ^{-/-}	A single band at 670bp
VWF ^{+/-}	A band at 965bp and 670bp

Table 2.5: *VWF* genotyping results

Step No	Temperature (°c)	Time	Note
1	94	2 min	
2	94	20 sec	
3	65	15 sec	-0.5°c per cycle
4	68	10 sec	
5			Repeat steps 2-4 for 10 cycles
6	94	15 sec	
7	60	15 sec	
8	72	10 sec	
9			Repeat steps 6-8 for 28 cycles
10	72	2 min	
11	4		Hold

Table 2.6: *VWF* genotyping PCR protocol

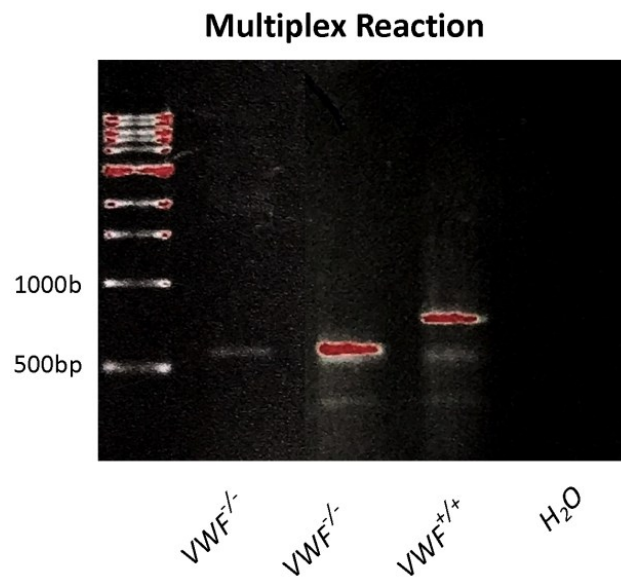


Figure 2.7: *VWF* genotyping reaction performed as a multiplex reaction

Primer Name	5'-3' Sequence	Band size
<i>Asgr1</i>/wild type	CAG TAG GCC CCA CAC CTT	500bp
<i>Asgr1</i>/mutant	GCC TGA AGA ACG AGA TCA GC	750bp
<i>Asgr1</i>/common	CAG GCT TGG GAG CAG ATA GG	

Table 2.7: *Asgr1* genotyping primer sequences.

Genotype	Wild type reaction result	Mutant reaction result
<i>Asgr1</i>^{+/+}	A single band at 500bp	Blank
<i>Asgr1</i>^{-/-}	Blank	A single band at 750bp
<i>Asgr1</i>^{+/-}	A single band at 500bp	A single band at 750bp

Table 2.8: *Asgr1* genotyping results

Step No	Temperature (°c)	Time	Note
1	95	2 min	
2	95	20 sec	
3	65	15 sec	-0.5°c per cycle
4	68	10 sec	
5			Repeat steps 2-4 for 10 cycles
6	95	15 sec	
7	60	15 sec	
8	72	10 sec	
9			Repeat steps 6-8 for 28 cycles
10	72	2 min	
11	4		Hold

Table 2.9: *Asgr1* genotyping PCR protocol

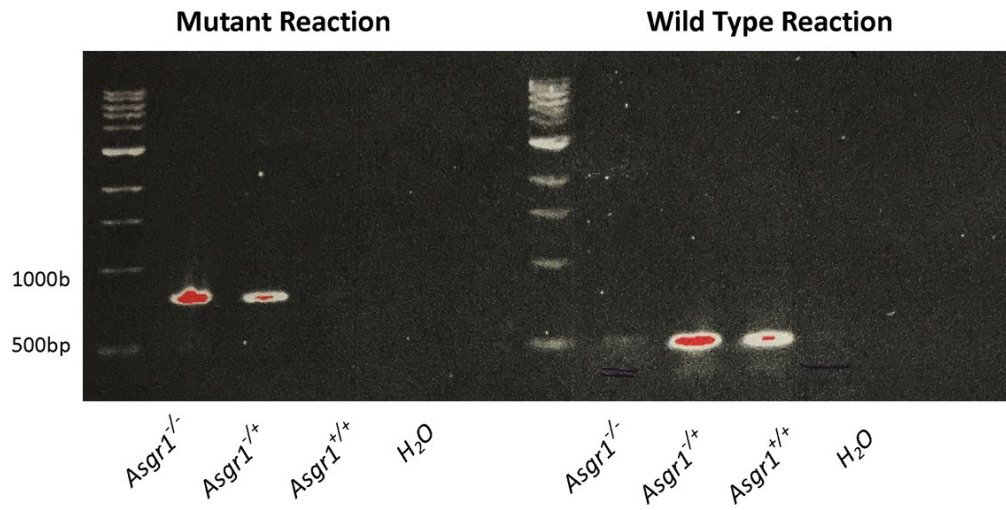


Figure 2.8: *Asgr1* genotyping reaction performed as a single plex reaction

2.8.3. Anaesthetic preparation

For non-recovery procedures tribromoethanol (TBE) was chosen as a suitable anaesthetic. This compound has a quick induction time (approx. 2mins) and thus is suitable for the time sensitive nature of blood collection for pharmacokinetic studies. The stock solution of TBE was prepared by dissolving 2,2,2 tribromoethanol (Sigma-Aldrich, Ireland) in T-amyl alcohol (Sigma-Aldrich, Ireland) to a final concentration of 1.6mg/ml. A working stock of 2.5% (v/v) in sterile saline was prepared for use in animal experiments; dose used was 20ml/kg body weight administered intraperitoneally.

2.8.4. Intravenous injection

For intravenous (IV) injection, mice were immobilised in a restraint unit and the tail was visualised using a led lamp up-light (Harvard Apparatus, UK). The tail was warmed gently in a gloved hand to encourage vasodilation. Tail veins were examined for quality and a suitable site for IV delivery was chosen. The tail was secured between forefinger and thumb of the non-dominant hand and the dominant hand was used to deliver desired dose of various compounds listed in Table 2.10.

Compound	Dose	Diluent
VWF/VWF glycoform protein	0.375mg/kg	Sterile PBS
Orsomuroid/Asialoorsomuroid	15mg/kg	Sterile PBS
Clodronate Liposome	1.875g/kg	Sterile PBS
Polyclonal IgG goat control	2mg/kg	Sterile PBS
anti MGL1/2 polyclonal IgG	2mg/kg	Sterile PBS
Oseltamivir Phosphate	10mg/kg	Sterile PBS
Biotin NHS	10mg/kg	5% DMSO

Table 2.10: Intravenous dosing

2.8.5. Intraperitoneal injection

For intraperitoneal (i.p) injection mice were held securely by the scruff in a gloved hand for restraint. The abdomen is exposed and using a 23 gauge needle the bottom right quadrant of the abdomen was injected.

2.8.6. Submandibular blood collection

This technique was used to collect small volumes of blood (20-250µl) for repeat sampling a subset of pharmacokinetic experiments. The mouse was securely held by its scruff and a 3mm lancet was used to prick the submandibular vein. Blood is collected into lithium heparin coated microtainers (BD Unitech, Ireland.) and gentle pressure was applied to arrest bleeding.

2.8.7. Subclavical blood collection

Mice were anaesthetised with i.p injection of 20ml/kg TBE, once pedal reflex was lost mice were placed on their side and forelimbs stretched. A deep incision was made, and the subclavian or axillary arteries were severed. Blood was collected into lithium heparin coated microtainers (BD Unitech, Ireland). Mice were sacrificed after collection.

2.8.8. Cardiac puncture blood collection

Mice were anaesthetised with 20ml/kg TBE, once pedal reflexes were lost a lateral incision through the integument and abdominal wall was made, just beneath the ribcage. The liver was carefully separated from the diaphragm and the pleural cavity was exposed. After carefully displacing the lungs, the rib cage was cut up the collarbone. Any tissue connected to the heart was carefully trimmed away. Using a 1ml syringe coated in 109mM sodium citrate attached to a 25-gauge needle blood was collected directly from the right atrium.

2.9. VWF clearance studies

2.9.1. Plasma preparation

Whole blood was collected into lithium heparin or sodium citrate containing microtainers (anticoagulant selection was dependant on downstream assay). Samples were processed within 1-4 hours of collection to ensure sample quality. Platelet poor plasma was prepared by centrifugation at 2,500 rpm for 15 minutes at 20°C and aliquots were stored at -20°C until analysis.

2.9.2. Clearance of infused VWF in *VWF*^{-/-} and *VWF*^{-/-}/*Asgr1*^{-/-} mice

All clearance experiments were performed on mice 8-10 weeks old, in accordance with the HPRA. Mice were infused with 0.75U of pd-VWF or glyco-variants thereof via tail-vein injection and subsequently anesthetized with 2.5% tribromoethanol prior to blood collection. Blood was collected via sub-clavicle/sub-mandibular incision into lithium-heparin coated microtainers. Three to five mice per time point were used. Residual plasma VWF:Ag levels were determined at specific time points by ELISA. In preliminary experiments, we demonstrated that infused pd-VWF is not subjected to enhanced ADAMTS13 proteolysis upon infusion (Figure 2.9).

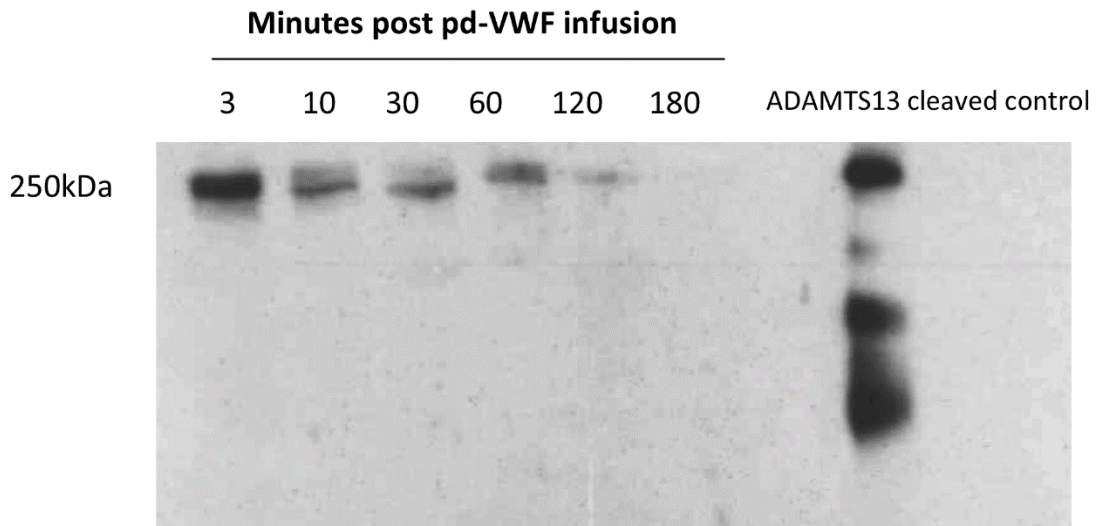


Figure 2.9: Clearance of human pd-VWF over time in $VWF^{-/-}$ mice without the presence of ADAMTS13 proteolytic bands.

2.9.3. VWF clearance curve analysis in *VWF*^{-/-} mice

Plasma VWF:Ag levels in the murine blood samples were measured by ELISA (Section 2.4.1). The starting concentration of VWF infused was corrected for the dilution factor of total mouse blood volume. Residual VWF:Ag values was expressed as a percentage of this injected amount. Clearance curves were graphed as percentage residual VWF as a function of time (minutes/hours). These 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 determine VWF half-life ($t_{1/2}$) and Mean Residence Time (MRT). The following equation was used to fit exponential clearance data:

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

Y = the amount of residual VWF:Ag in plasma relative to the amount injected

X= time.

K= rate constant

Plateau = 0

MRT equation: $MRT = 1/K \pm SEM$

Half-life equation: $t_{1/2} = \ln 2 / K \pm SEM$.

2.9.4. Clodronate induced macrophage depletion

Clodronate is a bisphosphate drug, intracellular accumulation inhibits mitochondrial function, resulting in cell death. Once encapsulated in a lipid bilayer of liposomes clodronate is specifically targeted to resident macrophages in the liver and spleen (Van Rooijen *et al*, 1989). For macrophage depletion, mice were injected intravenously with clodronate liposomes (100 μ l/10g body weight) 24 hours prior to injection of VWF. Control mice were injected with PBS liposomes. Previous verification studies performed in our lab have demonstrated through flow cytometry analysis that treatment with clodronate liposomes results in a 75%-reduction in F4/80+/CD11B+ cell populations in the liver and spleen (Figure 2.12).

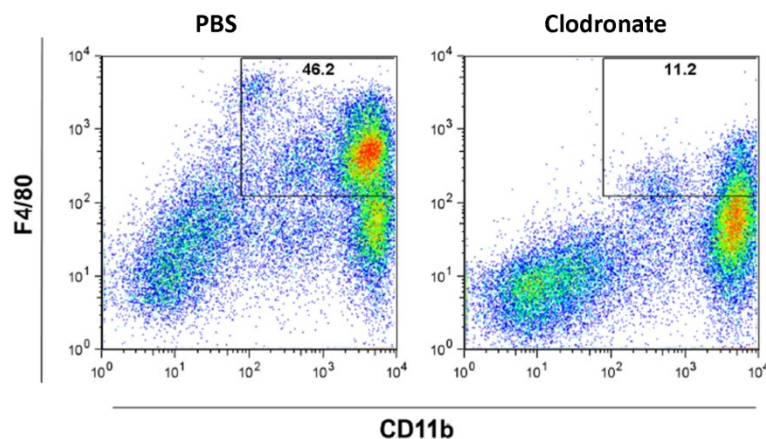


Figure 2.10: Flow cytometry scatter plot of WT mouse splenic F4/80 CD11B macrophages

Experiment performed by Dr Hendrik Nel (Inflammation and Immunity Research Group, Trinity College Dublin).

2.9.5. ASOR

Additionally, VWF clearance was performed in the presence of the antagonist asialo-orosomucoid (ASOR). For inhibition of galactose sensitive lectin receptors, mice were intravenously injected with 300µg ASOR in 100µl PBS, 1 minute prior to injection of VWF. To generate ASOR, orosomucoid protein (Sigma Aldrich, USA) was treated with 16mU α 2-3,6,8,9 Neuraminidase overnight at 37°C. Efficacy of sialic acid removal was confirmed with lectin binding assays.

2.9.6. Inhibition of endogenous neuraminidase

Oseltamivir phosphate (OP) was utilised to inhibit neuraminidase activity *in vivo*. OP was injected intravenously at a dose of 10mg/kg. Time-point zero was determined to be 2 hours post OP infusion as OP is an inactive form of the compound which is metabolised to its active form oseltamivir carboxylate (OC) within 2 hours (Holodniy *et al*, 2008). OC exhibits a 10-hour half-life in mice, thus for prolonged studies mice received a second dose of OP (40mg/kg) via IP injection 8 hours following initial OP infusion.

2.9.7. Specific inhibition of murine MGL1/2

For specific inhibition of MGL *in vivo*, polyclonal goat anti-mouse MGL1/2 antibody (R&D Systems, UK) was infused via intravenous injection 10 minutes prior to injection of VWF. This neutralizing antibody is targeted towards both the MGL1 and MGL2 homologs in mice. A non-specific polyclonal IgG antibody (R&D Systems, UK) was infused into control mice. A series of preliminary dose-titration experiments were first performed using a range of anti-MGL1/2 antibody concentrations (0.5mg/kg, 2mg/kg and 8mg/kg) co-infused with pd-VWF to determine optimal anti-MGL1/2 antibody concentrations. Based upon these experiments a dose of 2mg/kg was selected (Figure 2.13).

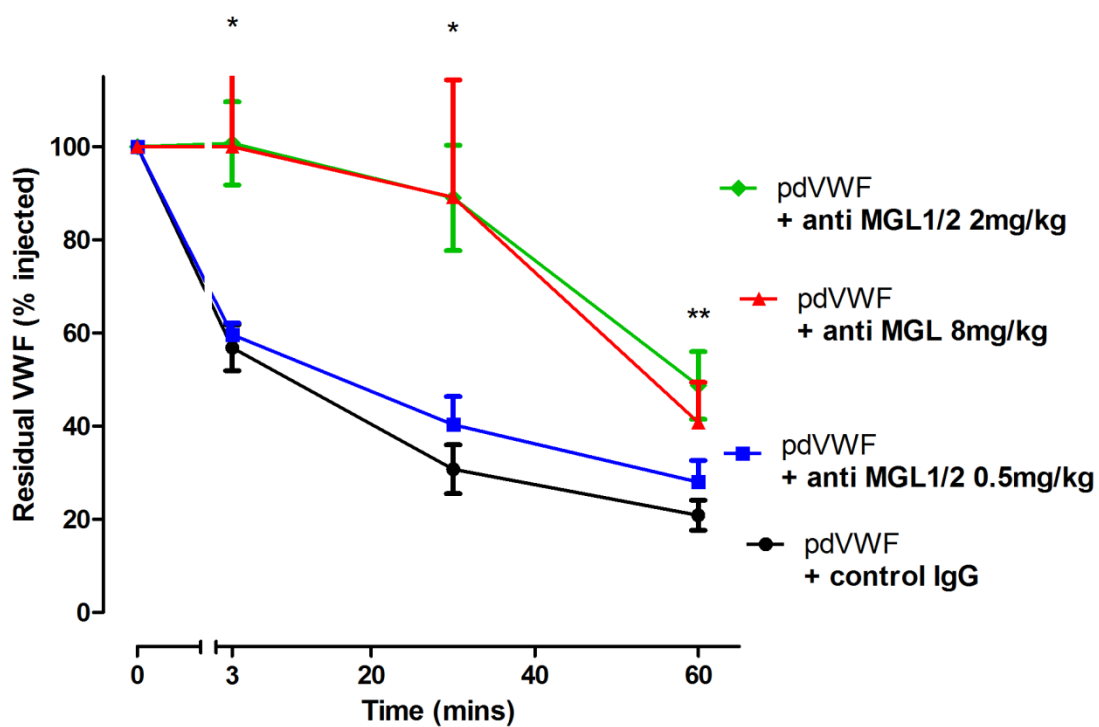


Figure 2.11: Optimisation of specific MGL1/2 inhibition

2.9.8. Clearance of endogenous murine VWF

Endogenous murine VWF (mVWF) was labeled with an intravenous injection of N-hydroxysuccinimide-biotin (Thermo Scientific™ Pierce™, UK) at a dose of 10 mg/kg of mouse body weight. Subsequently, blood was collected at specific time points into lithium-heparin containing microtainer tubes (BD Biosciences) via submandibular or subclavian vein blood draw. Residual biotinylated VWF was quantified using a modified VWF ELISA. Time 0 was defined as 10 min post biotin injection and mVWF clearance was calculated as a percentage of the biotin signal at time 0.

2.10. Analysis of murine VWF

2.10.1. Modified murine VWF:Ag ELISA

Murine plasma VWF:Ag levels were determined as per Section 2.4.1 with the following modifications. Maxisorp plates (Thermo Scientific™ Pierce™, UK) were coated with polyclonal anti-VWF antibodies (Dako, Denmark) diluted 1:500 in carbonate buffer (50mM Na₂CO₃, pH 9.6) overnight at 4°C. Wells were blocked for 1 hour at 37°C with 5% BSA in PBS-T. Murine VWF containing samples diluted (1:40) in PBS-T were added to wells. Pooled murine plasma (n=20), diluted 1:20 -1:1280) was used as a standard reference. This pool was generated using murine whole blood collection via cardiac puncture (into sodium citrate collection tubes) from 20 age and sex matched wild type C57Bl/6J mice, samples were centrifuged and plasma was aliquoted and stored at -80°C. Test samples and standard were incubated for 2 hours at 37°C, subsequently polyclonal anti-VWF-HRP antibody (Dako, Denmark) diluted 1:500 in PBS-T was added to the wells

and incubated for 1 hour at 37°C. HRP specific detection and analysis was performed as previously (Section 2.4.1).

2.10.2. Modified biotinylated murine VWF:Ag ELISA

Biotinylated murine plasma VWF:Ag levels were determined as per Section 2.4.1 with the following modifications. Reference plasma (Siemens, Germany) was biotinylated prior to the experiment. using NHS-Biotin according to manufacturer guidelines. Biotinylated- reference plasma was diluted and used as a standard reference. Plasma samples containing biotinylated murine VWF were diluted in PSB-T added to wells and incubated for 2 hours at 37°C. After washing, high-sensitivity streptavidin-HRP (Thermo Scientific™ Pierce™, UK) diluted 1;10,000 in PBS-T was added to the wells and incubated for 1 hour at 37°C. HRP specific detection and analysis was performed as previously (Section 2.4.1).

2.10.3. Determination of murine VWFpp:Ag ratio

Analysis of murine VWF propeptide to antigen (mVWFpp:Ag) ratio was performed in collaboration with Dr. Sandra Haberichter (BloodWorks Institute, Milwaukee, USA) as previously described (Jacobi *et al*, 2018).

2.10.4. Murine VWF lectin ELISA

Murine VWF lectin ELISA was performed as in Section 2.4.3 with some modifications. Polysorp 96 well plates (Nunc) were coated with N-deglycosylated polyclonal rabbit anti-VWF antibody (Dako, Denmark). Plates were blocked with protein free blocking solution (Thermo Scientific™ Pierce™, UK) for 1 hour at 37°C. Samples containing murine VWF

were diluted in PBS-T and were incubated for two hours at 37°C. Biotinylated plant lectins (Table 2.2) were also diluted (0.1 – 0.0125 U/mL) in PBS-T and incubated for 1 hour at 37°C. HRP specific detection and analysis was performed as previously. Murine VWF lectin binding was expressed as a percentage of untreated control pooled murine plasma.

2.11. Data Analysis and Statistics

Data analysis was performed using GraphPad Prism program (GraphPad Prism Version 5.0 for Windows). All experiments were performed in technical triplicate. Data is expressed as the mean \pm SEM, unless otherwise stated. To assess statistical differences in lectin binding single concentrations were analysed with Student's unpaired 2-tailed t-test. Apparent dissociation constant (KD) was calculated using nonlinear regression model for total and non-specific binding. For mouse clearance studies, half-life and MRT were defined as described in Section 2.9.3. For comparison of clearance data half-life and MRT were expressed \pm SD and statistical differences were assessed using a Student's unpaired 2-tailed t-test, with Bonferroni correction for multiple comparisons when applicable. Outliers were identified in murine population data using Grubbs' test for outliers, based upon the assumption of normality with a p value of 0.05 deemed significant.

3. Investigating the role of the asialoglycoprotein receptor in regulating VWF clearance

In studying the role of sialic acid in regulating the survival of glycoproteins in circulation, Ashwell and Morell discovered the hepatic ASGPR, also known as the Ashwell-Morell receptor (AMR). Morell *et al* first demonstrated that desialylated orsomucoid and fetuin were removed rapidly from the circulation in rats and were recovered in the liver (Morell *et al*, 1971). Concurrently, a key role for hepatocyte plasma membranes in mediating serum glycoprotein clearance was demonstrated (Pricer & Ashwell, 1971). ASGPR was isolated from rabbit liver and described as a protein with specific binding affinity for desialylated glycoproteins (Hudgin *et al*, 1974). Subsequently, human ASGPR was isolated and characterised (Baenziger & Maynard, 1980) (Figure 3.1).

ASGPR belongs to a family of mammalian lectin receptors termed C-type (calcium dependent) lectins. Members of this family contain a QPD consensus sequence within the carbohydrate recognition domain (CRD) (Taylor & Drickamer, 2003; Drickamer & Taylor, 2003). Extensive binding studies have demonstrated that ASGPR binding affinity for Gal/GalNAc terminating glycans is in the order of tetra-antennary > tri-antennary > bi-antennary > mono-antennary (Monestier *et al*, 2016; Zacco *et al*, 2015; Lodish, 1991; Connolly *et al*, 1982; Khorev *et al*, 2008).

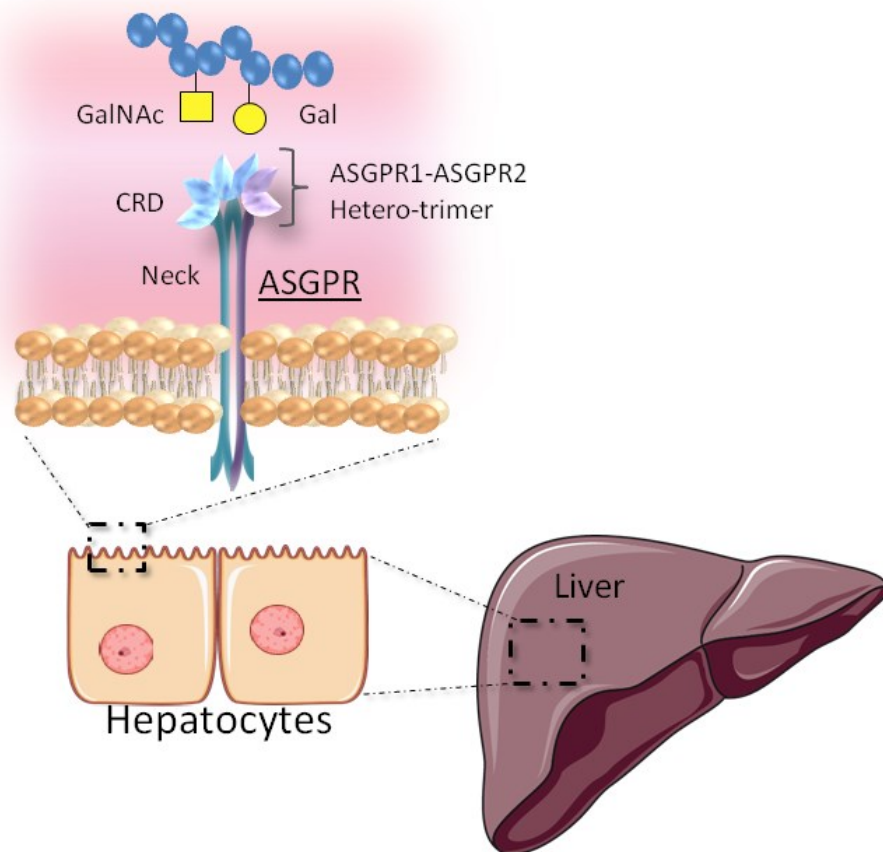


Figure 3.1: Asialoglycoprotein receptor

ASGPR is expressed exclusively on hepatocytes as a hetero-trimer, composed of a major (ASGPR1) and minor (ASGPR2) sub-units. The CRD of ASGPR has affinity for glycoproteins terminating in Gal/GalNAc.

More recent studies have identified that ASGPR can also bind to some glycoproteins expressing glycans terminating with sialic acid, albeit with a lower affinity compared to Gal/GalNAc moieties (Steirer *et al*, 2009; Park *et al*, 2005).

Grewal *et al* identified the hepatic ASGPR as the first clearance receptor for VWF *in vivo* (Grewal *et al*, 2008). In this study, it was demonstrated that bacterial neuraminidase-induced desialylation of VWF and platelets resulted in significantly enhanced circulatory clearance via ASGPR. Interestingly, in mice deficient in ASGPR2 subunit, endogenous VWF levels are not significantly different to wild type. However, mice deficient in ASGPR1 have a 1.5 fold increase in murine plasma VWF levels (Grewal *et al*, 2008). This may relate to the fact that *Asgr2*^{-/-} mice, residual ASGPR function is observed due to expression of ASGPR1 functional homo-trimers (Braun *et al*, 1996). Conversely, *Asgr1*^{-/-} mice have no assembly of ASGPR2 oligomers and thus no ASGPR function (Braun *et al*, 1996; Tozawa *et al*, 2001). The observed increase in endogenous plasma VWF levels in *Asgr1*^{-/-} mice was shown to be due to reduced VWF clearance.

Important questions regarding the biological mechanisms through which VWF sialylation regulates its clearance *in vivo* remain unclear. In particular, the relative importance of N-linked versus O-linked sialylation in regulating physiological and/or pathological clearance of VWF has not been defined. In addition to ASGPR, a number of other lectin receptors have been shown to bind with enhanced affinity to hyposialylated glycoproteins (Sørensen *et al*, 2012). Consequently, we first sought to re-examine the role of ASGPR in regulating mVWF clearance using *Asgr1*^{-/-} mice.

3.1. Hyposialylated VWF variants exhibit enhanced ASGPR binding *in vitro*.

To further investigate the role of VWF glycans in modulating interaction with ASGPR pd-VWF was treated with specific neuraminidases to generate VWF glycoforms (Figure 3.2 A). In previous studies, we and others have demonstrated that treatment with these neuraminidases has no significant effect upon pd-VWF multimer composition or collagen binding activity (McKinnon *et al*, 2008; McGrath *et al*, 2010a). Following each neuraminidase digestion, residual sialic acid expression on pd-VWF was characterized using a series of *Sambucus nigra* (specific affinity for α 2-6 linked) and *Maackia amurensis* (specific affinity for α 2-3 linked sialic acid) lectin-binding ELISAs (Figures 3.2 B and 3.2 C). Digestion with α 2-3,6,8,9 neuraminidase (α 2-3,6,8,9 Neu-VWF) resulted in loss of VWF sialic acid expression detected by both *Sambucus nigra* and *Maackia amurensis*. In contrast, VWF digestion with α 2-3 neuraminidase (α 2-3 Neu-VWF) resulted in a marked reduction in *Maackia amurensis* detectable sialic acid only. Previous studies suggest that terminal sialic acid expressed on the N-linked glycans of pd-VWF is predominantly α 2-6 linked to penultimate galactose residues (Canis *et al*, 2012; McGrath *et al*, 2013; Gashash *et al*, 2017). Conversely, O-linked sialic acid on pdVWF may be either α 2-6 or α 2-3 linked (Canis *et al*, 2010; McGrath *et al*, 2013; Solecka *et al*, 2016; Gashash *et al*, 2017). Unsurprisingly, following pdVWF digestion with α 2-3 or α 2-3,6,8,9 neuraminidase, binding of *Ricinus communis* to exposed terminal β -linked galactose residues was significantly increased (Figure 3.2 D). The absolute increase in *Ricinus communis* binding observed after α 2-3,6,8,9 neuraminidase was significantly greater than after α 2-3 neuraminidase digestion, which is consistent with the concept 80% of total sialic acid on pd-VWF is N-linked.

Following exoglycosidase digestion, the binding of each VWF glycoform to immobilised ASGPR1 *in vitro* was assessed. Interestingly, loss of α 2-3 linked sialic acid alone from VWF O-glycans resulted in a significant increase in VWF binding to ASGPR ($p < 0.05$) (Figure 3.3). Combined removal of VWF N-linked and O-linked sialylation resulted in a further marked increase in binding to ASGPR1 (Figure 3.3). These data are consistent with the reported preference of ASGPR for binding glycoproteins expressing tri-antennary complex glycans terminating in β 1-4 linked Gal residues.

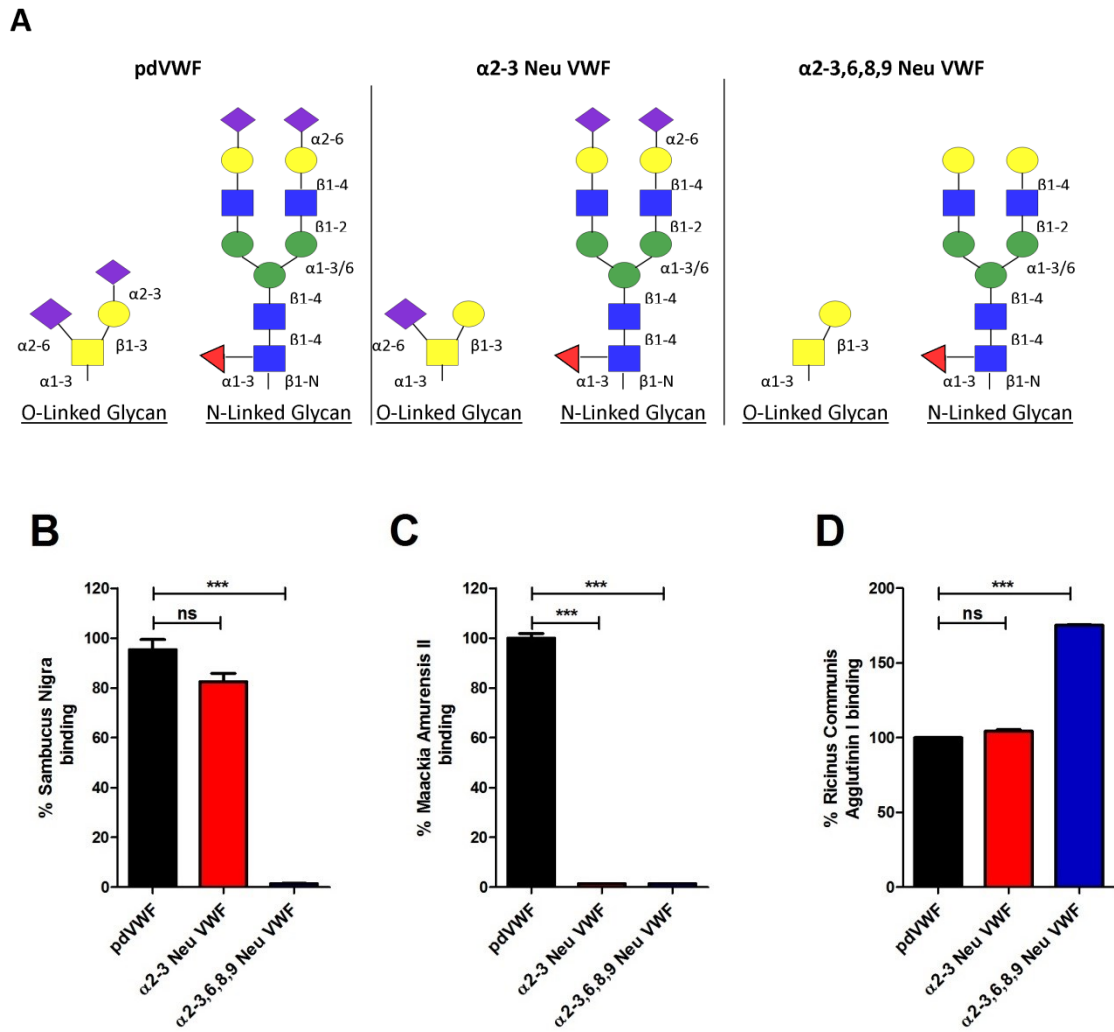


Figure 3.2: Generation of VWF glycoforms

Pd-VWF and glycoforms thereof (A) were generated using specific neuraminidases. (B) Loss of α 2-6 sialic acid (SNA binding), α 2-3 sialic acid (MAA II binding) and increase in galactose (RCA binding) were confirmed using plant lectin binding assays. All ELISAs were performed in triplicate, and results presented represent the mean values \pm SEM unless otherwise stated (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ respectively, ns = not significant).

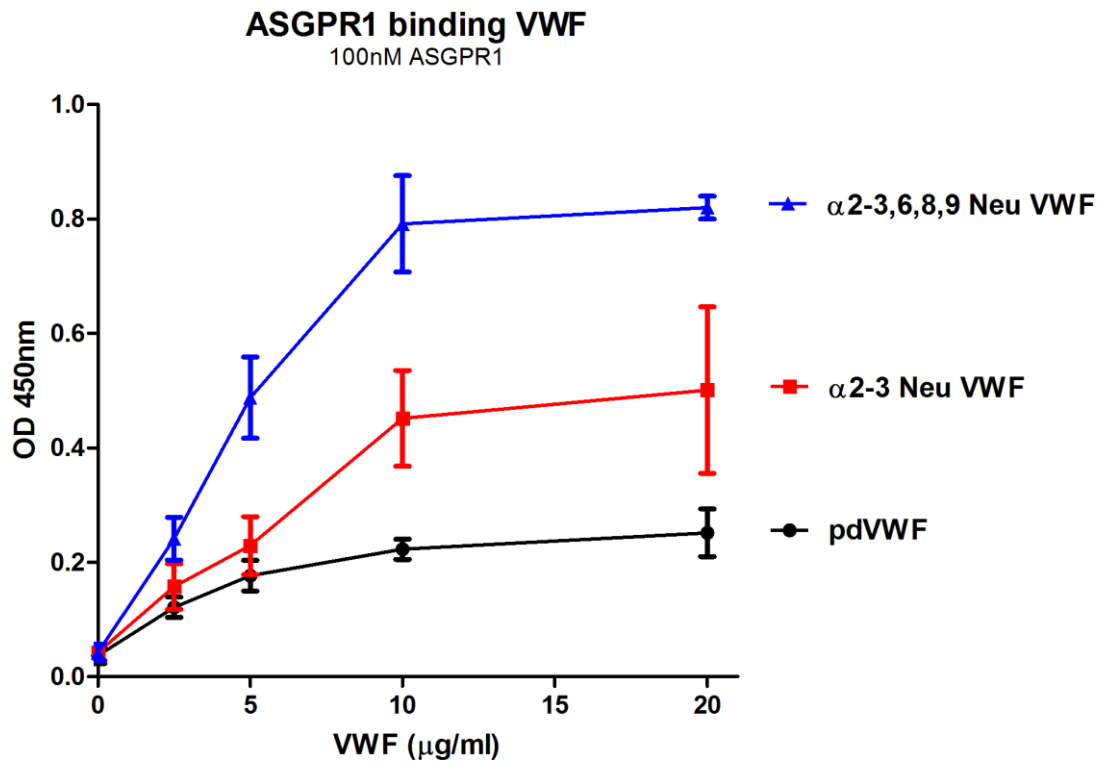


Figure 3.3: *In vitro* binding of VWF glycoforms to ASGPR1

Removal of α 2-3 sialylation significantly enhanced pdVWF *in vitro* binding to ASGPR1. Combined removal of α 2-3 and α 2-6 sialic acid further enhanced the binding of VWF to ASGPR1 *in vitro*.

3.2. *Asgr1*^{-/-} mice have increased endogenous VWF levels

To investigate whether ASGPR is involved in physiological clearance of VWF, we examined murine plasma VWF levels in *Asgr1*^{-/-} mice. In keeping with previous findings (Grewal *et al*, 2008), we observed that plasma VWF levels were significantly elevated in *Asgr1*^{-/-} mice compared to wild type controls ($p < 0.01$) (Figure 3.4).

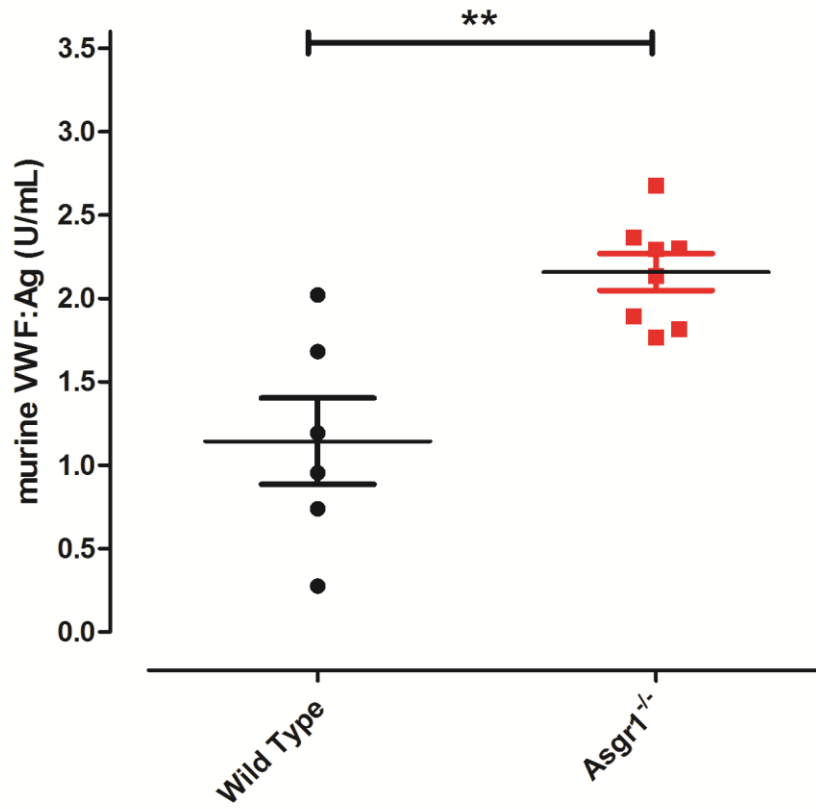


Figure 3.4: Murine plasma VWF levels were significantly increased in *Asgr1*^{-/-} mice compared to wild type controls

(**; p<0.01)(wild type n=6, *Asgr1*^{-/-} n=8).

3.3. Enhanced clearance of hyposialylated VWF variants is inhibited by asialosomucoid (ASOR).

To further investigate the role of ASGPR in regulating the clearance of hyposialylated VWF we assessed the clearance of hyposialylated human pd-VWF glycoforms (Figure 3.5) in *VWF*^{-/-} mice. In keeping with previous studies, we observed that combined removal of N- and O-linked sialic acid by digestion with α 2-3,6,8,9 neuraminidase resulted in markedly enhanced clearance (α 2-3,6,8,9 Neu-VWF $t_{1/2} = 3.7 \pm 0.7$ mins) (Figure 3.5 A).

Interestingly, specific removal of only α 2-3-linked sialic acid was also sufficient to markedly enhance VWF clearance (α 2-3 Neu-VWF $t_{1/2} = 8.2 \pm 1.4$ mins) (Figure 3.5 A).

The enhanced clearance of α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF were both significantly attenuated in the presence of a hyposialylated inhibitor glycoprotein (ASOR) (α 2-3 Neu-VWF $t_{1/2} = 8.2 \pm 1.4$ mins versus 12.4 ± 2.4 mins; $P < 0.05$ and α 2-3,6,8,9 Neu-VWF $t_{1/2} = 3.7 \pm 0.7$ mins versus 14.4 ± 2.7 mins; $p < 0.005$; respectively). (Figure 3.5 B). These results demonstrate that loss of sialylation is a key regulator of VWF clearance. Moreover, these novel data demonstrate that despite only accounting for 20% of total VWF sialylation, α 2-3 linked sialic acid on VWF O linked glycans plays a critical role in protecting VWF from enhanced lectin mediated clearance.

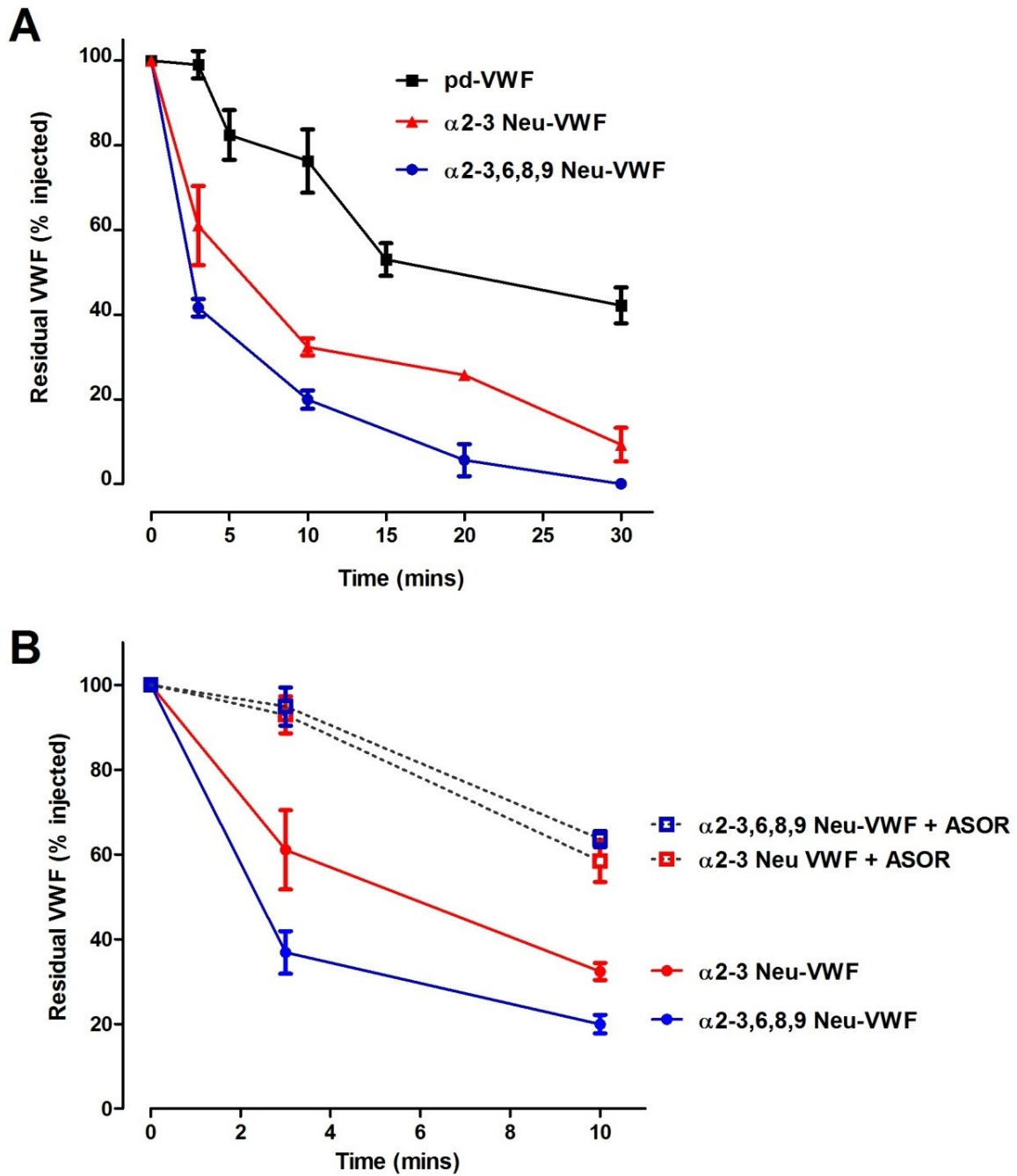


Figure 3.5: Clearance of hyposialylated VWF glycoforms in $VWF^{-/-}$ mice

(A) *In vivo* clearance of $\alpha 2-3$ Neu VWF and $\alpha 2-3,6,8,9$ Neu VWF in $VWF^{-/-}$ mice. (B) In the presence of ASOR, the enhanced *in vivo* clearance of both $\alpha 2-3$ Neu-VWF and $\alpha 2-3,6,8,9$ Neu-VWF was significantly attenuated. Adapted from Ward *et al*, Blood 2018.

3.4. Generation of *VWF*^{-/-}/*Asgr1*^{-/-} double knockout mice

The widely used inhibitor ASOR is not ASGPR specific and has the potential to inhibit other lectin receptors. In addition, ASOR has a short plasma half-life (<5mins) which limits interpretation of experimental data (Wall *et al*, 1980). To address these questions, we first generated a *VWF*^{-/-}/*Asgr1*^{-/-} double knockout mouse model using a standard breeding protocol. Subsequent *VWF*^{-/-}/*Asgr1*^{-/-} mice are viable and do not exhibit a pathological phenotype.

Previous studies have reported that ASGPR regulates the clearance of VWF in mice (Grewal *et al*, 2008). As such, we sought to investigate whether ASGPR regulates the clearance of human pd-VWF. *In vivo* clearance of human pd-VWF was studied in *VWF*^{-/-}/*Asgr1*^{-/-} mice compared to *VWF*^{-/-} mice. Interestingly, despite the reported role for ASGPR in regulating murine VWF clearance, the clearance of human pd-VWF was unchanged in *VWF*^{-/-}/*Asgr1*^{-/-} mice. pdVWF was cleared in a biphasic pattern in both *VWF*^{-/-} and *VWF*^{-/-}/*Asgr1*^{-/-} mice with mean residence times (MRT) of 67 ± 20.8 and 73.1 ± 8.2 minutes respectively (Figure 3.6).

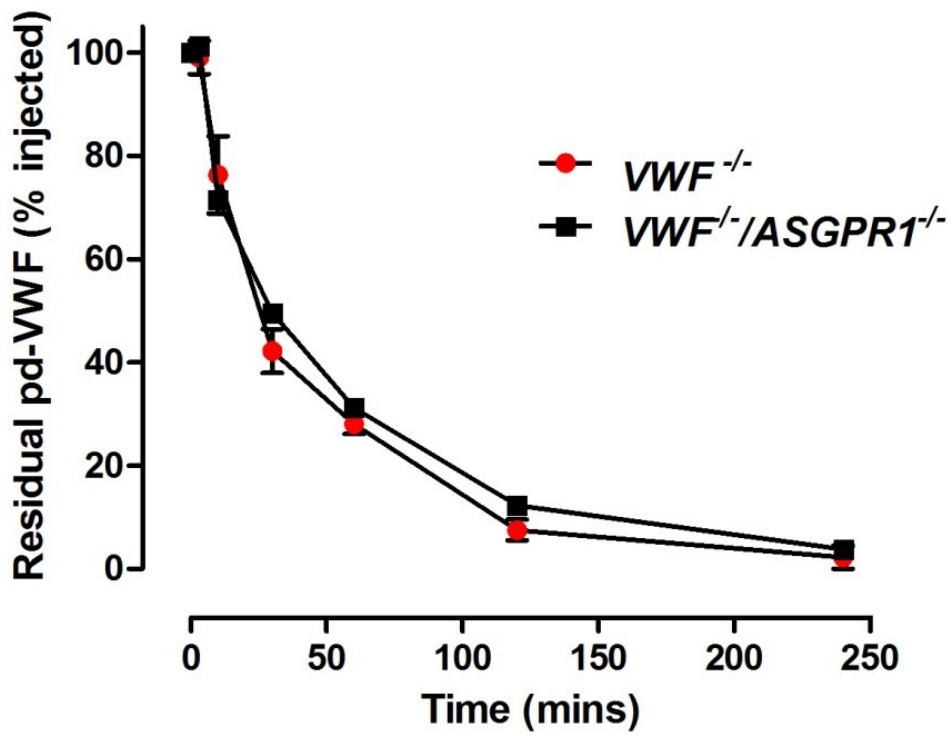


Figure 3.6: Human pd-VWF clearance in *VWF*^{-/-} compared to *VWF*^{-/-}/*Asgr1*^{-/-} mice.

3.5. Clearance of hyposialylated VWF remains enhanced in the absence of ASGPR.

To study the importance of ASGPR in regulating the clearance of hyposialylated VWF, clearance of VWF glycoforms was examined in *VWF^{-/-}/Asgr1^{-/-}* dual knockout mice. Importantly, markedly enhanced clearance of both α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF persisted in *VWF^{-/-}/Asgr1^{-/-}* mice ($t_{1/2}$ = 8.2 ± 0.6 and 3.2 ± 0.4 versus 50.6 ± 2 mins for pd-VWF; $P < 0.05$) (Figure 3.7). Interestingly, the rapid clearance of hyposialylated VWF was not significantly different in the presence or absence of the ASGPR (Figures 3.8 A). Indeed, the half-life of α 2-3 Neu-VWF in *VWF^{-/-}* single knockout mice was 8.2 ± 1.4 mins compared to 8.2 ± 0.6 mins in *VWF^{-/-}/Asgr1^{-/-}* double knockout mice. Similarly, α 2-3,6,8,9 Neu-VWF exhibited a half-life of 3.7 ± 0.7 mins in *VWF^{-/-}* single knockout mice compared to 3.2 ± 0.4 mins in *VWF^{-/-}/Asgr1^{-/-}* double knockout mice (Figure 3.8 B). These novel data suggest that alternate ASGPR-independent pathways contribute to the enhanced clearance of hyposialylated VWF.

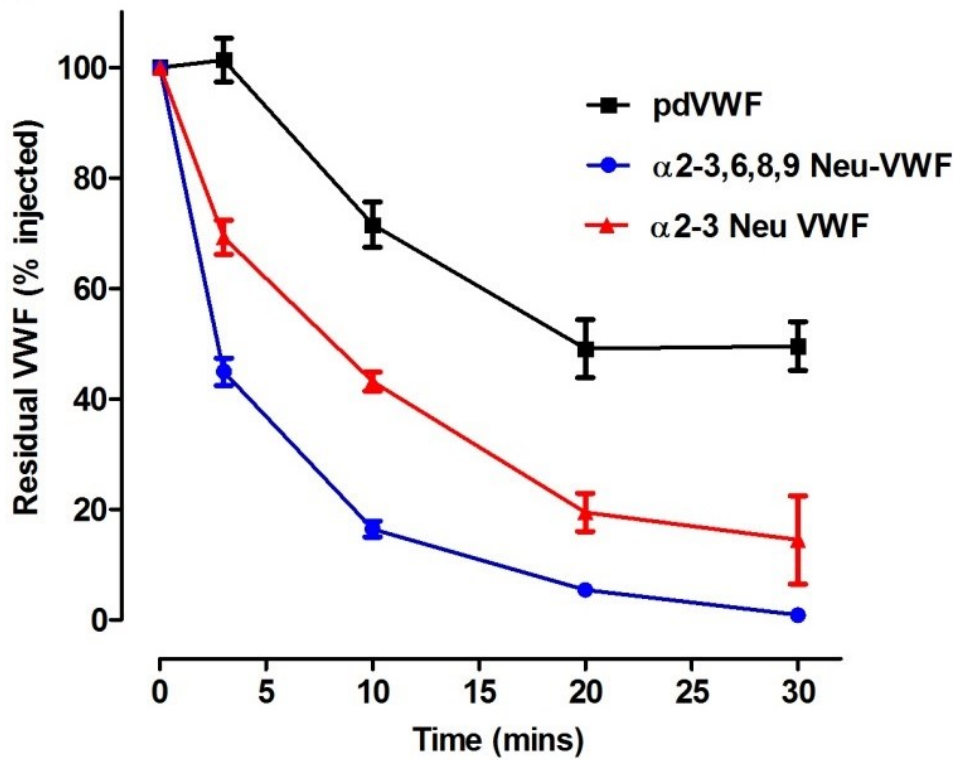


Figure 3.7: Clearance of hyposialylated VWF glycoforms in $VWF^{-/-}/Asgr1^{-/-}$ mice

The markedly enhanced clearance of both α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF was still evident in the absence of the ASGPR ($t_{1/2} = 8.2 \pm 0.6$ and 3.2 ± 0.4 compared to 50.6 ± 2 mins for pd-VWF; $P < 0.05$).

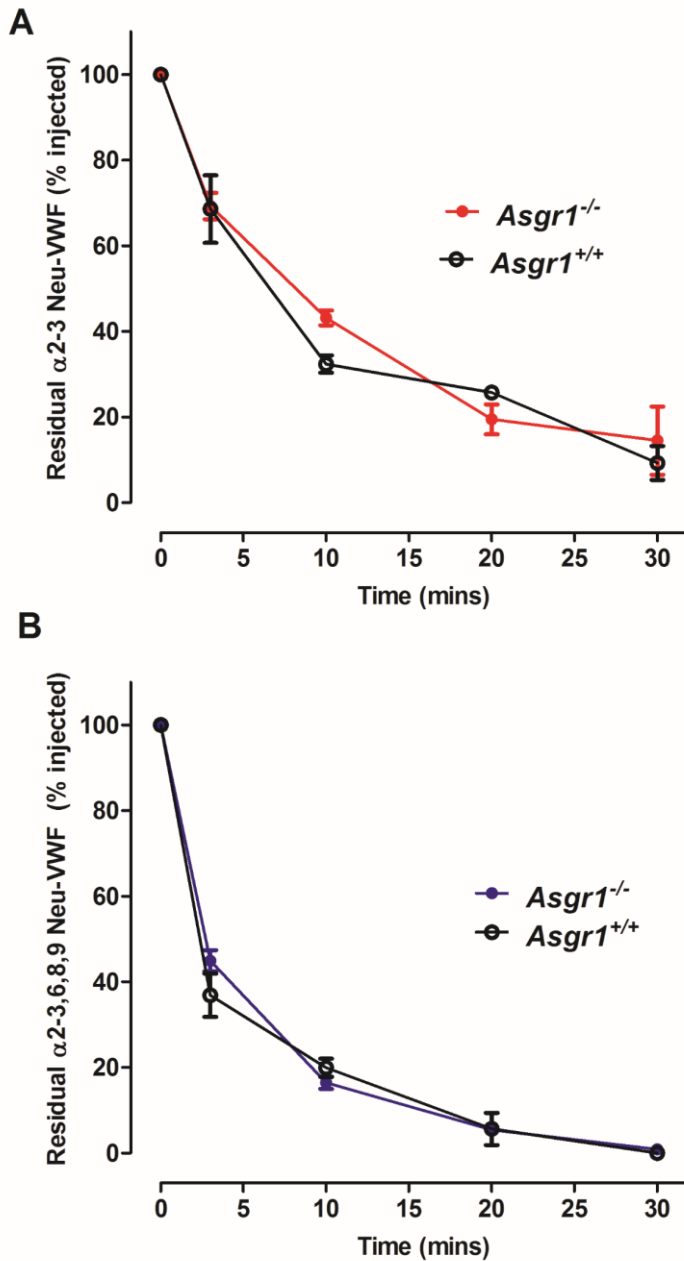


Figure 3.8: Absence of ASGPR has no effect on the rapid clearance of hyposialylated VWF glycoforms.

The reduced half-life observed for $\alpha 2-3$ Neu-VWF (A) and $\alpha 2-3,6,8,9$ Neu-VWF (B) were not significantly different in the presence or absence of the ASGPR ($\alpha 2-3$ Neu-VWF $t_{1/2} = 8.2 \pm 1.4$ mins versus 8.2 ± 0.6 mins; $P=0.96$ and $\alpha 2-3,6,8,9$ Neu-VWF $t_{1/2} = 3.7 \pm 0.7$ mins versus 3.2 ± 0.4 mins; $P=0.42$; respectively). Adapted from Ward *et al*, Blood 2018.

3.6. Investigation into ASGPR-independent clearance of hyposialylated VWF

The rapid clearance of hyposialylated VWF in the absence of ASGPR clearly suggests that additional lectin and or scavenger receptors may play important roles in modulating clearance of hyposialylated VWF. To further examine the putative role of other galactose sensitive receptors in regulating the clearance of hyposialylated-VWF, clearance experiments were repeated in our novel *VWF^{-/-}/Asgr1^{-/-}* dual knockout mice in the presence or absence of ASOR. Importantly, even in the absence of ASGPR, pre-infusion of ASOR significantly attenuated clearance of both α 2-3Neu-VWF (Figure 3.9 A) and α 2-3,6,8,9 Neu-VWF (Figure 3.9 B) in *VWF^{-/-}/Asgr1^{-/-}* mice. This finding is consistent with a role of other Gal/GalNAc sensitive clearance receptors.

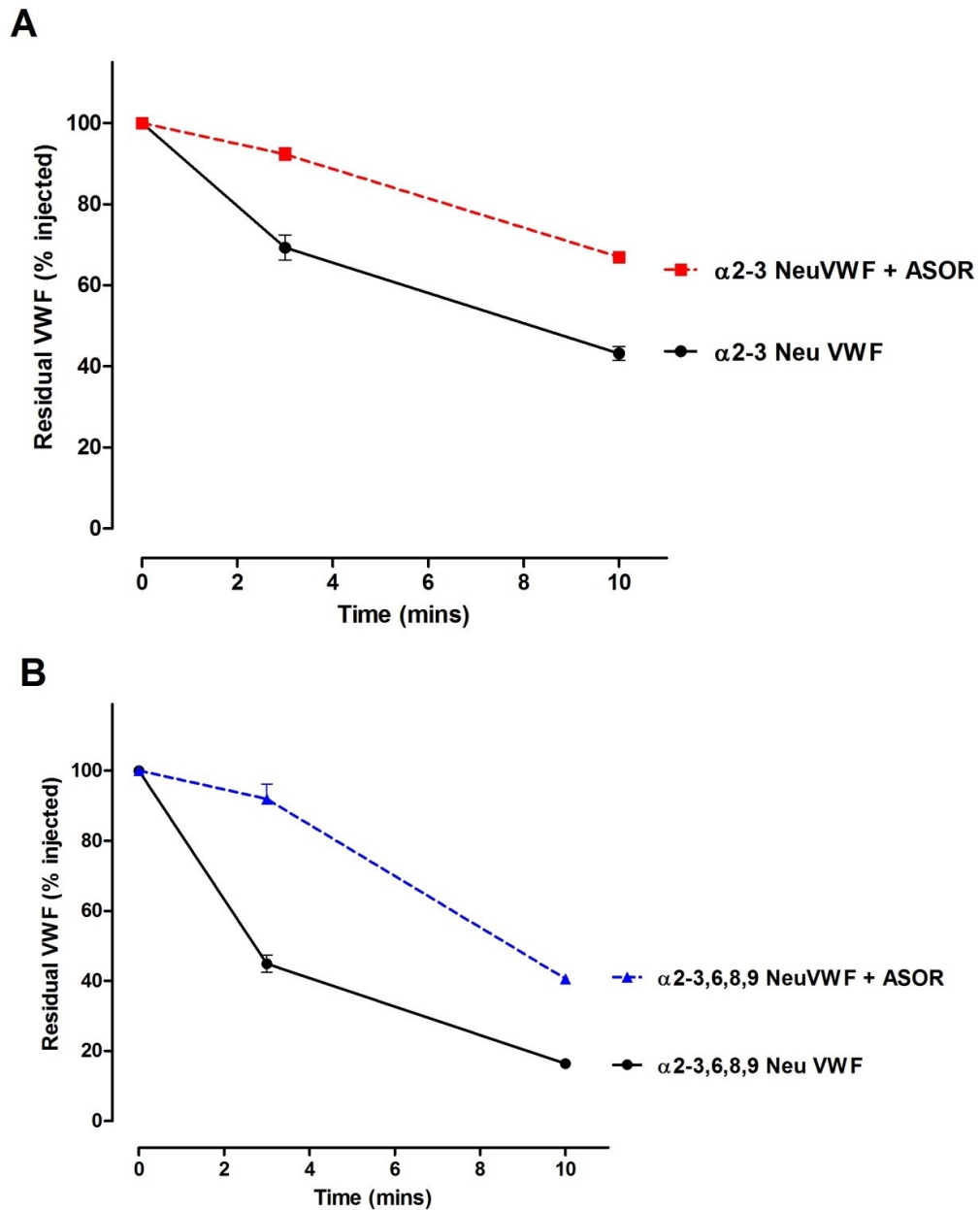


Figure 3.9: ASOR attenuates the enhanced clearance of hyposialylated VWF in *VWF^{-/-}/Asgr1^{-/-}* mice

The enhanced clearance of $\alpha 2-3$ Neu-VWF (A) and $\alpha 2-3,6,8,9$ Neu-VWF (B) was still inhibited by ASOR (dashed lines) even in the absence of ASGPR ($\alpha 2-3$ Neu-VWF $t_{1/2} = 8.2 \pm 1.4$ mins versus 16.8 ± 1.6 mins; $P < 0.005$ and $\alpha 2-3,6,8,9$ Neu-VWF $t_{1/2} = 3.7 \pm 0.7$ mins versus 7.7 ± 1.3 mins; $P < 0.05$; respectively). Adapted from Ward *et al*, Blood 2018.

3.7. Discussion

ASGPR was the first endogenous receptor implicated in regulating VWF clearance. In particular, ASGPR was reported to be involved in clearing hyposialylated VWF under pathological conditions such as *Streptococcus pneumoniae* sepsis. Furthermore, plasma VWF levels were also significantly increased in *Asgr1*^{-/-} mice, suggesting that ASGPR is also important in the constituent physiological clearance of VWF. In this context, it is interesting that recent studies have demonstrated that ASGPR not only binds hyposialylated glycoproteins but can bind glycoproteins expressing terminal sialic acid (Park *et al*, 2005; Steirer *et al*, 2009). However, ASGPR binds with highest affinity to glycoproteins with exposed terminal Gal and GalNAc moieties (Khorev *et al*, 2008; Zacco *et al*, 2015). Our *in vitro* binding experiments support these previous findings. Although, pd-VWF binds to ASGPR *in vitro*, this binding is markedly increased following total removal of sialic acid (Figure 3.3). Furthermore, our findings highlight that loss of terminal sialylation from either N- or O-linked glycans of VWF can trigger enhanced ASGPR-mediated clearance. Interestingly, removal of O linked sialylation (which constitutes only 20% of total sialic acid on VWF) was sufficient to increase ASGPR binding 2-fold, and markedly enhance *in vivo* clearance.

Collectively, our data support the previous conclusion of Grewal *et al* that ASGPR is involved in physiological and pathological VWF clearance. Nevertheless, it is important to consider that other lectin receptors can also bind to hyposialylated glycoproteins. Moreover, although pre-infusion of ASOR significantly attenuated the enhanced circulatory clearance of hyposialylated VWF variants in *VWF*^{-/-} mice, ASOR has limitations in that it is not an ASGPR-specific inhibitor. Rather, ASOR can inhibit a number

of Gal/GalNAc sensitive receptors. To further investigate the hypothesis that additional receptors may contribute to the enhanced clearance of hyposialylated VWF, a dual *VWF^{-/-}/Asgr1^{-/-}* mouse model was developed. Critically, the clearance of α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF remained markedly elevated in these mice, even in the absence of the ASGPR. Indeed, the rate of clearance of hyposialylated VWF glycoforms was not significantly different in the presence or absence of the ASGPR.

Pre-infusion of ASOR in *VWF^{-/-}/Asgr1^{-/-}* dual knockout mice still resulted in attenuated clearance of α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF even in the absence of ASGPR. Taken together, these results support the hypothesis that other galactose-sensitive lectin receptors also contribute to the regulation of VWF clearance *in vivo*.

4. Identification of the macrophage galactose lectin as a novel VWF clearance receptor

4.1. Introduction

In addition to the ASGPR, a number of other lectin receptors have been shown to bind with enhanced affinity to hyposialylated glycoproteins (Sørensen *et al*, 2012) (Figure 4.1). These lectin receptors recognise terminal Gal and/or GalNAc moieties, and thus can all be inhibited to varying degrees by ASOR. Based on the data in Chapter 3, we hypothesised that some of these receptors may be involved in regulating VWF clearance. In initial studies, we examined whether the Macrophage Galactose Lectin (MGL) might be important.

The MGL receptor was first identified as a macrophage specific C-type lectin, distinct from ASGPR (Kawasaki *et al*, 1986; Sato *et al*, 1992). MGL is a type II membrane protein which is expressed as a homo-trimer on macrophages and immature dendritic cells, but not on circulating monocytes (Figure 4.2) (Valladeau *et al*, 2001; Jégouzo *et al*, 2013). MGL and ASGPR have sequence homology for a shared GalNAc/GAL binding sequence in the carbohydrate recognition domain (CRD) of both receptors. In addition, MGL and ASGPR share similar internalisation signals in their cytoplasmic domains.

The CRD of MGL binds with high affinity to glycoproteins expressing terminal Gal and GalNAc residues. Glycan profiling has demonstrated human MGL has particular affinity for the T and Tn antigen structures (Yamamoto *et al*, 1994; Kawakami *et al*, 1994; Iida *et al*, 1999; van Vliet *et al*, 2005, 2008). Critically, mass spectrometry studies have shown that the T antigen accounts for 70% of the total O-glycan population on VWF (Canis *et*

al, 2012, 2010). As demonstrated in Section 3, removal of terminal O-linked sialylation from VWF has been associated with a markedly reduced plasma half-life *in vivo*. Similarly, genetic inactivation of ST3GalIV sialyltransferase (which catalyses the addition of α 2-3 linked sialic acid to glycoproteins) in a transgenic mouse model causes enhanced VWF clearance (Ellies *et al*, 2002). Furthermore, van Schooten *et al* reported an inverse correlation between aberrant sialylation of T antigen and plasma VWF:Ag levels, suggesting that O-linked sialylation on VWF may be of particular importance (van Schooten *et al*, 2007). On the basis of these data we hypothesised that MGL may play a role in mediating the clearance of VWF.

Asialo orsomucoid (ASOR)

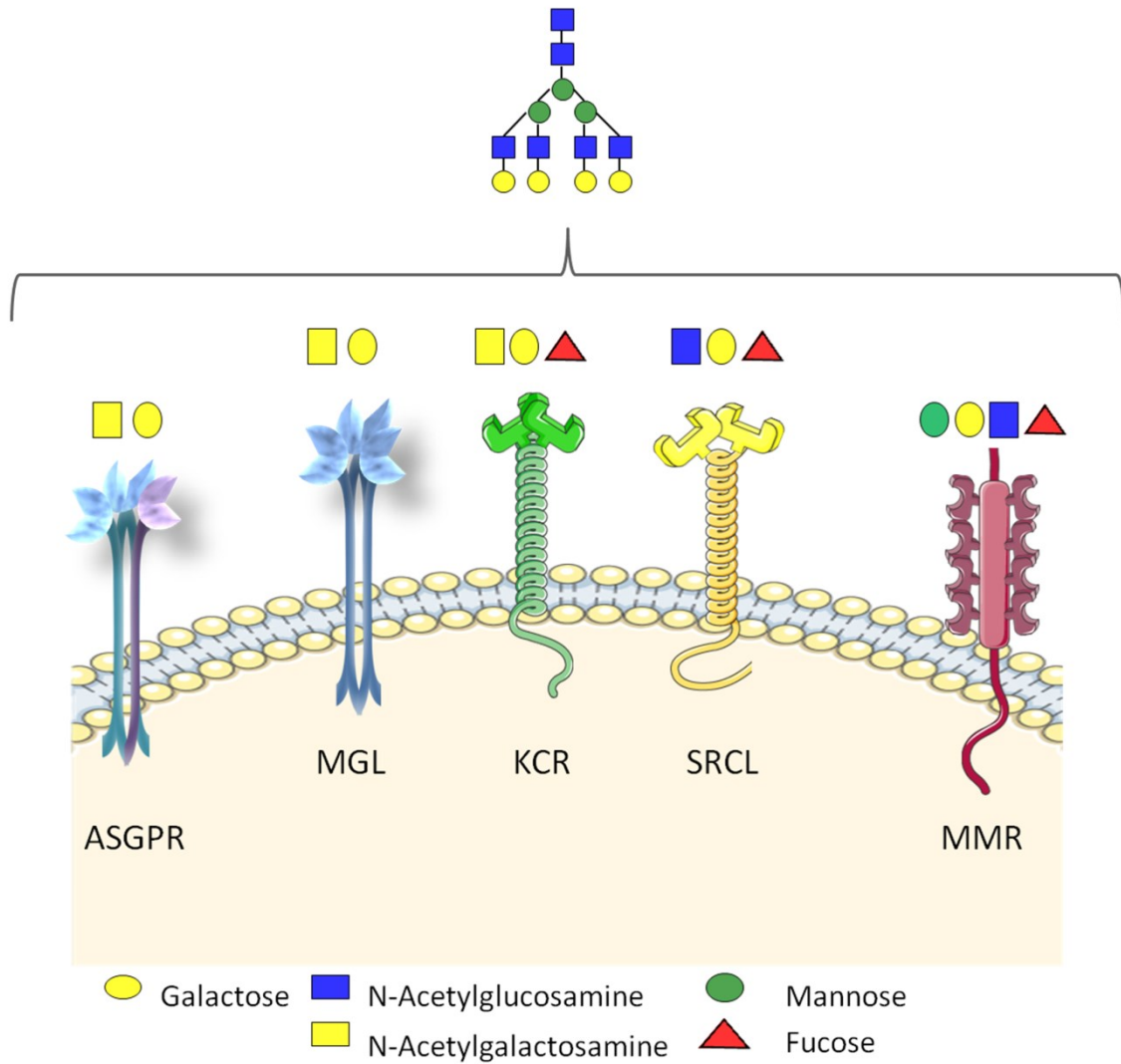


Figure 4.1: ASOR can inhibit clearance via multiple lectin receptors

ASOR has affinity for multiple galactose sensitive receptors; asialoglycoprotein receptor (ASGPR), macrophage galactose lectin (MGL), Kupffer cell receptor (KCR), scavenger receptor C-type lectin (SRCL), macrophage mannose receptor/CD206 (MMR).

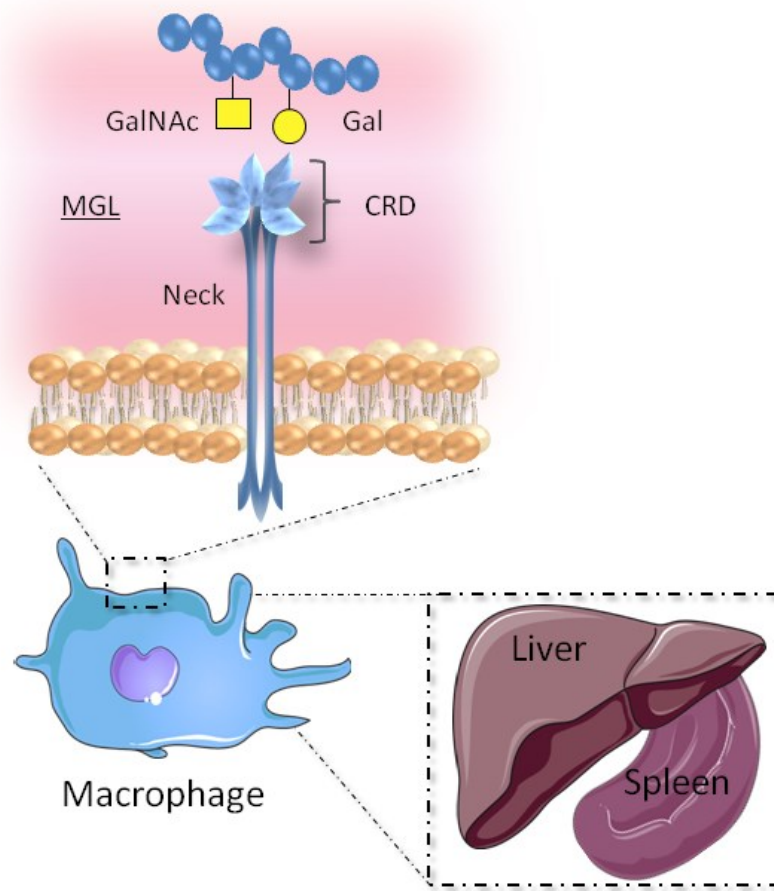


Figure 4.2: Macrophage Galactose Lectin

MGL is expressed on resident macrophages of the liver and spleen. The CRD has affinity for Gal/GalNAc terminating glycans.

4.2. Desialylated VWF binds to hepatic macrophages *in vivo*

Clearance studies in $VWF^{-/-}/Asgr1^{-/-}$ mice (Figure 3.9) demonstrated that ASGPR independent clearance pathways are important in the clearance of hyposialylated VWF *in vivo*. To investigate the biological mechanisms involved, the cellular basis of the enhanced clearance of hyposialylated VWF was examined by immunohistochemistry.

In brief, $VWF^{-/-}$ and $VWF^{-/-}/Asgr1^{-/-}$ mice were injected with either PBS control or α 2-3,6,8,9 Neu-VWF. Murine livers were then harvested ten minutes post infusion, fixed and stained (in collaboration with the Department of Histopathology, St James' Hospital Dublin). As expected, no anti-VWF staining was observed in PBS treated mice (Figure 4.3). In contrast, liver sections from both $VWF^{-/-}$ mice, and $VWF^{-/-}/Asgr1^{-/-}$ mice treated with α 2-3,6,8,9 Neu-VWF demonstrated significant VWF staining along liver sinusoids, characteristic of Kupffer cell staining (Figure 4.3 A and B). Collectively, these data demonstrate that in the presence or absence of ASGPR, hyposialylated VWF co-localises with hepatic macrophages *in vivo*. These findings are interesting given that the ASGPR is not expressed on macrophages (Li *et al*, 2017).

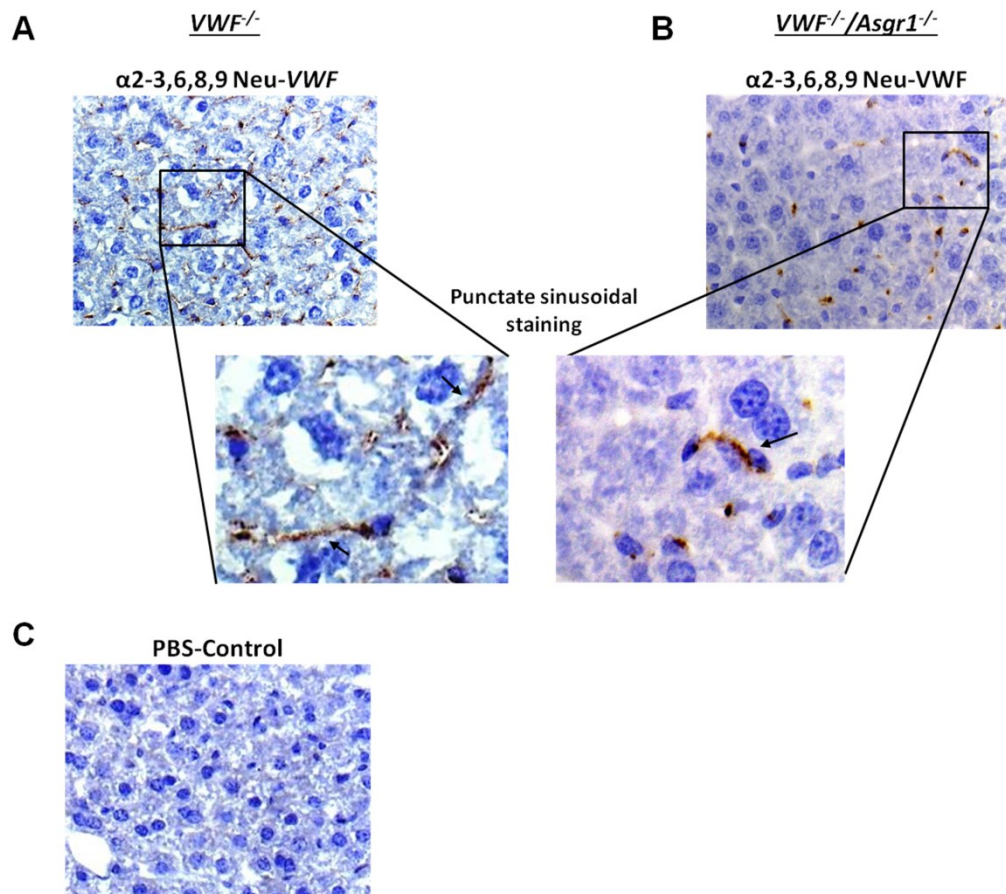


Figure 4.3: $\alpha 2-3,6,8,9$ Neu-VWF is cleared by macrophages *in vivo*.

$VWF^{-/-}$ and $VWF^{-/-}/Asgr1^{-/-}$ mice were injected with either PBS control or $\alpha 2-3,6,8,9$ Neu-VWF. Ten minutes following infusion tissues were perfused and livers collected into 10% buffered formalin. VWF was detected with anti-VWF HRP and tissues were counterstained with haematoxylin (magnification 400x).

4.3. Desialylated VWF binds differentiated THP1 macrophages.

To further investigate a putative role for macrophages in modulating the enhanced clearance of hyposialylated VWF, *in vitro* binding studies were performed to assess binding of pd-VWF and α 2-3,6,8,9 Neu-VWF to differentiated THP1 macrophage cells. Consistent with our *in vivo* data, binding of both α 2-3,6,8,9 Neu-VWF and pd-VWF was observed. Furthermore, binding of α 2-3,6,8,9 Neu-VWF was significantly increased compared to pd-VWF (* P<0.05, ** P<0.01 respectively) (Figure 4.4).

Together with the *in vivo* immunohistochemistry data, these findings suggest that in addition to the hepatic ASGPR, macrophages may also play a role in regulating the clearance of hyposialylated VWF.

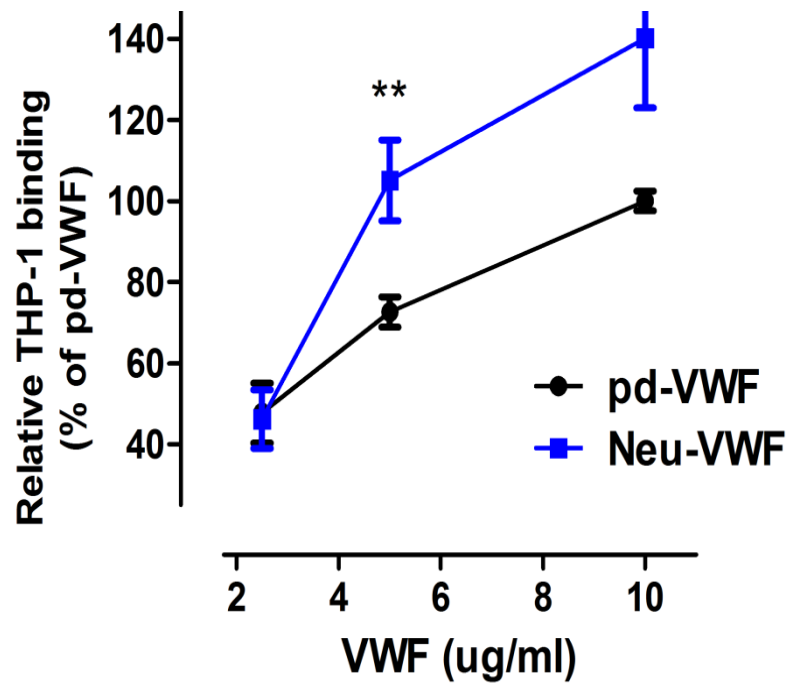


Figure 4.4: α 2-3,6,8,9 Neu-VWF binds THP1 macrophages *in vitro*.

α 2-3,6,8,9 Neu-VWF binds to THP1 macrophages *in vitro* with greater affinity than pd-VWF (*; $p < 0.05$, **; $p < 0.01$ respectively). Experiments were performed in triplicate, and results presented represent the mean values \pm SEM. Adapted from Ward *et al*, Blood 2018. Experiment performed by Mr Clive Drakeford.

4.4. Macrophage depletion corrects the fast clearance of desialylated VWF *in vivo*.

To assess the role of macrophages in regulating the enhanced clearance of hyposialylated VWF, *in vivo* clearance experiments were repeated in *VWF^{-/-}/Asgr1^{-/-}* mice before and after clodronate-induced macrophage depletion. Previous studies have shown that clodronate depleted macrophages in liver and spleen resulting in a 70-90% reduction in F4/80 positive cells (Van Rooijen *et al*, 1989; Rawley *et al*, 2015; O'Sullivan *et al*, 2016a).

Interestingly, the enhanced clearance of α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF in *VWF^{-/-}/Asgr1^{-/-}* mice were both significantly attenuated following macrophage depletion (Figure 4.5 A) (α 2-3 Neu-VWF $t_{1/2} = 8.2 \pm 1.4$ mins versus 24.0 ± 1.1 mins; $P < 0.05$) (α 2-3,6,8,9 Neu-VWF $t_{1/2} = 3.7 \pm 0.7$ mins versus 9.6 ± 4.1 mins; $P < 0.05$) (Figure 4.5 B). Together, these data demonstrate that additional asialo-receptors, at least in part expressed on macrophages, are involved in regulating the enhanced clearance of hyposialylated VWF *in vivo*.

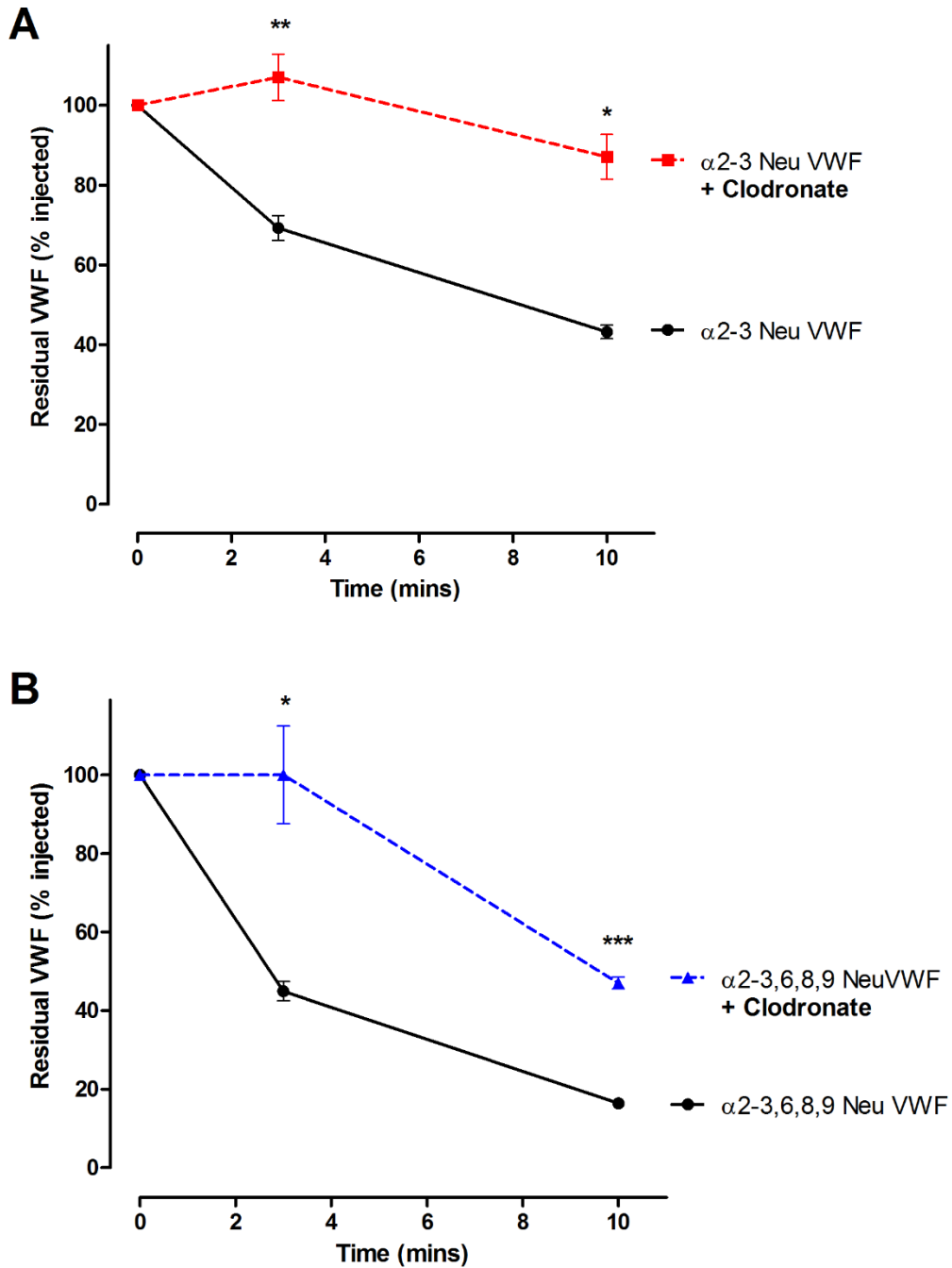


Figure 4.5: Macrophage depletion attenuates hyposialylated VWF clearance in $VWF^{-/-}/Asgr1^{-/-}$ mice

$\alpha 2-3$ Neu-VWF (A) and $\alpha 2-3,6,8,9$ Neu-VWF (B) clearance (dashed lines) were significantly attenuated following macrophage depletion. Three to five mice were used per timepoint and data are represented as mean \pm SEM (*; $p < 0.05$, **; $p < 0.01$, *** $p < 0.001$ respectively). Adapted from Ward *et al*, Blood 2018.

4.5. Macrophage Galactose lectin binds immobilised pd-VWF in a dose dependant manner in surface plasmon resonance studies

A number of potential macrophage surface receptors may play a role in promoting clearance of hyposialylated VWF. As discussed in the introduction to this chapter, a potential candidate receptor is MGL.

Surface plasmon resonance (SPR) was used to evaluate MGL binding to pd-VWF. Pd-VWF was immobilised on a CM5 sensor chip, over which varying concentrations of recombinant human MGL was subsequently perfused.

Dose-dependent binding of recombinant human MGL to human pd-VWF was confirmed (Figure 4.6). An approximate dissociation constant was calculated to be $18.4\mu\text{g/mL} \pm 3.3\mu\text{g/mL}$ or $73.6\text{nM} \pm 13.2\text{nM}$.

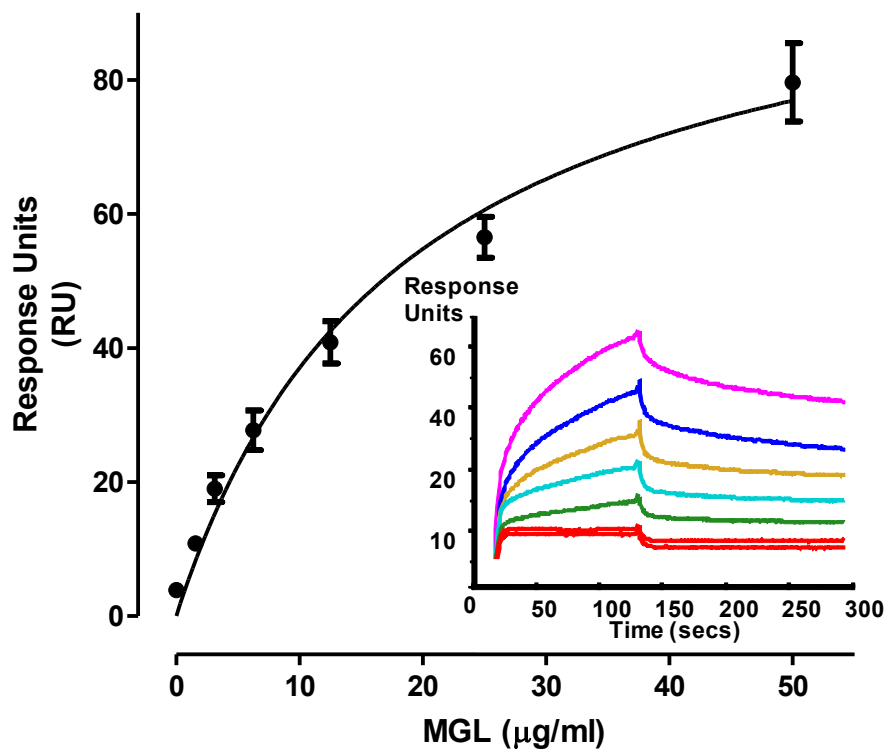


Figure 4.6: MGL binds pdVWF in SPR

Surface plasmon resonance (SPR) was used to evaluate the binding of immobilised purified pd-VWF to recombinant human MGL. Adapted from Ward *et al*, Blood 2018.

4.6. VWF Co-localizes with MGL on the surface of THP1 macrophages

In order to further study whether MGL may be implicated in regulating macrophage binding to VWF, proximity ligation assays (Duolink-PLA, Sigma Aldrich) were performed using differentiated THP1 macrophages in the presence of VWF/PBS. PLA utilises species-specific secondary antibodies conjugated with a short DNA probe, which when in close proximity (less than 40nm) then hybridise and undergo amplification to reveal discrete fluorescent red dots.

Following incubation with PBS, no fluorescent staining was observed on THP1 macrophages (Figure 4.7). Critically, PLA analysis demonstrated that on the surface of differentiated THP1 macrophages VWF co-localises with MGL as evidenced by the appearance of fluorescent red dots visualised by immunofluorescence microscopy (Figure 4.7). These data further demonstrate that VWF interacts with MGL, further supporting the hypothesis that MGL may play a role in regulating the clearance of hyposialylated VWF *in vivo*.

THP1- Macrophages

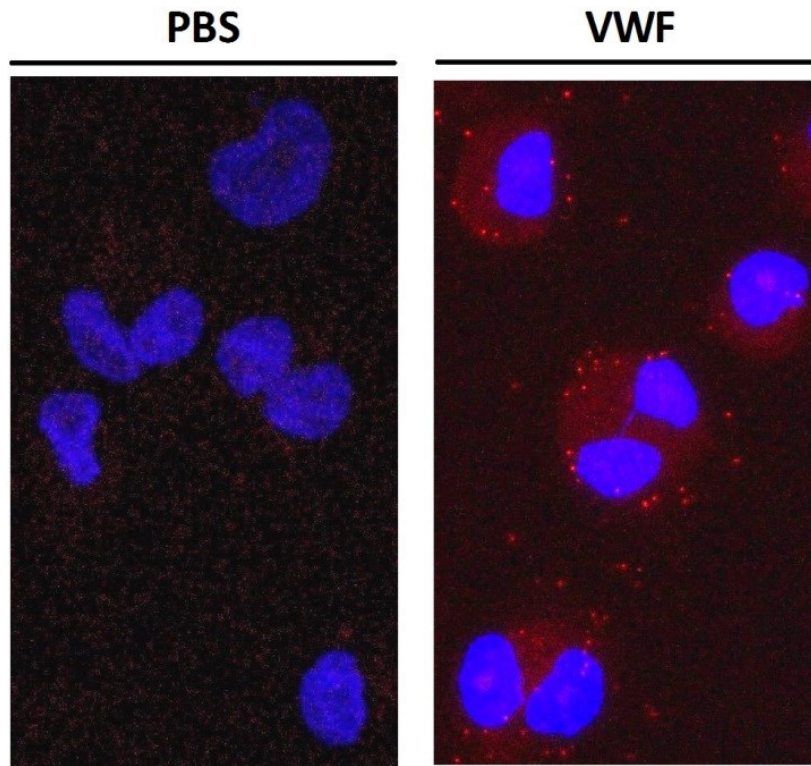


Figure 4.7: pdVWF co-localises with MGL on the surface of THP1 macrophages

THP1 macrophages incubated with VWF demonstrated VWF-MGL co-localisation detected by Duolink-proximity ligation assay, visualized as red spots via immunofluorescence microscopy. No signal was observed from cells incubated with PBS alone. Adapted from Ward *et al*, Blood 2018. Experiment performed by Mr Clive Drakeford.

4.7. MGL inhibition attenuates the clearance of hyposialylated VWF.

To investigate whether MGL may contribute to macrophage-mediated clearance of hyposialylated VWF *in vivo*, clearance experiments were repeated in *VWF^{-/-}/Asgr1^{-/-}* in the presence or absence of polyclonal goat anti-mouse-MGL1/2 antibodies.

Pre-infusion with polyclonal goat IgG control had no significant effect on α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF clearance (Figure 4.8 A & B). Interestingly however, the enhanced clearance of both α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF in *VWF^{-/-}/Asgr1^{-/-}* mice were significantly attenuated in the presence of the anti mMGL1/2 inhibitory antibody (α 2-3 Neu-VWF $t_{1/2} = 21.9 \pm 11.8$ mins versus 9.1 ± 1.5 mins; $P < 0.05$ and α 2-3,6,8,9 Neu-VWF $t_{1/2} = 24.4 \pm 8.1$ mins versus 5.7 ± 2.1 mins; $P < 0.05$; respectively) (Figure 4.8 C).

Cumulatively, these *in vitro* and *in vivo* data demonstrate a novel role for MGL in regulating macrophage-mediated clearance of hyposialylated VWF.

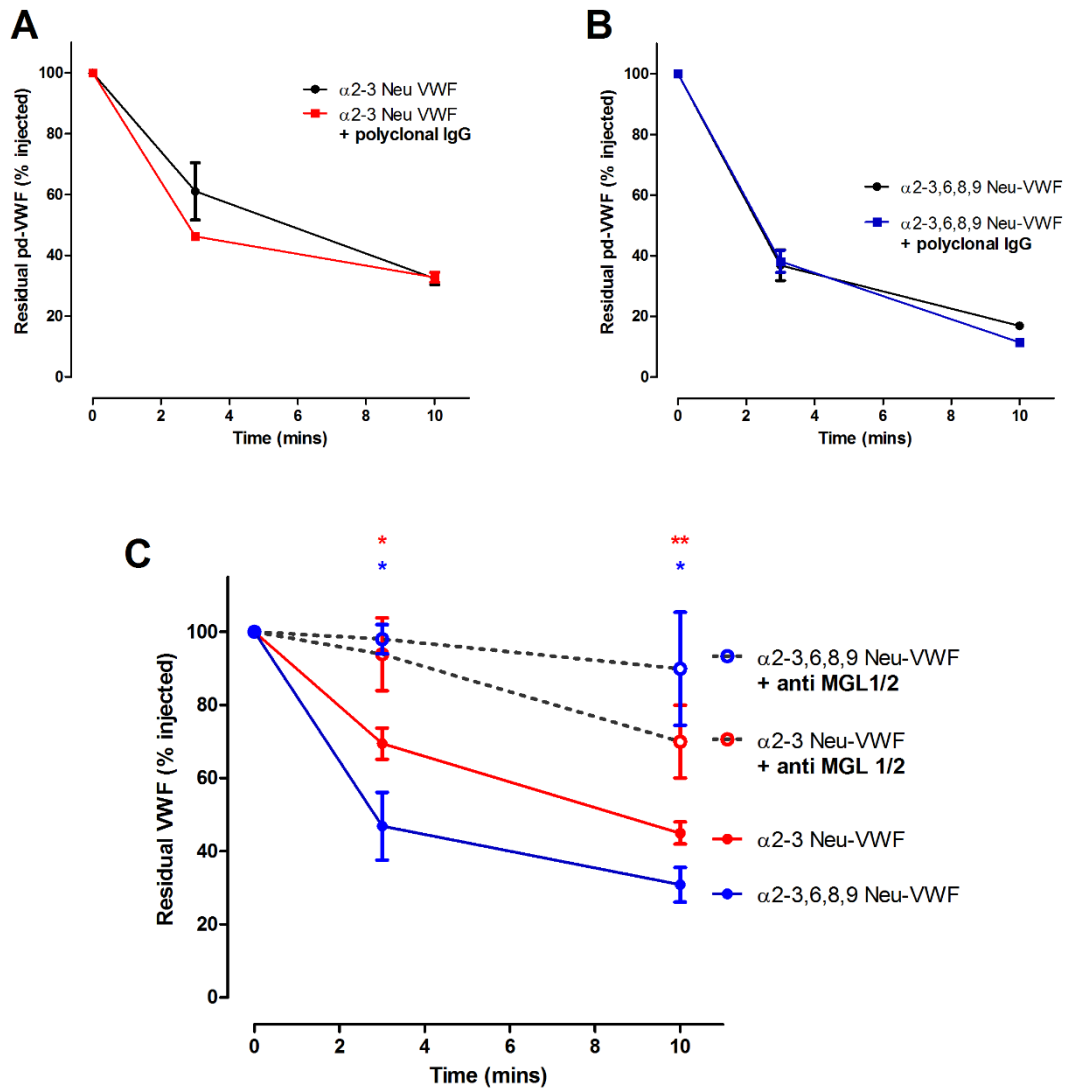


Figure 4.8: Inhibition of MGL attenuated clearance of hyposialylated VWF

Pre-infusion of IgG control has no effect on (A) $\alpha 2-3$ Neu-VWF and (B) $\alpha 2-3,6,8,9$ NeuVWF VWF half-life. (C) Enhanced clearance of both $\alpha 2-3$ Neu-VWF (red lines) and $\alpha 2-3,6,8,9$ Neu-VWF (blue lines) mice was significantly attenuated in the presence of an mMGL blocking antibody (*; $p < 0.05$, **: $p < 0.01$ respectively). Adapted from Ward *et al*, Blood 2018.

4.8. *MGL1*^{-/-} mice have increased endogenous plasma VWF:Ag levels

In mice, there are two homologs of human MGL, mMGL1 and mMGL2. Murine MGL1 shares significant sequence homology with human MGL and binds oligosaccharides with multiple terminal Gal residues including the T antigen. To determine whether MGL may be involved in regulating physiological clearance of pd-VWF plasma VWF:Ag levels were determined in mice deficient in *mMGL1* compared to wild type littermate controls.

Significantly elevated endogenous plasma VWF:Ag levels were present in *mMGL1*^{-/-} mice compared to wild type controls (Figure 4.9). Interestingly, this 1.5-fold increase ($152.6 \pm 15.7\%$ versus $100 \pm 16.9\%$; $P < 0.05$) in endogenous VWF is observed in mice sufficient in ASGPR, suggesting that MGL-mediated VWF clearance is important even in the presence of ASGPR.

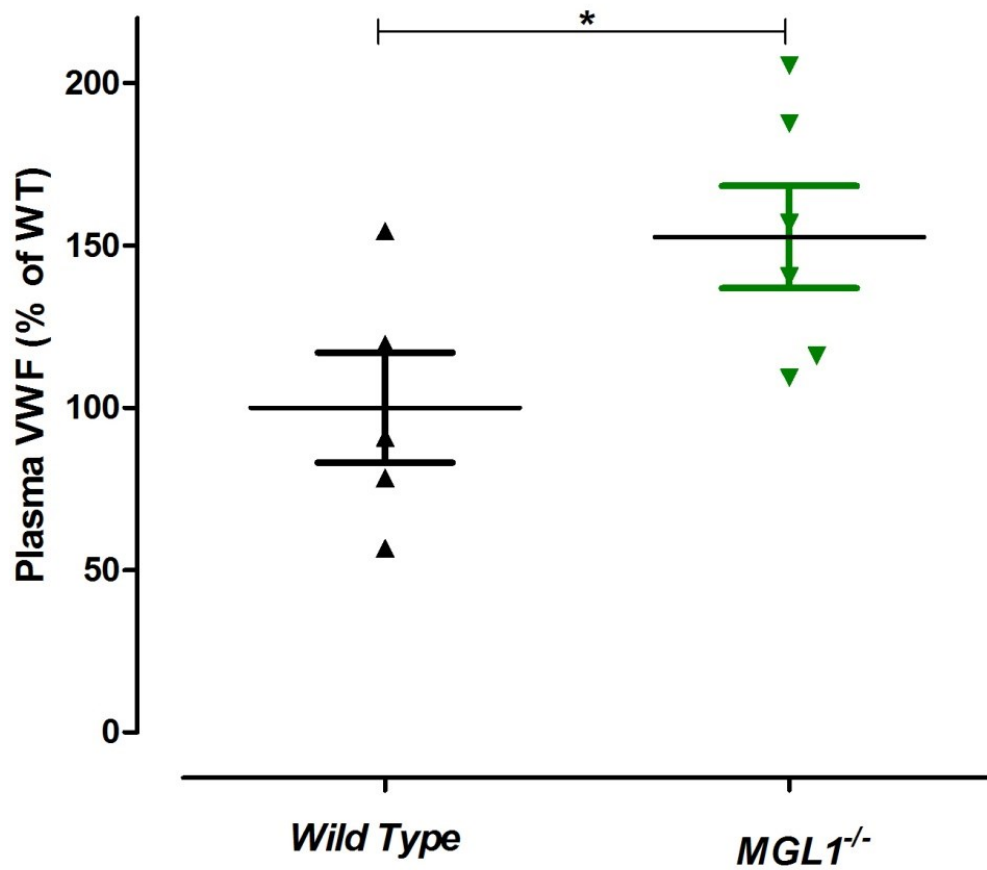


Figure 4.9: Plasma VWF:Ag levels are significantly increased in *mMLG1*^{-/-} mice.

Wild type mice n=5, *MGL1*^{-/-} n=6 (*; p<0.05). Adapted from Ward *et al*, Blood 2018.

4.9. Plasma VWF:Ag levels are increased in *mMGL1*^{-/-} mice due to reduced VWF clearance

Mature murine VWF is secreted from ECs in a 1:1 molar ratio with the VWFpp. However, VWFpp exhibits a significantly shorter half-life compared to full length VWF (2-3 hours versus 8-12 hours respectively). As such the ratio of VWFpp to antigen can be used as a surrogate marker to assess VWF clearance rates (Haberichter *et al*, 2006). An increased VWFpp:Ag ratio is indicative marker of enhanced circulatory clearance (Haberichter *et al*, 2006). Conversely, a significantly decreased VWFpp:Ag ratio indicates reduced VWF clearance or prolonged survival of VWF in circulation.

When compared to wild type littermate controls, mice deficient in *mMGL1* exhibited a 1.3-fold decrease in VWFpp:Ag ratio (2.26 ± 0.26 versus 3.1 ± 0.34 respectively, $p=0.09$) (Figure 4.10). While this decrease in VWFpp:Ag ratio did not achieve statistical significance with the current population size, the data are in keeping with the observed increase in endogenous mVWF levels in *mMGL1*^{-/-} mice and suggest a role for MGL in regulating physiological VWF circulatory clearance.

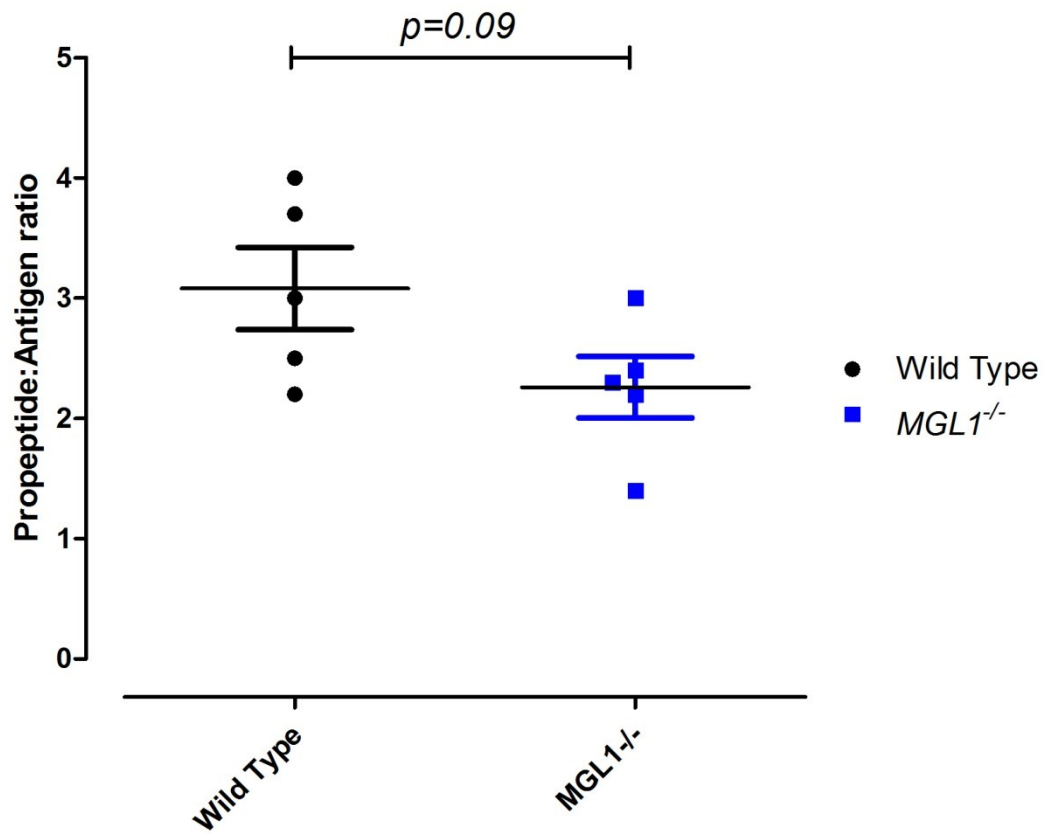


Figure 4.10: *MGL1*^{-/-} mice exhibit decreased VWFpp:Ag ratio

mVWF pp:Ag ratio is modestly reduced in *MGL1*^{-/-} mice compared to Wild Type controls (n=5).

4.10. Clearance of endogenous VWF is prolonged in *mMGL1*^{-/-} mice

To further investigate the biology underlying the elevated plasma VWF:Ag levels observed in *mMGL1*^{-/-} mice, endogenous mVWF clearance was assessed in *MGL1*^{-/-} mice compared to wild type controls. Briefly, endogenous VWF was biotinylated by infusion of NHS biotin. Subsequently, endogenous mVWF clearance rate was assessed by measuring residual biotin signal over-time in a modified VWF Elisa.

Interestingly, at all time-points, residual biotin signal on captured VWF was significantly greater in *mMGL1*^{-/-} mice compared to wild type controls ($p < 0.05$) (Figure 4.11). Collectively, these data are consistent with the VWFpp:Ag results and confirm that the elevated plasma VWF levels observed in *mMGL1*^{-/-} mice results from a reduction in VWF clearance, and further suggest that MGL may regulate the clearance of both normally sialylated, and hyposialylated VWF *in vivo*.

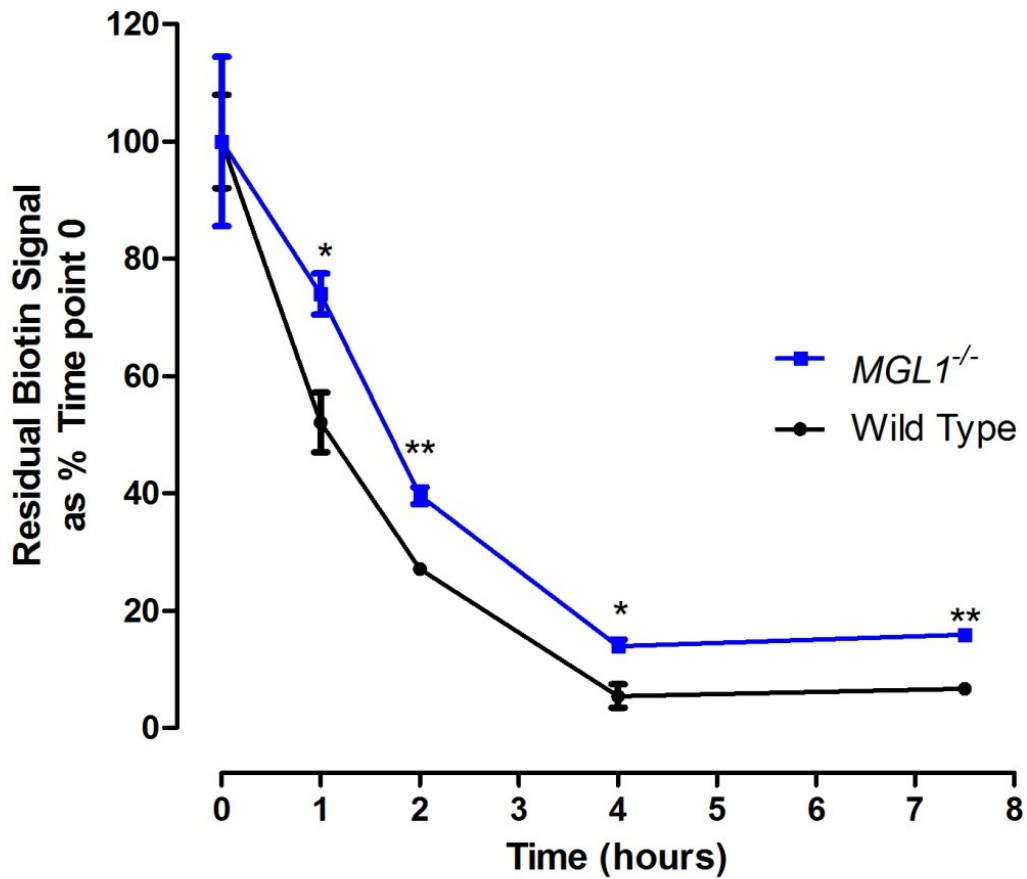


Figure 4.11: *MGL1*^{-/-} mice exhibit prolonged VWF clearance

The clearance of endogenous murine VWF in *MGL1*^{-/-} mice was significantly attenuated compared to WT controls at all time points measured (*; $p < 0.05$, **; $p < 0.01$ respectively). Adapted from Ward *et al*, Blood 2018.

4.11. Clearance of pdVWF is prolonged following inhibition of MGL *in vivo*

While human pdVWF is highly sialylated, a proportion of the glycan antennae on circulating pdVWF express exposed Gal moieties, evidenced by the binding of RCA to pdVWF (Ellies *et al*, 2002; Millar *et al*, 2008; Aguila *et al*, 2019). Moreover, *in vitro* binding studies here demonstrate the pdVWF can bind MGL in an immobilised system and in cell-based assays. Furthermore, mice deficient in *mMGL1* exhibit prolonged VWF half-life *in vivo*. As such, we sought to further the role of MGL in regulating physiological VWF clearance *in vivo*.

Specific inhibition of murine MGL was again performed utilising anti-MGL1/2 blocking antibodies. Pre-infusion of isotype control IgG had no effect on the clearance of pdVWF in *VWF^{-/-}/Asgr1^{-/-}* mice (pdVWF $t_{1/2}$ = 31.4 ± 2.2 mins versus 31.1 ± 2.3 mins respectively) (Figure 4.11 A). In contrast, specific inhibition of MGL mediated clearance resulted in a significant increase in pdVWF half-life ($t_{1/2}$ = 64.6 ± 18.4 mins versus 31.4 ± 2.2 mins; $P < 0.005$) (Figure 4.11 B).

Interestingly, these data demonstrate that MGL can mediate the clearance of both hyposialylated and normally sialylated VWF *in vivo*. Given that the molecular mechanisms underpinning the physiological clearance of VWF are poorly understood, these data are of direct translational significance.

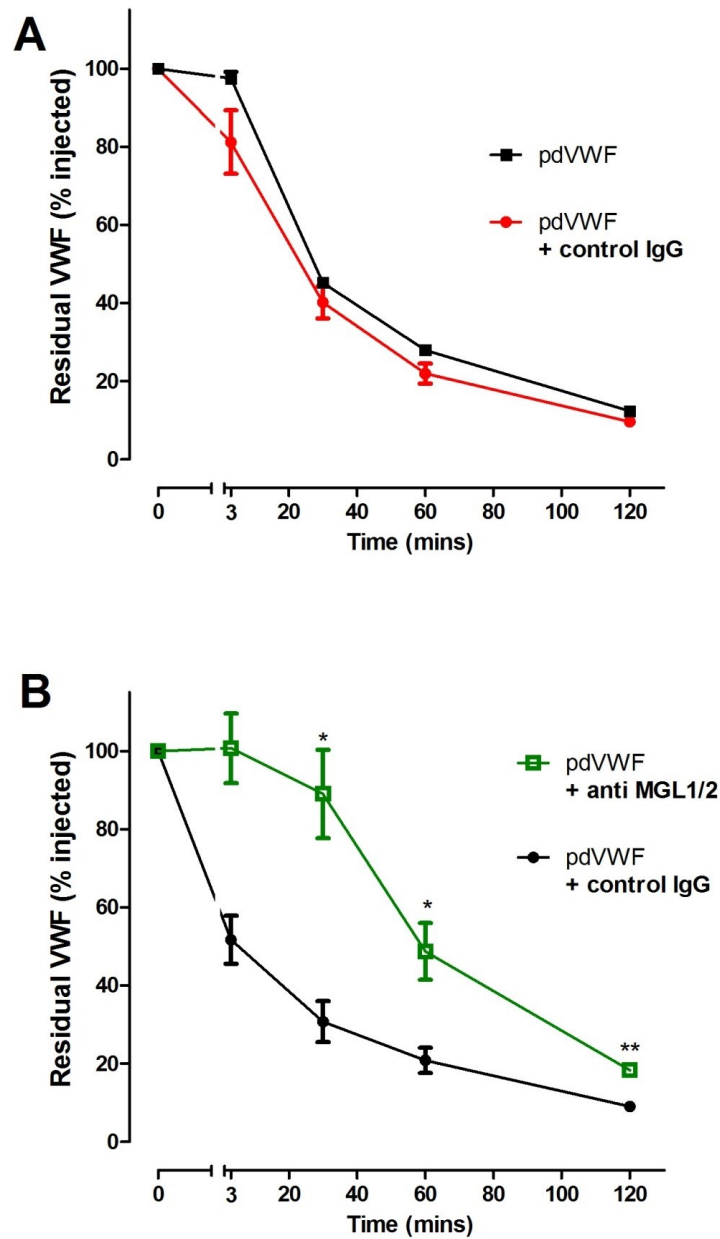


Figure 4.12: pdVWF half-life is prolonged following inhibition of MGL *in vivo*

(A) Pre-infusion of control IgG had no effect on the clearance of pdVWF (pdVWF $t_{1/2}$ = 31.4 ± 2.2 mins versus 31.1 ± 2.3 mins respectively; $p > 0.05$). (B) *In vivo* clearance of wild type pd-VWF in $VWF^{-/-}Asgr1^{-/-}$ mice was significantly attenuated in the presence of an mMGL blocking antibody compared to isotype control IgG ($t_{1/2}$ = 64.6 ± 18.4 mins versus 31.2 ± 3.2 mins; $P < 0.005$). A minimum of three mice were used per time point, data is plotted as mean \pm SEM. Adapted from Ward *et al*, Blood 2018.

4.12. Discussion

Grewal *et al* previously demonstrated that plasma VWF clearance is significantly attenuated in *Asgr1*^{-/-} knockout mice (Grewal *et al*, 2008). However, the data presented in this Chapter demonstrates that in *Asgr1* deficient mice, the enhanced circulatory clearance associated with VWF desialylation persists. As such, important questions remain regarding the biological mechanisms through which other galactose sensitive receptor/s may influence the enhanced clearance of hyposialylated VWF.

Lenting *et al* initially demonstrated that the liver and spleen are the primary sites of VWF clearance following injection of radiolabelled VWF (Lenting *et al*, 2004). Accounting for the organ size and proportionate blood flow, it is understood that the liver is the predominant site for VWF clearance. Immunohistochemistry performed on murine livers post infusion of α 2-3,6,8,9 Neu-VWF demonstrate that in the presence and absence of ASGPR, hyposialylated VWF co-stains with elongated Kupffer cells in liver sinusoids (Figure 4.3). Furthermore, differentiated THP1 macrophages demonstrate enhanced affinity to α 2-3,6,8,9 Neu-VWF compared to pdVWF *in vitro* (Figure 4.4). To investigate whether VWF sialylation influences macrophage-mediated clearance, studies were repeated in *VWF*^{-/-}/*Asgr1*^{-/-} mice following clodronate administration. We found that the increased clearance phenotypes of both α 2-3, Neu-VWF and α 2-3,6,8,9 Neu-VWF were significantly attenuated following macrophage depletion (Figure 4.5). These results confirm that macrophages play a critical role in modulating the enhanced clearance of hyposialylated VWF.

We hypothesised that MGL may play a role in regulating macrophage-mediated clearance of hyposialylated VWF. Importantly, we observed dose-dependent binding of human pd-VWF to purified recombinant human MGL using surface plasmon resonance (Figure 4.6). Moreover, Duolink-proximity ligation assay (PLA) analysis demonstrated that VWF co-localises with MGL on the surface of THP1 macrophages, as indicated by the distinct red fluorescent dots (Figure 4.7).

Interestingly, the enhanced clearance of both α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF in *VWF^{-/-}/Asgr1^{-/-}* mice was significantly attenuated in the presence of anti mMGL1/2 inhibitory antibody, suggesting a novel role for MGL in regulating macrophage-mediated clearance of hyposialylated VWF (Figure 4.8 C). Plasma VWF:Ag levels were significantly elevated in *MGL1^{-/-}* mice compared to wild type controls ($152.6 \pm 15.7\%$ versus $100 \pm 16.9\%$; $P < 0.05$) (Figure 4.9). In addition, VWFpp:Ag ratio was decreased in *mMGL1^{-/-}* mice when compared to wild type controls (Figure 4.10), indicating that the increased endogenous mVWF observed in *mMGL1^{-/-}* mice is due to prolonged circulatory half-life of mVWF. Furthermore, *in vivo* clearance of endogenous murine VWF was attenuated in *MGL1^{-/-}* mice (Figure 4.11), suggesting that MGL-mediated VWF clearance is important even in the presence of ASGPR. In addition, clearance of human pd-VWF in *VWF^{-/-}/Asgr1^{-/-}* mice was attenuated in the presence of mMGL1/2 inhibitory antibody (Figure 4.12).

Collectively, these findings reveal MGL as a novel macrophage lectin receptor for VWF that contributes to the clearance of both wild-type and hyposialylated VWF. Further studies will be required to determine the importance of MGL compared to other recently described receptors involved in regulating VWF clearance (Pipe *et al*, 2016).

Nevertheless, the role of MGL in modulating VWF clearance has direct translational relevance in that quantitative variations in N- and O-linked sialylation have been described in patients with type 1 VWD (Ellies *et al*, 2002; van Schooten *et al*, 2007). In addition, desialylation of VWF has been described with glycoprotein ageing in plasma (Yang *et al*, 2015) and can also occur during infections with specific pathogens that are associated with significantly enhanced neuraminidase activity (e.g. Streptococcus pneumoniae)(Grewal *et al*, 2008).

5. Characterisation of the interaction between VWF and MGL

5.1. Introduction

In Chapter 4, we identified a novel role for the Macrophage Galactose Lectin (MGL) in regulating clearance of VWF (Ward *et al*, 2018). Consequently, we next proceeded to investigate the molecular mechanisms underpinning the interaction between MGL and VWF.

A critical role for VWF sialylation in modulating VWF half-life was first described by Sodetz *et al* who observed that desialylated VWF was rapidly removed from circulation. In Chapter 3, we further demonstrated that removal of α 2-3 linked sialylation from VWF O-linked glycans alone was sufficient to cause markedly enhanced clearance *in vivo* (Ward *et al*, 2018). These data highlight that O-linked sialylation has a specific role in regulating VWF clearance. Nevertheless, the mechanisms through which O-linked glycans modulate VWF half-life have not been clearly defined.

Previous carbohydrate profiling studies have demonstrated that MGL binds glycoproteins expressing desialylated T antigen moieties. Interestingly, T antigen structures account for 70% of total O glycan structures on pd-VWF and has also been shown to correlate with VWF:Ag in a number of patient groups (van Schooten *et al*, 2007). Given these previous data, in this chapter we have further investigated the specific role of O-linked sialylation in regulating VWF clearance via the MGL receptor (Figure 5.1).

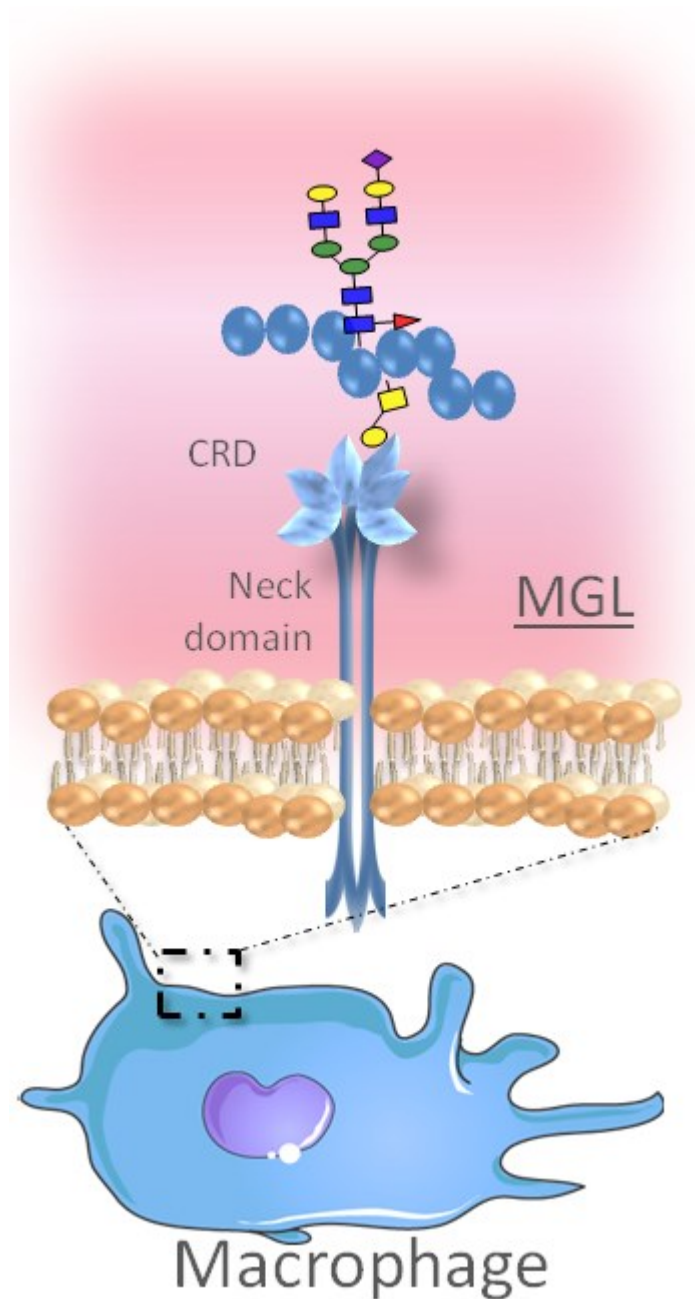


Figure 5.1: Macrophage galactose lectin glycan affinity

MGL is expressed as a homo-trimer on macrophages and dendritic cells. The MGL CRD is specific for exposed Gal/GalNAc terminal moieties; MGL preferentially binds desialylated O-linked glycan structures, with particular affinity for the T antigen.

5.2. VWF binding to MGL is ristocetin and calcium dependent

To further investigate the binding of pd-VWF to MGL *in vitro*, a solid phase binding assay was developed. Briefly, recombinant human MGL was coated onto 96 well polystyrene plates and exposed to VWF. Using this assay, we confirmed that pd-VWF binds to recombinant human MGL in a saturable and dose-dependent manner (Figure 5.2 A). In keeping with the fact that MGL is a C-type lectin, we observed that VWF binding was calcium dependent and thus inhibited by EDTA (Figure 5.2 A). Interestingly, the binding of VWF to MGL was significantly enhanced in the presence of ristocetin (Figure 5.2 A), suggesting that VWF A domain conformation may be important in regulating MGL binding.

To investigate whether VWF multimer distribution influences MGL interaction, pd-VWF preparations with varying multimer distribution were purified by gel filtration. Similar binding was observed for HMW and LMW VWF fractions suggesting that VWF multimer distribution does not influence MGL binding *in vitro* (Figure 5.2 B).

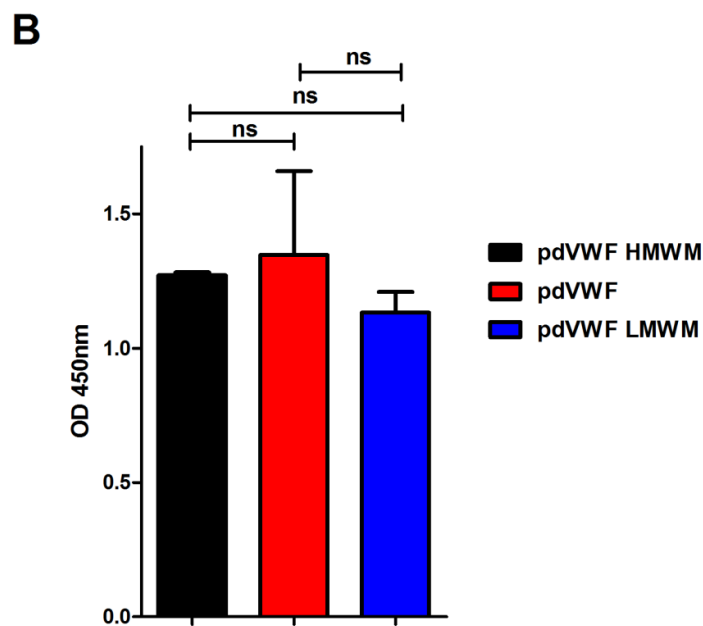
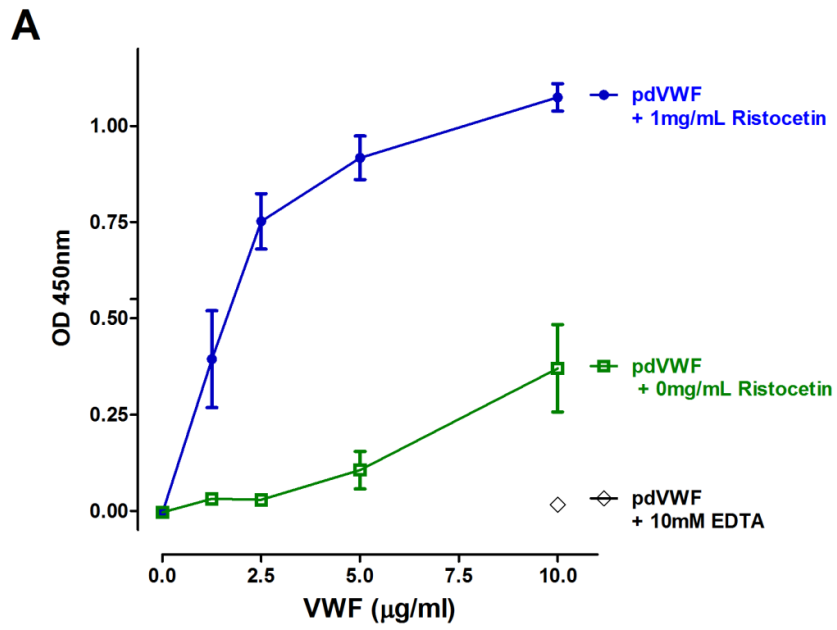


Figure 5.2: pd-VWF binds MGL in a ristocetin dependant, and multimerisation independent manner.

(A) pd-VWF binds MGL in a dose and calcium dependant manner. (B) VWF multimer distribution has no effect on MGL binding (ns = not significant).

5.3. Removal of α 2-3 sialylation causes enhanced MGL binding *in vitro*.

To further investigate VWF-MGL interaction, plate binding assays were repeated after removal of α 2-3 linked sialylation or combined removal of α 2-3,6,8,9 sialylation from pd-VWF. *In vitro* plate-binding studies confirmed that α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF both demonstrated significantly enhanced binding to purified MGL compared to wild type pd-VWF ($p = 0.0172$ and 0.006) (Figure 5.3).

Importantly, additional removal of α 2-6 linked sialic acid (which accounts for 80% of total sialic acid and is predominantly located on the N-linked glycans of pd-VWF) did not further enhance MGL binding compared to the α 2-3 Neu-VWF (Figure 5.3). These data suggest that VWF O-linked sialic acid plays a critical role in protecting human pd-VWF from MGL mediated clearance.

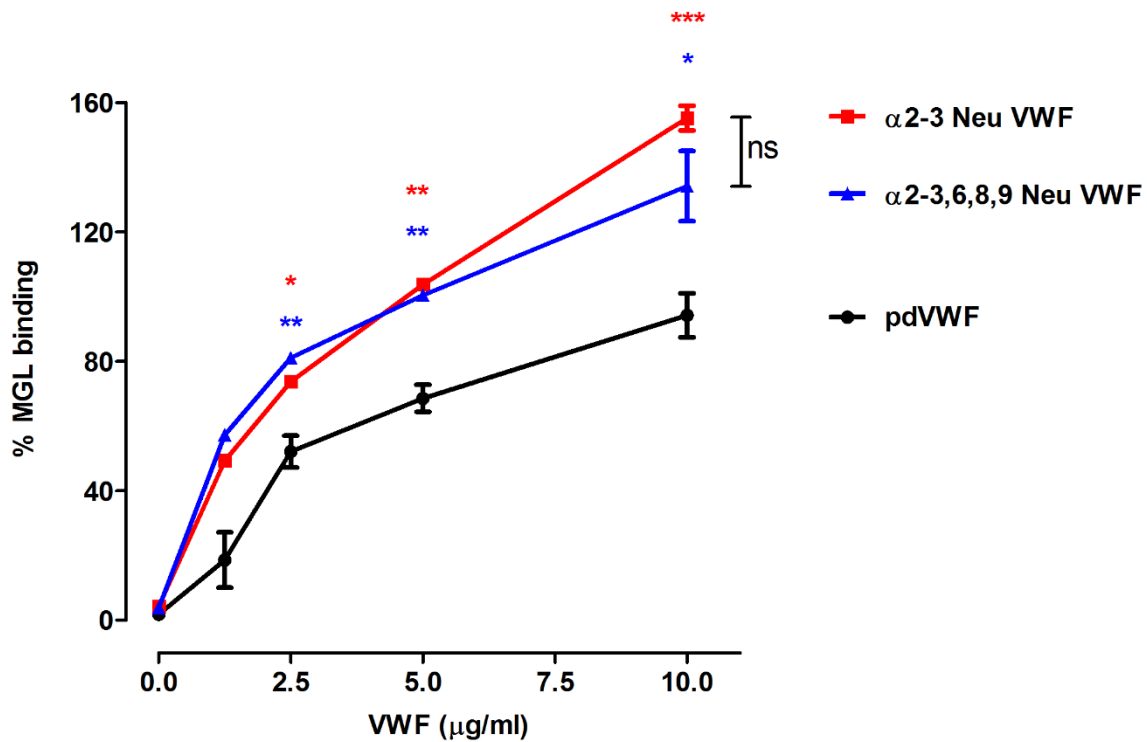


Figure 5.3: Hyposialylated VWF binds MGL with enhanced affinity compared to pd-VWF

Binding of α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF to immobilised MGL were significantly increased compared to untreated pd-VWF. Binding of α 2-3,6,8,9 Neu-VWF was not significantly enhanced compared to α 2-3 Neu-VWF (*; $p < 0.05$, **; $P < 0.01$, ***; $p < 0.001$). All binding was conducted in the presence of 1mg/mL ristocetin. Percentage calculated based upon the optical density (OD) reading for 10 μ g/mL pdVWF.

5.4. MGL is more important than ASGPR in modulating enhanced clearance of α 2-3 Neu-VWF

To investigate the relative contribution of α 2-3 linked sialylation in regulating VWF clearance through the MGL compared to ASGPR receptors *in vivo*, clearance studies were therefore repeated in *VWF^{-/-}/Asgr1^{+/+}* and *VWF^{-/-}/Asgr1^{-/-}* mice in the presence or absence of mMGL1/2 inhibitory antibodies.

Inhibition of MGL1/2 resulted in attenuation of the rapid clearance of α 2-3-Neu-VWF in *VWF^{-/-}/Asgr1^{-/-}* mice (Half-life; 40.2 ± 1.3 mins vs 45.7 ± 2.2 mins, $p=0.076$) (Figure 5.3 A). Importantly, MGL inhibition was also able to block the enhanced clearance of α 2-3-Neu-VWF even in the presence of ASGPR (Figure 5.3 B). Collectively, these data suggest that MGL is probably more important than ASGPR in modulating the increased clearance of α 2-3 Neu-VWF and thus that O linked glycans play a critical role in protecting VWF against MGL-mediated clearance *in vivo*.

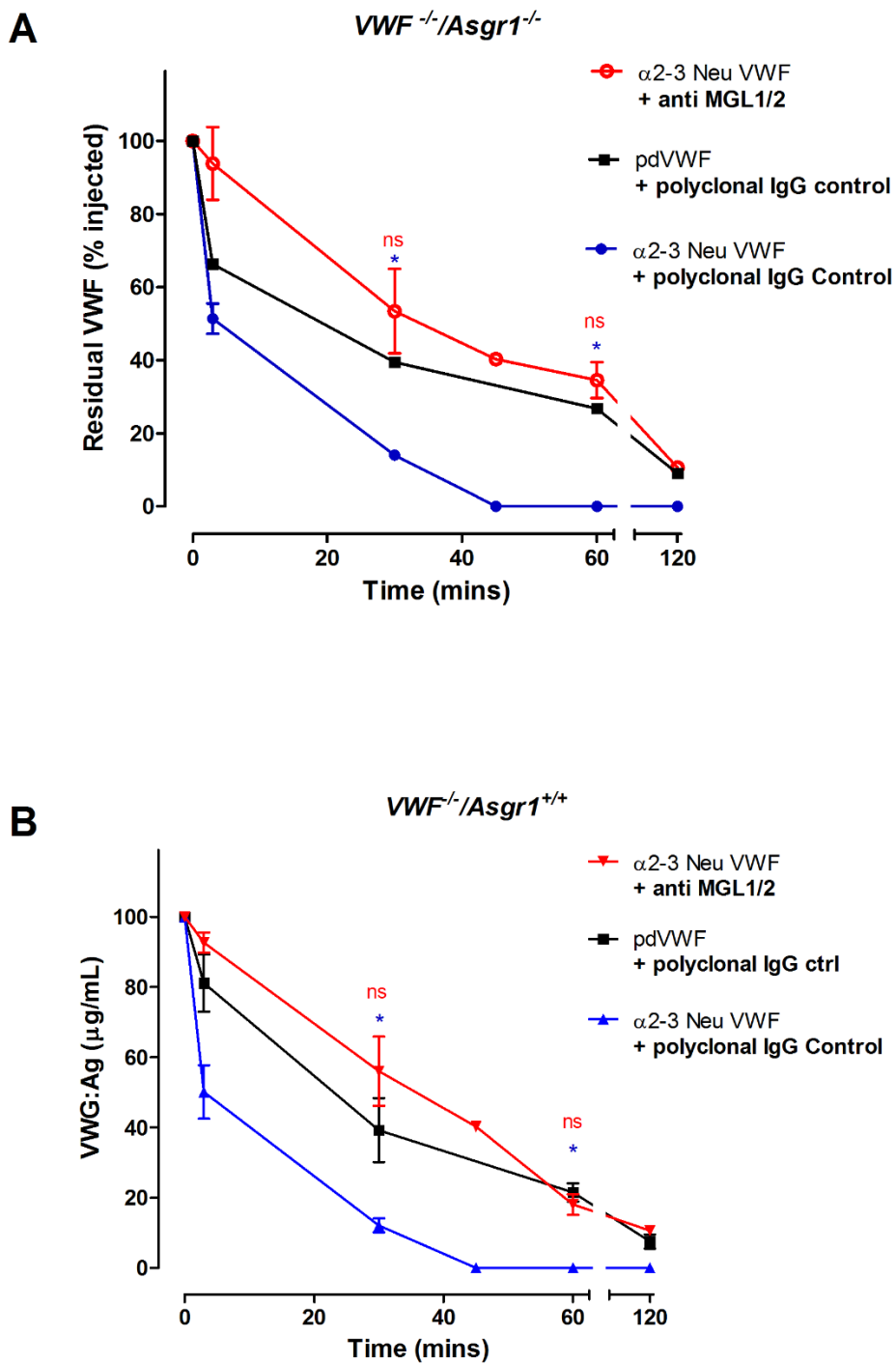


Figure 5.4: Clearance of $\alpha 2-3$ Neu-VWF is regulated by MGL alone.

In (A) *VWF^{-/-}/Asgr1^{-/-}* and (B) *VWF^{-/-}* mice clearance of $\alpha 2-3$ Neu-VWF is significantly greater compared to untreated pd-VWF (blue stars) while inhibition of murine mMGL1/2 corrects the attenuates clearance of $\alpha 2-3$ Neu-VWF (red text) (*; $p < 0.05$, ns; not significant).

5.5. Both MGL and ASGPR modulate the enhanced clearance of α 2-3,6,8,9 Neu-VWF *in vivo*.

To compare the relative roles of MGL and ASGPR in regulating α 2-3,6,8,9 Neu-VWF clearance, *in vivo* clearance studies were performed in both $VWF^{-/-}/Asgr1^{-/-}$ and $VWF^{-/-}/Asgr1^{+/+}$ mice in the presence or absence of MGL inhibition.

In $VWF^{-/-}/Asgr1^{-/-}$ double knockout mice, murine-MGL1/2 inhibition resulted in attenuation of the enhanced clearance of α 2-3,6,8,9 Neu-VWF compared to pd-VWF (27.87 ± 10.7 mins vs 38.83 ± 4.5 mins respectively, $p > 0.05$) (Figure 5.4 A). Interestingly however, in mice expressing ASGPR ($VWF^{-/-}/Asgr1^{+/+}$), MGL inhibition had minimal effects in reducing the fast clearance of α 2-3,6,8,9 Neu-VWF (Figure 5.5 B). These findings contrast significantly with the observation that MGL inhibition successfully inhibits the clearance of α 2-3 Neu-VWF in the presence or absence of ASGPR (Figure 5.4). Together these data suggest that MGL may have a particular role in modulating the clearance of α 2-3 Neu-VWF.

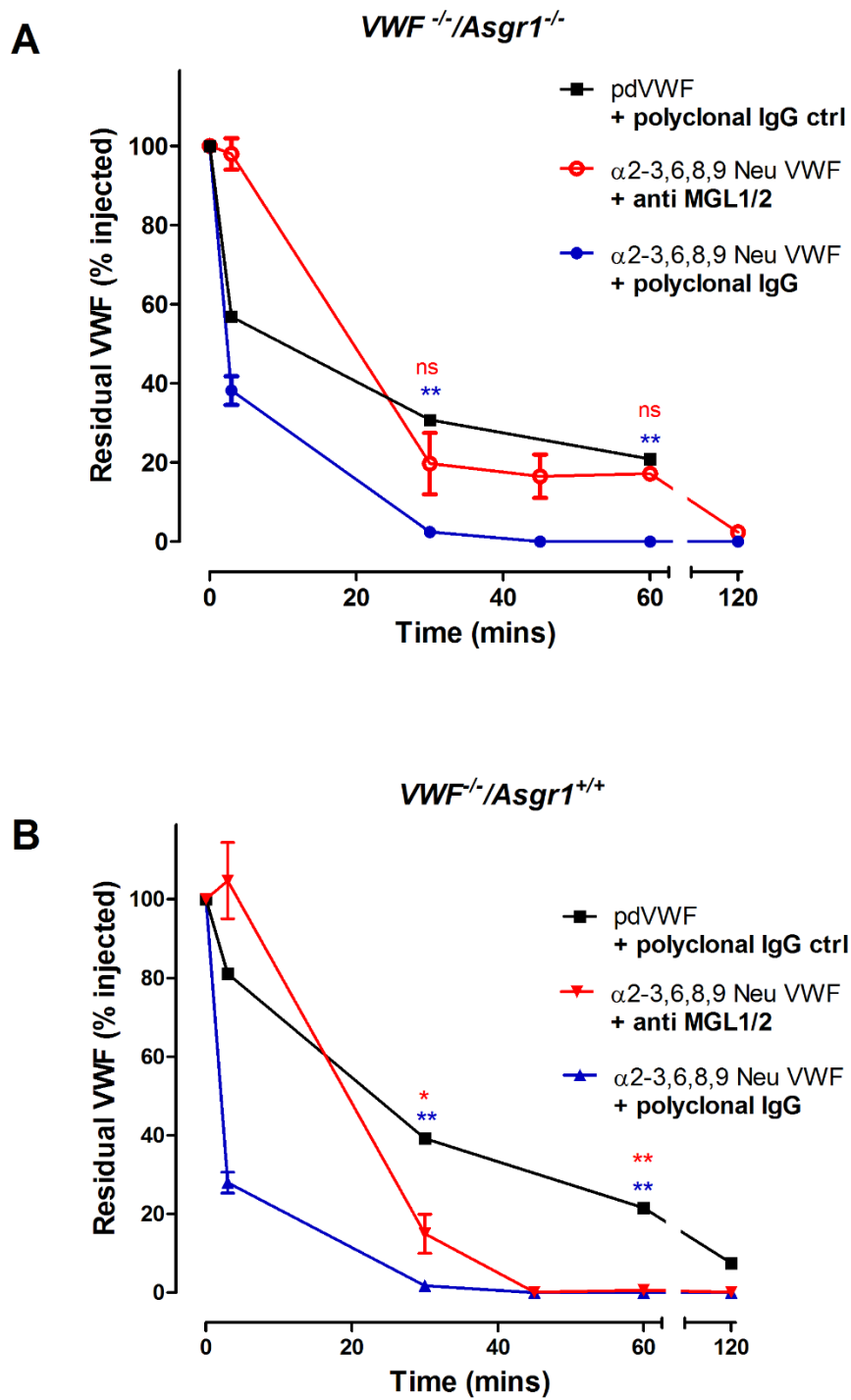


Figure 5.5: Clearance of α -3,6,8,9 Neu-VWF is modulated by MGL and ASGPR

In (A) *VWF^{-/-}/Asgr1^{-/-}* and (B) *VWF^{-/-}* mice clearance of α 2-3,6,8,9 Neu-VWF exhibits enhanced clearance compared to untreated pd-VWF (blue stars) while inhibition of murine mMGL1/2 attenuates the clearance of α 2-3 Neu-VWF (red text) in *VWF^{-/-}/Asgr1^{-/-}* mice alone (*; $p < 0.05$, **; $p < 0.01$, ns; not significant).

5.6. VWF O glycans modulate MGL binding *in vitro*.

α 2-3 linked sialylation predominantly expressed in the O-linked glycans of pd-VWF. To further investigate the specific role of VWF O-glycan in determining MGL-mediated clearance, pd-VWF was treated with specific exoglycosidases to generate glycoforms from which either the N-linked glycans alone (PNGase VWF) or combined N- and O-linked glycans (PNGase, O Glycosidase VWF) were removed from pd-VWF.

Removal of VWF glycans was confirmed with lectin binding assays (Figure 5.6). PNGase VWF digestion was confirmed by a complete loss in SNA and RCA binding (α 2-6 linked sialic acid and β 1-4 Gal respectively) with a retention of MAA II binding (α 2-3 linked sialic acid) compared to untreated pd-VWF (Figure 5.6 A). Further removal of VWF O-linked glycans was confirmed by a loss of MAA II and PNA lectin binding (α 2-3 linked sialic acid and desialylated T antigen respectively) (Figure 5.6 B).

Interestingly, removal of VWF N-linked glycans alone had no significant effect on MGL binding (Figure 5.7) ($p > 0.05$). Additional removal of VWF O-linked glycans however, resulted in a marked reduction in MGL binding (Figure 5.7) ($p < 0.05$). Collectively, these data are consistent with the concept that O glycans of VWF play a key role in modulating MGL interaction.

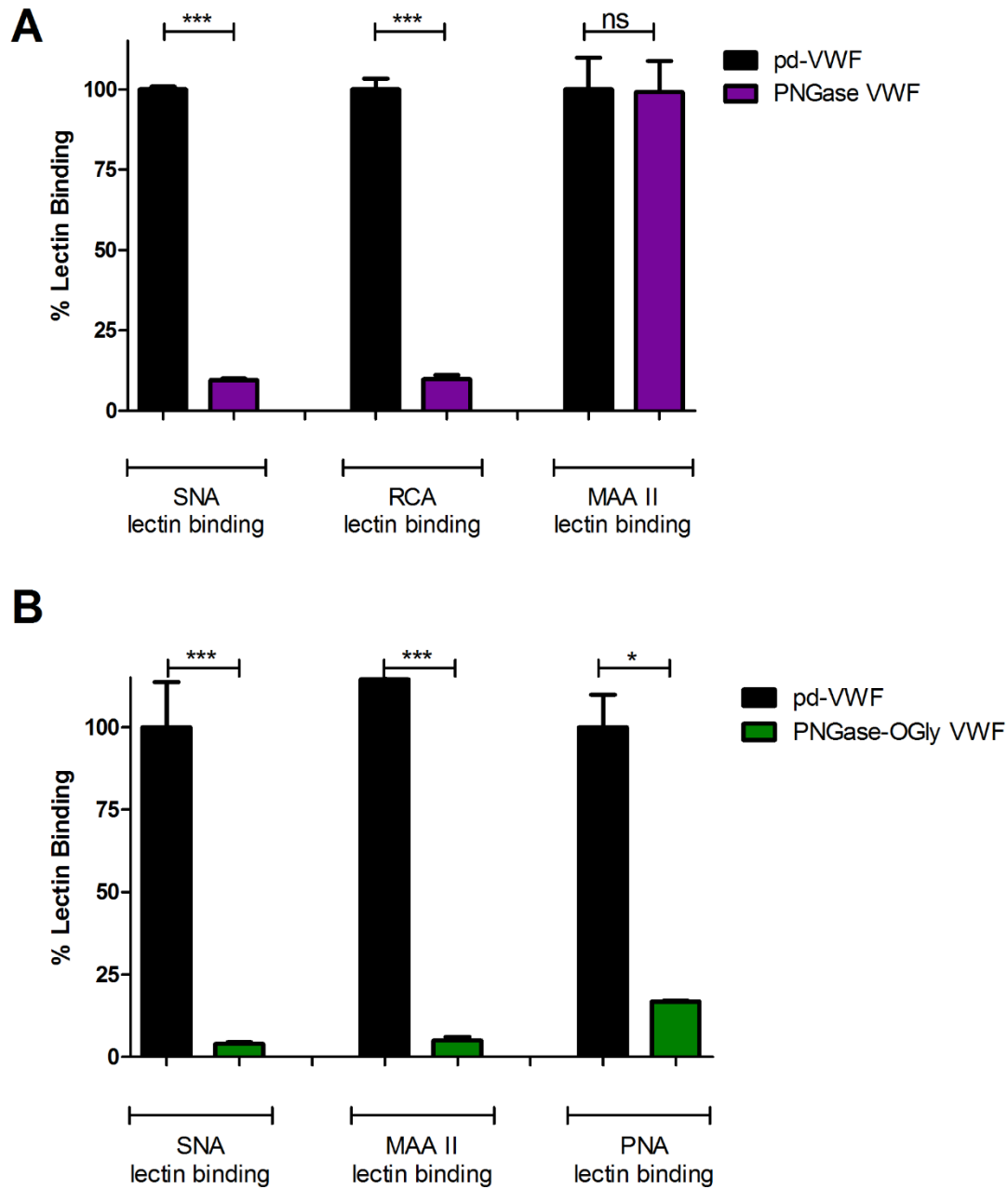


Figure 5.6: Confirmation of exoglycosidase digestion

(A) PNGase VWF digestion was confirmed by assessing binding to SNA, RCA and MAA II plant lectins. (B) Further removal of VWF O-linked glycans was confirmed by assessing SNA, MAA II and PNA lectin binding (*; $p < 0.05$, ***; $p < 0.0001$, ns; not significant).

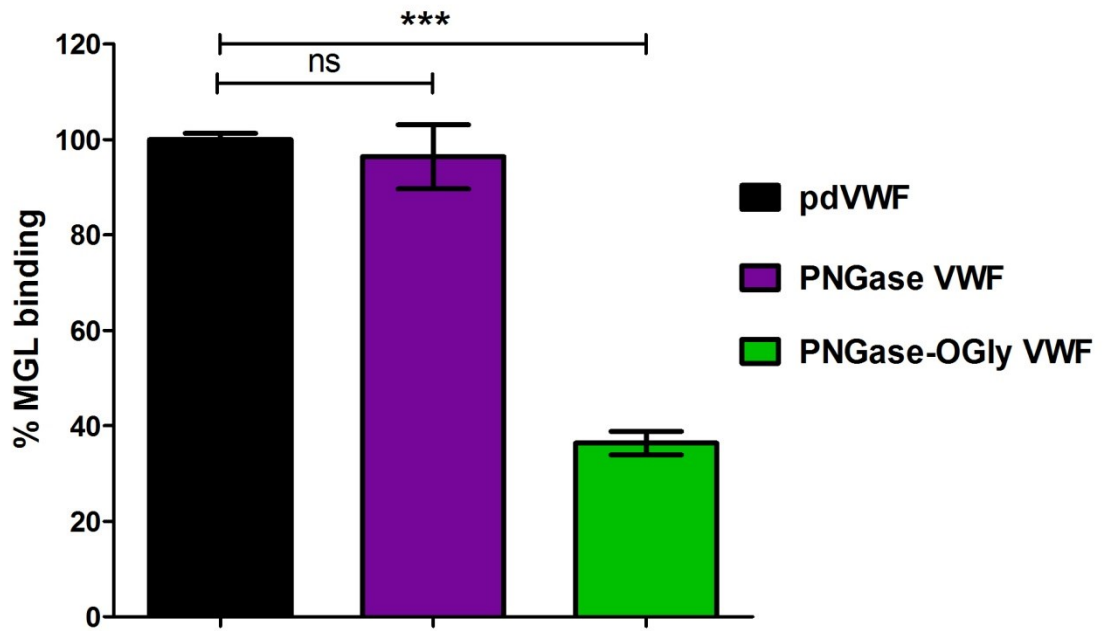


Figure 5.7: VWF O glycans modulate MGL binding *in vitro*

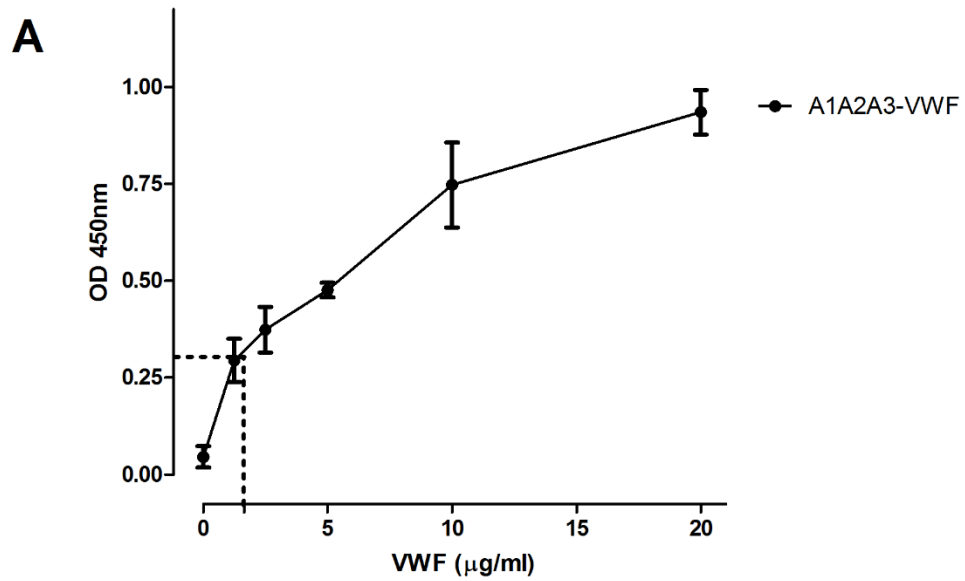
PNGase-VWF binds MGL with the same affinity as pdVWF. Additional removal of O-linked glycans results in a 64% reduction in MGL binding (***, $p < 0.0001$, ns; not significant).

5.7. The VWF A1 domain regulated MGL interaction.

Pd-VWF binding to MGL is significantly enhanced by ristocetin (Figure 5.2). Moreover, the O-linked glycans of VWF, which are concentrated in A1A2A3 region contain key MGL binding sites. To investigate the role of VWF A domains in regulating MGL binding, the binding of a truncated VWF-A1A2A3 fragment to MGL was assessed *in vitro*.

Importantly, we observed dose dependant binding of VWF-A1A2A3 to MGL *in vitro*, with an estimated apparent KD of 1.7 μ g/mL (22.8nM) (Figure 5.8 A). In keeping full length pd-VWF, the binding of VWF-A1A2A3 was also ristocetin and calcium-dependant (Figure 5.8 B).

To further characterise the role of the A domains in regulating VWF binding to MGL, isolated VWF-A1, VWF-A2 and VWF-A3 domains were expressed and purified. Interestingly, no binding was observed for the isolated VWF-A2 or VWF-A3 domains (Figure 5.9). In contrast VWF-A1 domain demonstrated significant binding to MGL *in vitro* in the presence and absence of ristocetin. Importantly, eight of 10 O-linked glycans of VWF are clustered around the A1 domain.



Kd app: 1.7 µg/mL or 22.8 nM

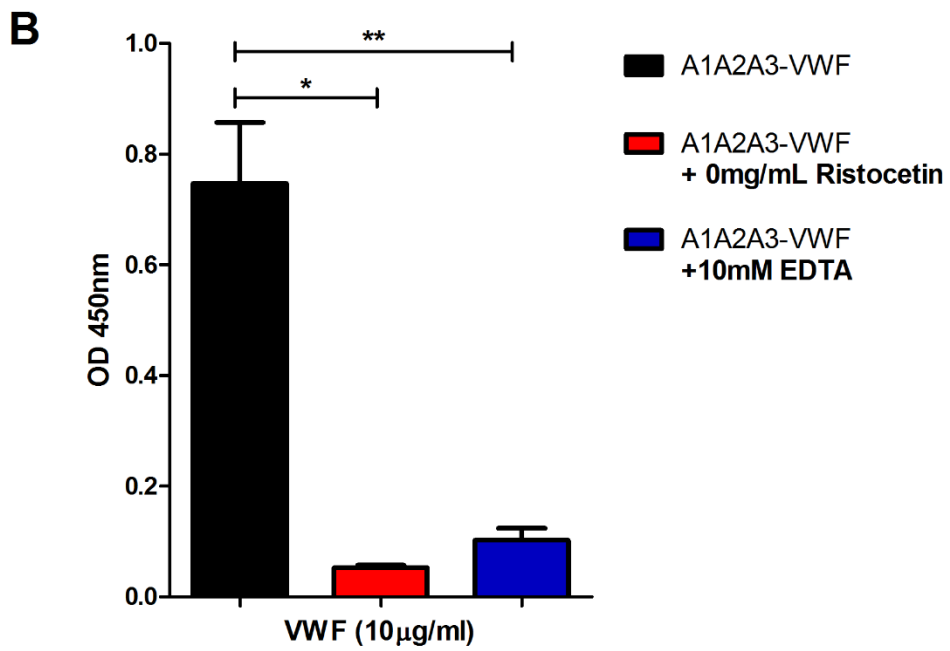


Figure 5.8: VWF-A1A2A3 contains critical MGL binding sites.

(A) VWF-A1A2A3 binds MGL with the high affinity *in vitro*. (B) A1A2A3-VWf binding to MGL is ristocetin and calcium dependent (*; $p < 0.05$, ** $p < 0.01$).

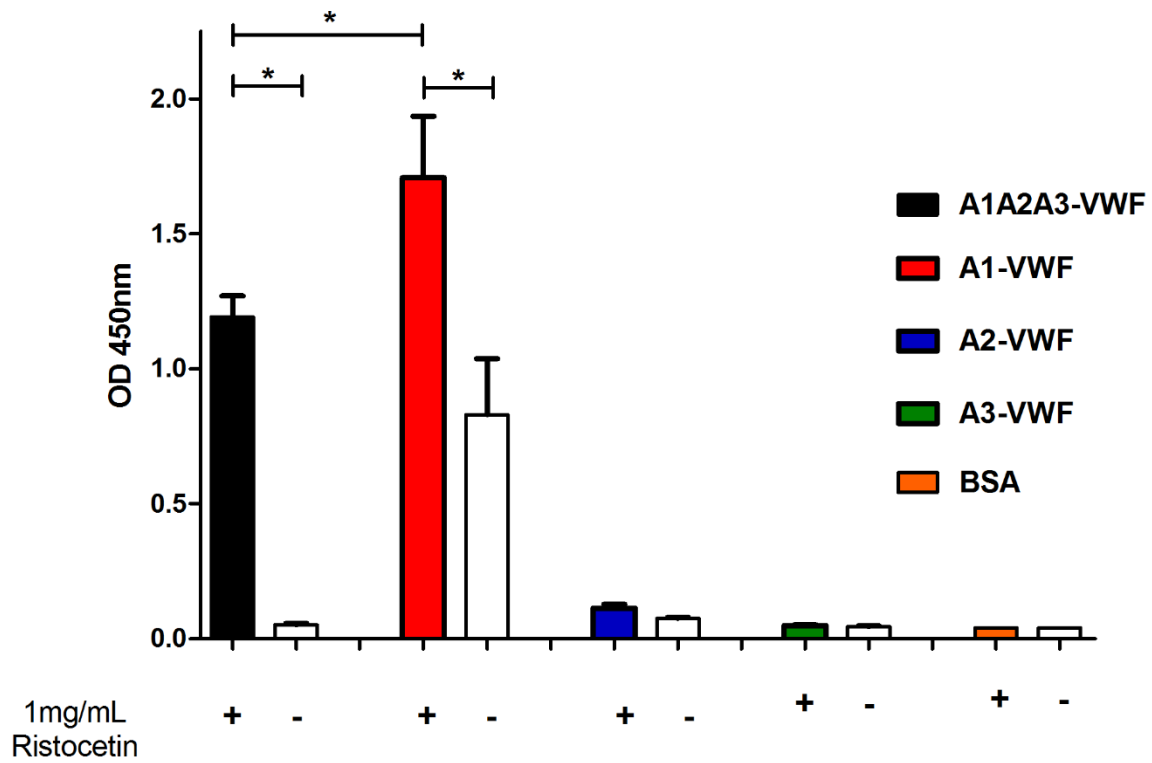


Figure 5.9: VWF-A1 binds with high affinity to MGL *in vitro*.

At equimolar concentrations, VWF A1 domain binds MGL with high affinity *in vitro* in the presence and absence of ristocetin. No measurable binding was observed for VWF-A2, VWF-A3 and a 10mg/mL BSA control (*; $p < 0.05$).

5.8. O linked glycans within VWF-A1 are critical for MGL binding

The VWF-A1A2A3 truncation contains two N-linked glycans (sites N1515, N1574) and eight O-linked glycans (Cluster 1; T1248, T1255, T1256, S1263 and Cluster 2; T1468, T1477, S1486, T1487) (Canis *et al*, 2010, 2012). We sought to further characterise the role of N-linked and O-linked glycans in mediating the binding of truncated VWF-A1A2A3 to MGL.

Truncated A1A2A3-VWF was digested with exoglycosidases to generate PNGase A1A2A3-VWF and O Glycosidase A1A2A3-VWF glycoforms. Glycosidase digestions were confirmed using plant lectin binding analysis as previously (Figure 5.10). Of note, removal of O-linked glycans from truncated A1A2A3-VWF was successful without prior removal of N-linked glycans (Figure 5.10 A).

Consistent with previous studies for full length pd-VWF, removal of N-linked glycans within VWF-A1A2A3 had no significant effect on MGL binding ($p>0.05$) (Figure 5.10 C). In contrast, removal of O-linked glycan significantly reduced binding to MGL *in vitro* (Figure 5.10 C). Given that there was no measurable binding for the isolated A3 domain which contains O-linked glycan T1679, these data suggest that the two O glycan clusters flanking the A1 domain are important regulators of MGL binding.

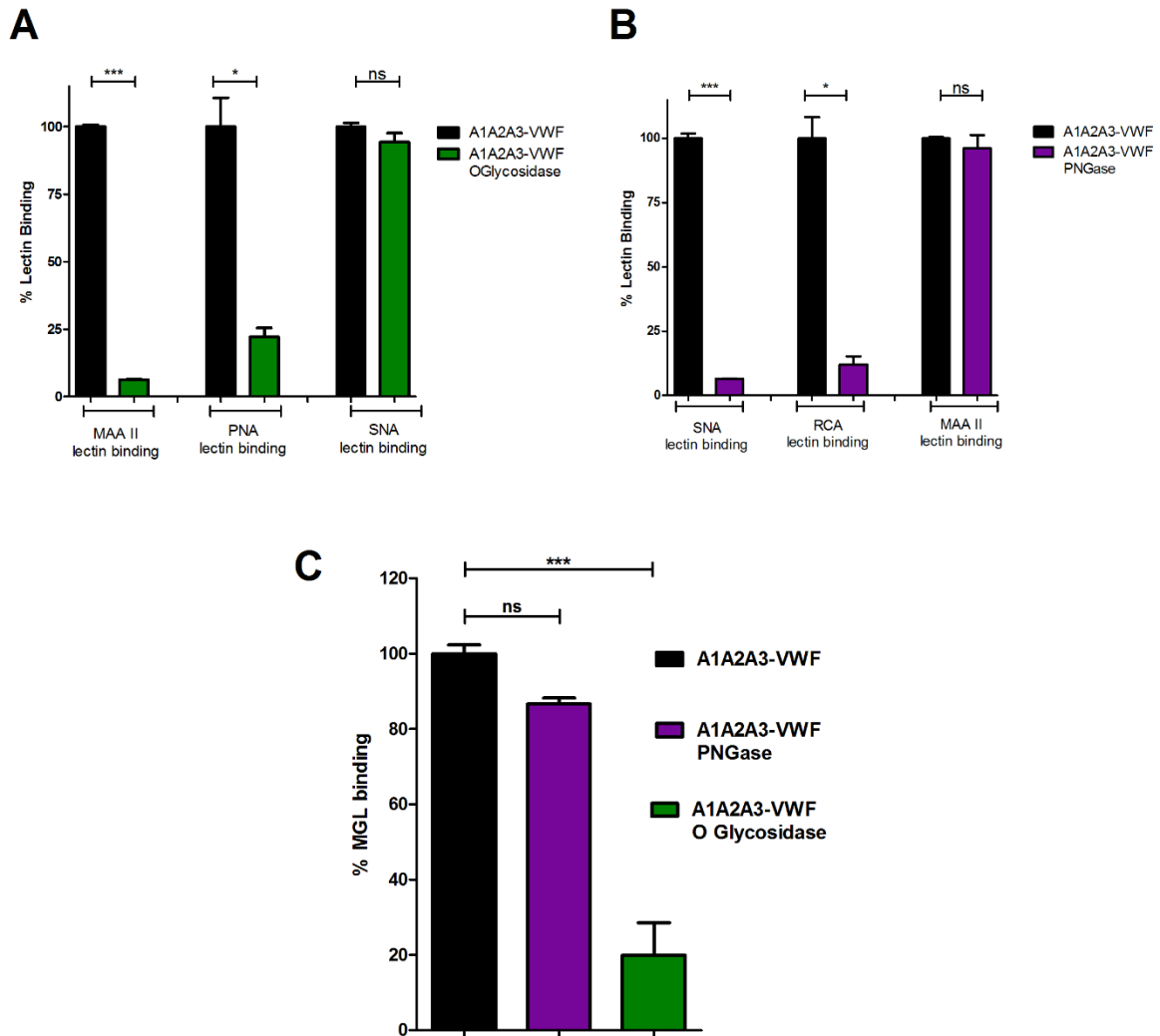


Figure 5.10: VWF-A1A2A3 O-linked glycans modulate MGL *in vitro* binding.

(A) O glycosidase digestion was confirmed with MAA II, PNA and SNA lectin binding. (B) Removal of N-linked glycans was confirmed with SNA, RCA and MAA II binding. (C) *In vitro* binding of VWF-A1A2A3 to MGL is mediated by O linked glycans (*; $p < 0.05$, ***; $p < 0.001$).

5.9. VWF O linked glycan clusters modulate MGL binding

To further investigate the role of the two O-linked glycan clusters either side of the A1 domain in regulating VWF-MGL interaction, site directed mutagenesis was used to generate full length VWF variants lacking either the cluster 1 (Δ Cluster1; T1248A, T1255A, T1256A, T1263A), or cluster 2 (Δ Cluster2; T1468A, T1477A, S1486A, T1487A) or both clusters (Δ Double Cluster; T1248A, T1255A, T1256A, T1263A, T1468A, T1477A, S1486A, T1487A).

Interestingly, the binding of Δ Cluster 1 VWF and Δ Cluster 2 VWF to MGL was not significantly different (KD app 1.285 μ g/mL and 1.577 μ g/mL respectively) (Figure 5.11). In contrast, the binding of binding of the Δ Double Cluster VWF variant to MGL was markedly reduced (KD app 3.4 μ g/mL).

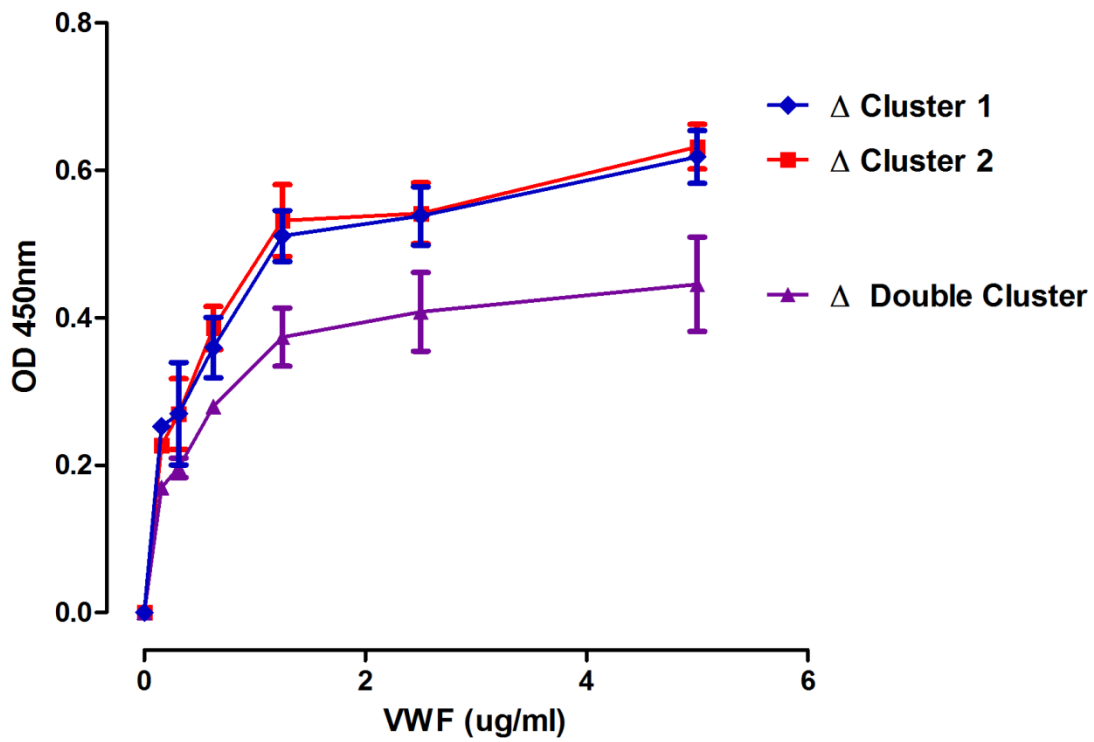


Figure 5.11: Loss of VWF O-linked glycan clusters reduces MGL binding

VWF variants Δ Cluster1 and Δ Cluster2 bind MGL with a similar affinity, however Δ Double Cluster VWF binds MGL with lower affinity.

5.10. Identification of murine MGL2 as a VWF clearance receptor

In mice there are two distinct MGL homologs; murine MGL1 (mMGL1) and murine MGL2 (mMGL2). The carbohydrate binding profile of mMGL2 is more similar to that of human MGL. Consequently, we further investigated whether murine MGL2 could also bind to VWF. Similar to human MGL, dose-dependent binding of pd-VWF to both MGL1 and MGL2 was observed in vitro. Interestingly, binding of pdVWF to MGL2 was greater than to mMGL1 (1.9-fold increase, $p=0.0317$) (Figure 5.12 A).

As previously shown (Figure 4.9), mice deficient in *mMGL1* demonstrate significantly elevated VWF:Ag levels (1.5 fold). To study whether MGL2 may contribute to the clearance of VWF in *MGL1* deficient mice, studies were performed in *mMGL1*^{-/-} mice using an MGL2 specific inhibitory antibody. Interestingly, 24 hours after treatment with anti-MGL2 blocking antibodies, murine VWF:Ag levels were increased significantly compared to polyclonal IgG treated *mMGL1*^{-/-} controls (2.78 ± 0.04 U/mL versus 1.75 ± 0.5 U/mL respectively) (Figure 5.12 B).

In addition, MGL2 inhibition in *mMGL1*^{-/-} mice significantly extended the half-life of endogenous murine VWF (1.915 ± 0.15 hrs vs 2.5 ± 0.2 hrs, $p=0.028$) (Figure 5.13 A). Furthermore, combined inhibition of both MGL1 and MGL2 resulted in a significant 2.4-fold increase in murine VWF mean residence time (MRT) (1.55 ± 0.2 hours versus 3.74 ± 0.3 hours respectively; $p<0.001$) (Figure 5.14 B).

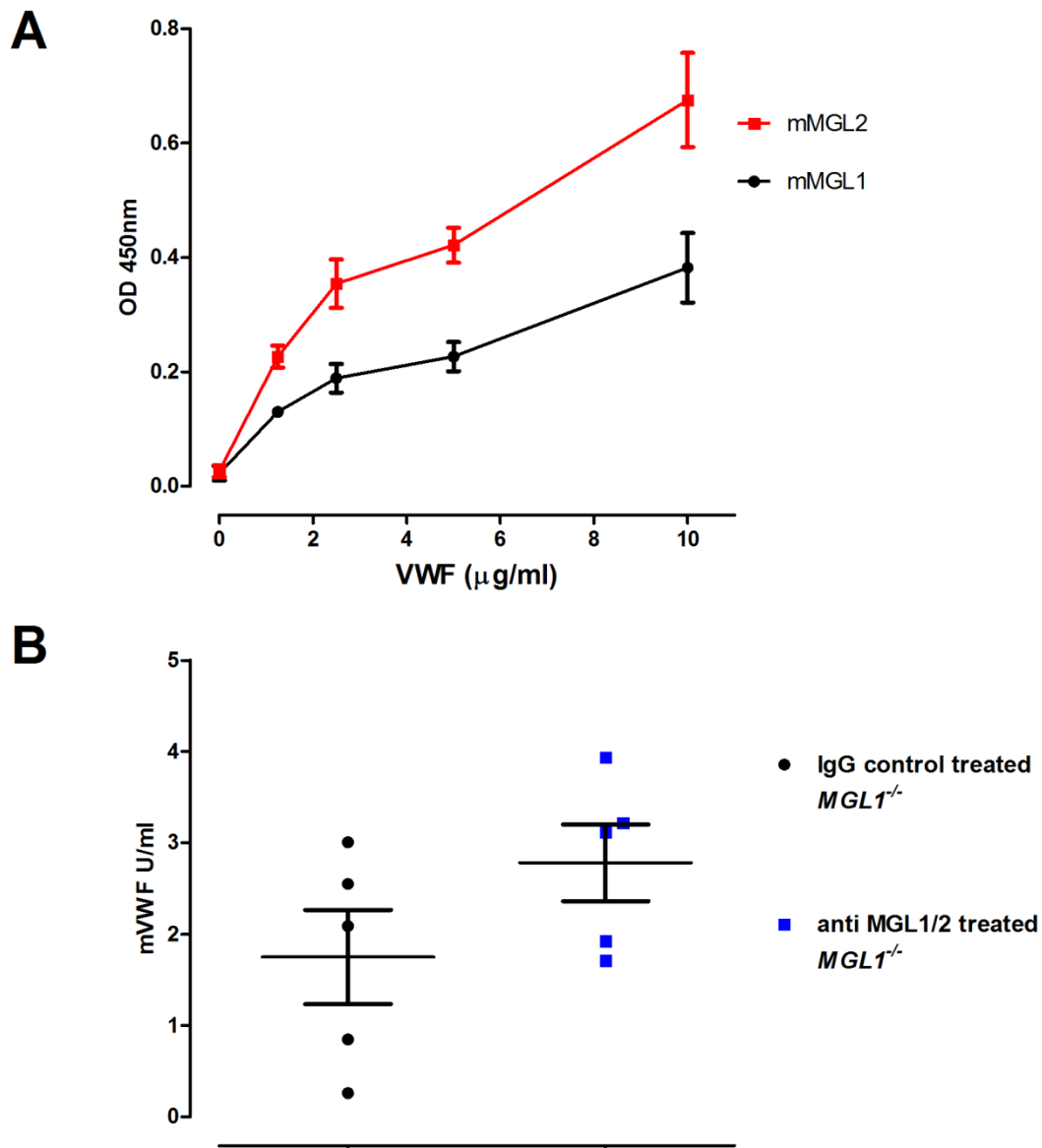


Figure 5.12: mMGL2 binds VWF and regulates plasma VWF:Ag levels

(A) pd-VWF binds mMGL2 with a 1.9 fold higher affinity than mMGL1 *in vitro*. (B) Inhibition of mMGL2 in $MGL1^{-/-}$ mice resulted in a further 1.6-fold increase of murine VWF:Ag levels

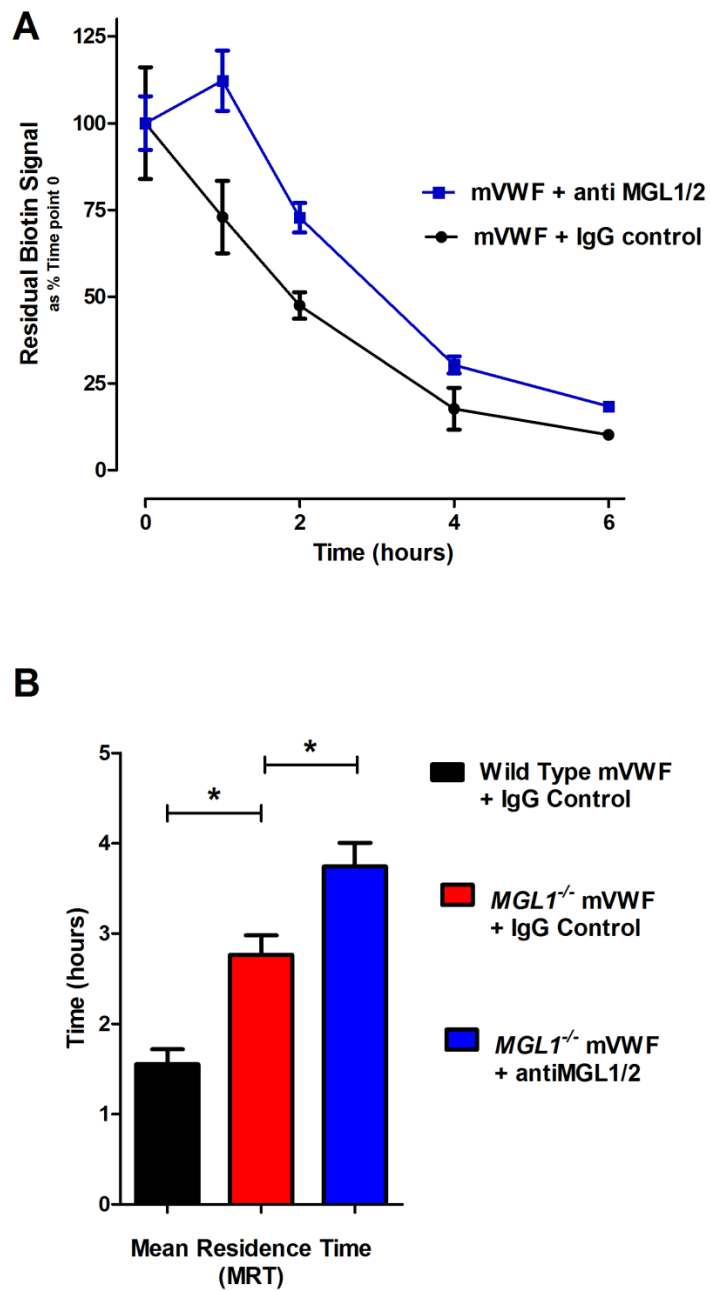


Figure 5.13: mMGL2 mediates VWF clearance

(A) Clearance of mVWF is significantly attenuated upon inhibition of mMGL2 in *MGL1*^{-/-} mice. (B) MRT upon combined inhibition of mMGL1/2 is increased 2.4-fold compared to wild type.

5.11. Discussion

Having established MGL as a novel clearance receptor for VWF, we sought to further define the molecular mechanisms underpinning the interaction between VWF and MGL. *In vitro* binding studies demonstrated that while human pd-VWF can bind MGL in the absence of ristocetin, supplementation with ristocetin significantly enhanced VWF binding (Figure 5.2 A). These data demonstrate that VWF has a higher affinity for MGL when in an active conformation i.e. under shear stress or following incubation with ristocetin. In addition, we also observed that VWF multimer distribution had no effect on MGL binding (Figure 4.2 B), which is in keeping with studies demonstrating that VWF clearance rate is unchanged respective to multimer distribution (Lenting *et al*, 2004).

Approximately 80% of the total sialic acid on human VWF is expressed on N-glycans where it is predominantly α 2-6 linked. In contrast, the other 20% of VWF sialylation is expressed O-linked glycans where it can be α 2-3, α 2-6 or α 2-8 linked (McGrath *et al*, 2010a). Interestingly, *in vivo* clearance of α 2-3 Neu-VWF in was almost as rapid as that of asialo VWF (Figure 3.7). *In vitro* plate-binding studies confirmed that α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF both demonstrated significantly enhanced binding to purified MGL compared to wild type VWF (155% and 134% versus 100%; $p = 0.0172$ and 0.006) (Figure 5.3). Importantly however, combined removal of α 2-3 and α 2-6 linked sialic acid from the N-glycans of VWF did not further enhance α 2-3 Neu-VWF binding to MGL. These data indicate that α 2-3 linked sialylation on the O glycans of VWF plays a specific crucial role in mediating MGL binding.

Besides MGL, the ASGPR has also been implicated in regulating the clearance of hyposialylated VWF. However, the relative importance of these two receptors has not been defined. Interestingly, we observed that inhibition of murine MGL1/2 resulted in significant attenuation of the enhanced clearance of α 2-3 Neu-VWF in mice deficient (Figure 5.4 A) and sufficient (Figure 5.4 B) for ASGPR. These data indicate that the enhanced clearance observed upon loss of VWF O-linked sialylation occurs through MGL. In contrast, additional removal of VWF N-linked sialylation resulted in an enhanced clearance which involved both MGL and ASGPR (Figure 5.5 A).

To further understand the contribution of VWF O-linked glycans in regulating MGL-VWF interactions we assessed binding of a number of VWF truncations and glycoforms to MGL *in vitro*. We have previously shown that removal of N-linked glycans resulted in markedly enhanced clearance through a number of mechanisms (O'Sullivan *et al*, 2016a; Chion *et al*, 2016). However, we observed that PNGase removal of VWF N-glycans did not affect MGL binding *in vitro* (Figure 5.7). Conversely, treatment with O-glycosidase to cause removal of VWF O-linked glycans significantly attenuated MGL binding (Figure 5.7). These novel data further confirm that O-linked rather than N-linked glycans represent key MGL ligands. Consistent with this hypothesis we observed that the A1 domain, which contains 8 of the 10 O-linked glycans in VWF, modulated MGL binding (Figure 5.9).

Interestingly, we observed a significant reduction in MGL binding for a full length VWF variant lacking both Cluster 1 and Cluster 2 O-linked glycans (Figure 5.11). Extensive profiling of VWF O-linked glycan clusters has identified significant differences between the glycan structures expressed within these two clusters, VWF cluster 2 glycans

comprise a significantly higher proportion of core 2 structures. In addition, Johnsen *et al* reported that a rare variant which disrupts the O-linked glycan at position S1486 results in a 35-40 IU/dL decrease in VWF:Ag level (Johnsen *et al*, 2013).

In recent years galactose receptors ASGPR and MGL, have been implicated in VWF clearance, however the relative importance of these receptors remains elusive. *ASGPR*^{-/-} and *MGL1*^{-/-} mice have been shown to have 1.5-fold endogenous VWF, suggesting that both receptors contribute to VWF clearance. Interestingly, extensive carbohydrate profiling has identified that while the binding profile of both mMGL1 and mMGL2 overlap with human MGL, mMGL2 is in fact more homologous to human MGL than mMGL1. In keeping with this, *in vitro* binding studies demonstrate that human pd-VWF binds mMGL2 with significantly higher affinity than mMGL1 *in vitro* (Figure 5.12 A). Moreover, upon inhibition of mMGL2 in *MGL1*^{-/-} mice, plasma VWF:Ag is increased (Figure 5.12 B) and the clearance of endogenous murine VWF is further attenuated (Figure 5.13 A). Furthermore, upon combined inhibition of both mMGL1 and mMGL2, murine plasma VWF:Ag is increased 2.8-fold and murine VWF MRT is increased 2.4-fold when compared to wild type (Figure 5.13 B). The increase in VWF half-life and VWF:Ag levels observed upon complete mMGL1/2 inhibition presented here are markedly greater than that observed for ASGPR. As such, these data indicate that MGL may have a larger contribution to the clearance of VWF compared to ASGPR.

6. Further studies into the mechanisms through which sialylation may influence VWF biology

Through the previous studies described in this thesis, we have identified pathways through which VWF sialylation influenced its biology and in particular, *in vivo* clearance. Collectively, these data clearly demonstrate that sialylation protects VWF against clearance through both the ASGPR and MGL receptors. With respect to MGL clearance, the α 2-3 linked sialylation on the O-glycans of VWF appear to have a specific important role. In addition to these novel data, previous studies from our laboratory and others have shown that terminal sialylation also influenced other aspects of VWF function, including interaction with platelets and susceptibility to proteolysis by ASAMTS13. In this final Chapter, additional studies into VWF sialylation are presented in three distinct sections:

- (i) A role for the macrophage mannose receptor in the clearance of VWF
- (ii) The effect of endogenous neuraminidase activity on plasma VWF half-life
- (iii) The sialylation of commercial recombinant VWF (Vonvendi[®], Takeda)

6.1. Macrophage mannose receptor; a putative VWF clearance receptor

Macrophages and LSECs have been shown to be important modulators of VWF clearance. Interestingly, both LSECs and macrophages express another C-type lectin receptor, the macrophage mannose receptor (MMR) (Roseman & Baenziger, 2003). MMR consists of eight extracellular CRDs on a single polypeptide backbone (Taylor *et al*, 1990) (Figure 6.1). These MMR CRDs have affinity for glycoproteins terminating in N-acetylglucosamine (GlcNAc), fucose (Fuc) and mannose (Man) residues (Taylor & Drickamer, 1993; Taylor *et al*, 1992), all of which are exposed following desialylation. Complex VWF N-linked glycan structures are rich in GlcNAc and Man residues. In addition, human VWF is abundantly fucosylated (Canis *et al*, 2012). Moreover, truncation of VWF N-linked glycans and exposure of these sub-terminal moieties has been shown to markedly enhance VWF clearance (O'Sullivan *et al*, 2016a). Consequently, in search of lectin receptors that might influence VWF clearance through glycan dependant mechanisms, we examined whether MMR may play a role.

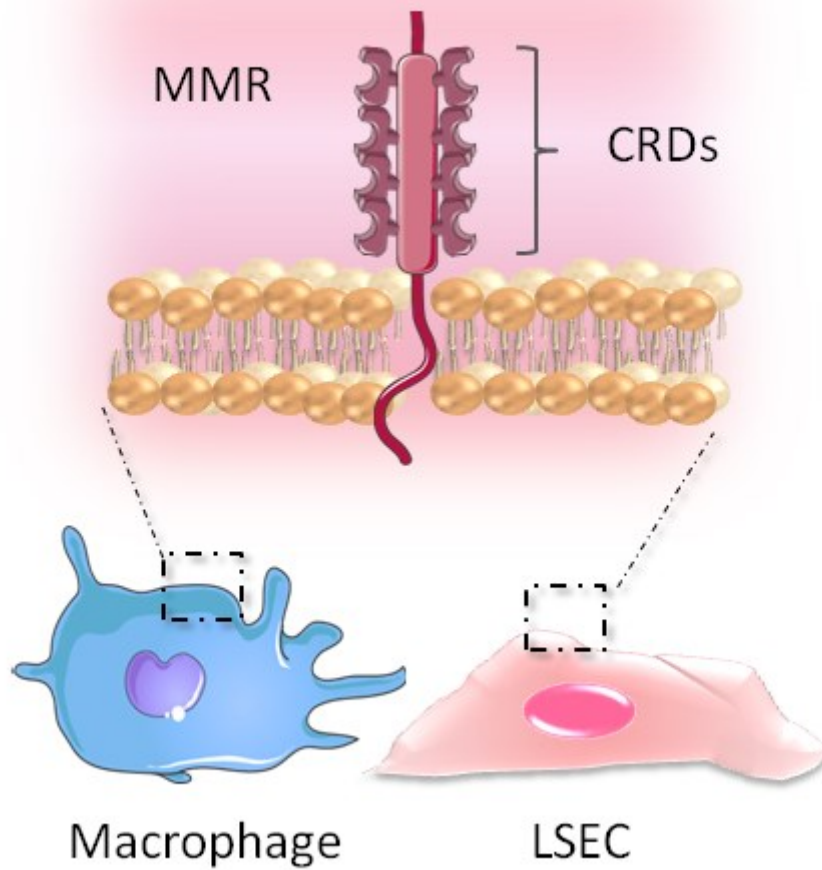


Figure 6.1: Macrophage mannose receptor

MMR is expressed on both macrophages and LSECs has eight CRDs with affinity for GlcNAc, Fuc and Man residues

6.1.1. Mice deficient in *MMR* have increased plasma VWF:Ag levels.

Having identified MMR as a putative VWF clearance receptor we first sought to investigate endogenous plasma VWF:Ag levels in *MMR* deficient mice. *MMR*^{-/-} mice on a C57/Bl6J background were obtained and plasma VWF:Ag was assessed compared to age and sex matched wild type controls. Interestingly, we observed that deficiency in *MMR* was associated with a significant increase in murine VWF:Ag levels (2.1 ± 0.27 U/mL versus 1.01 ± 0.17 U/mL respectively, $p < 0.01$) (Figure 6.2).

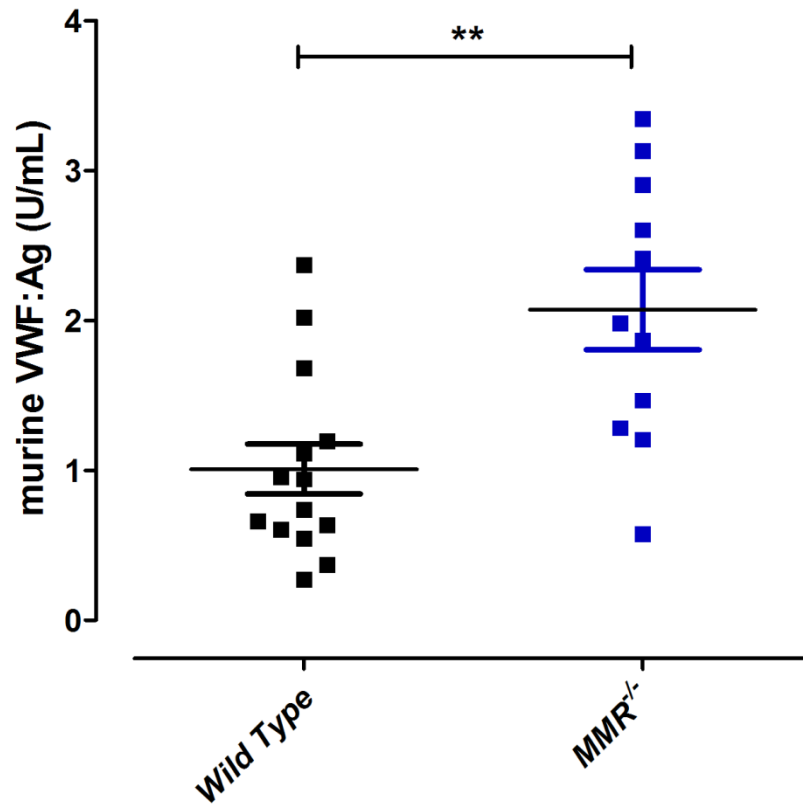


Figure 6.2: Plasma VWF:Ag levels are significantly increased in *MMR*^{-/-} mice

MMR^{-/-} mice (n=11) exhibit significantly increased endogenous VWF compared to wild type controls (n=14) (**; p<0.01.)

6.1.2. Clearance of VWF is not altered in *MMR*^{-/-} mice

To investigate the mechanism responsible for the elevated plasma VWF:Ag levels in *MMR* deficient mice, we first examined whether MMR may play a role in regulating VWF clearance *in vivo*.

To test this hypothesis, the half-life of endogenous murine VWF (mVWF) was measured in *MMR*^{-/-} compared to wild type controls. Surprisingly, despite the significant increase in VWF levels, no alteration in endogenous VWF clearance was observed in *MMR*^{-/-} mice (Figure 6.3).

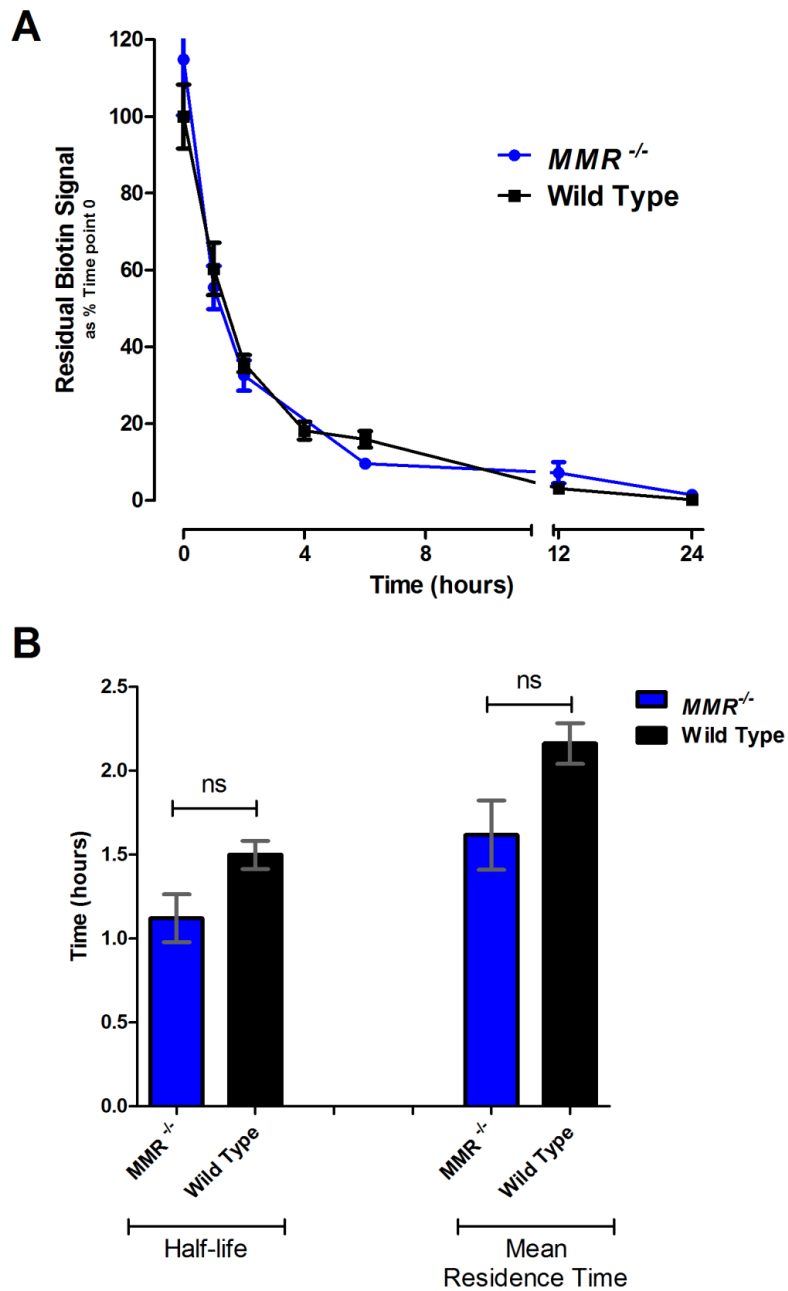


Figure 6.3: Endogenous VWF clearance in $MMR^{-/-}$ mice

(A) Endogenous mVWF was biotinylated with injection of NHS-Biotin and VWF clearance was measured by the fall off in biotin signal over time in a modified VWF:Ag ELISA. (B) mVWF half-life and mean residence time were not significantly prolonged in MMR deficiency (ns= not significant, $p > 0.05$).

6.1.3. The extracellular domain of MMR does not bind pd-VWF *in vitro*

To examine whether pd-VWF can bind MMR, an *in vitro* solid phase binding assay was developed. The extracellular domain of MMR (including all eight CRDs) was assessed for VWF binding. Mannan (a known MMR ligand) was used as a positive control.

In keeping with the *in vivo* clearance data, no binding of pd-VWF to the extracellular domain of MMR was observed in either the presence or absence of ristocetin (Figure 6.5). The positive control confirms that the extracellular domain of MMR retained its ability to bind its preferred ligand (Figure 6.5).

Collectively these data indicate that *MMR*^{-/-} mice exhibit significantly increased VWF:Ag levels (Figure 6.2). However physiological clearance of murine VWF is unchanged in *MMR*^{-/-} mice (Figure 6.3). In addition, pd-VWF does not bind MMR *in vitro* (Figure 6.4). Further studies will be required to define the mechanism underpinning the increase in mVWF:Ag levels in *MMR*^{-/-} mice.

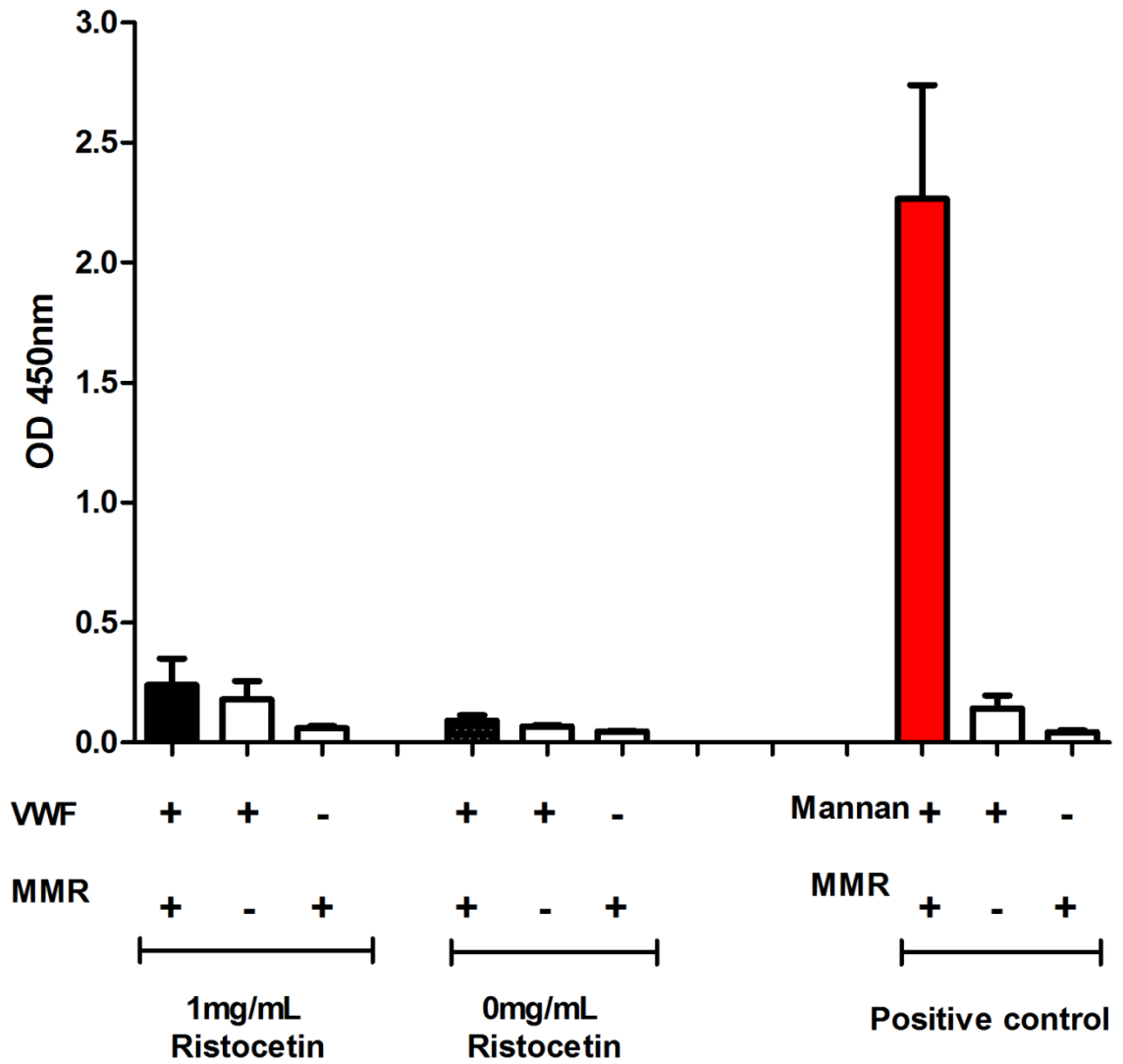


Figure 6.4: pdVWF does not bind to MMR *in vitro*.

Solid phase binding assay demonstrates that MMR extracellular domain does not bind to VWF *in vitro* in the presence of absence of ristocetin. Mannan as positive control exhibited high affinity binding to MMR.

6.2. *In vivo* neuraminidase activity and VWF clearance

Emerging evidence has demonstrated that VWF glycosylation is dynamic in nature and changes with protein ageing. This is evidenced by lectin studies performed on samples collected pre- and post DDAVP stimulated VWF release. Studies have shown that α 2-6 sialylation and blood group antigen expression on VWF is significantly enhanced post-DDAVP, demonstrating that VWF stored within WP bodies and steady state circulating VWF have different glycosylation status (Brown *et al*, 2002; Aguila *et al*, 2019).

In keeping with this, a recent study by Yang *et al* demonstrated that glycoprotein ageing and turnover is linked to the activity of endogenous plasma neuraminidases (Yang *et al*, 2015). Plasma glycoproteins were demonstrated to undergo trimming of glycan chains *in vivo* (catalysed by plasma neuraminidases), commencing with the removal of sialic acid residues (Figure 6.5). This mechanism of plasma glycoprotein ageing is not only associated with loss of sialic acid, but also progressive stepwise loss of sugar residues from N-glycan chains. Critically, inhibition of endogenous neuraminidase activity arrested this process, resulting in an increase in half-life of a number of glycoproteins (Yang *et al*, 2015).

Oseltamivir phosphate (OP) is a neuraminidase inhibitor used in the treatment of influenza. Interestingly case reports have described that OP administration was associated with a decrease of platelet glycoprotein de-sialylation, and a subsequent increase in platelet half-life in patients with idiopathic thrombocytopenia (Alioglu *et al*, 2010; Shao *et al*, 2014). In light of these observations, we sought to identify whether

endogenous neuraminidases contribute to VWF clearance and whether murine VWF half-life can be prolonged through treatment with OP.

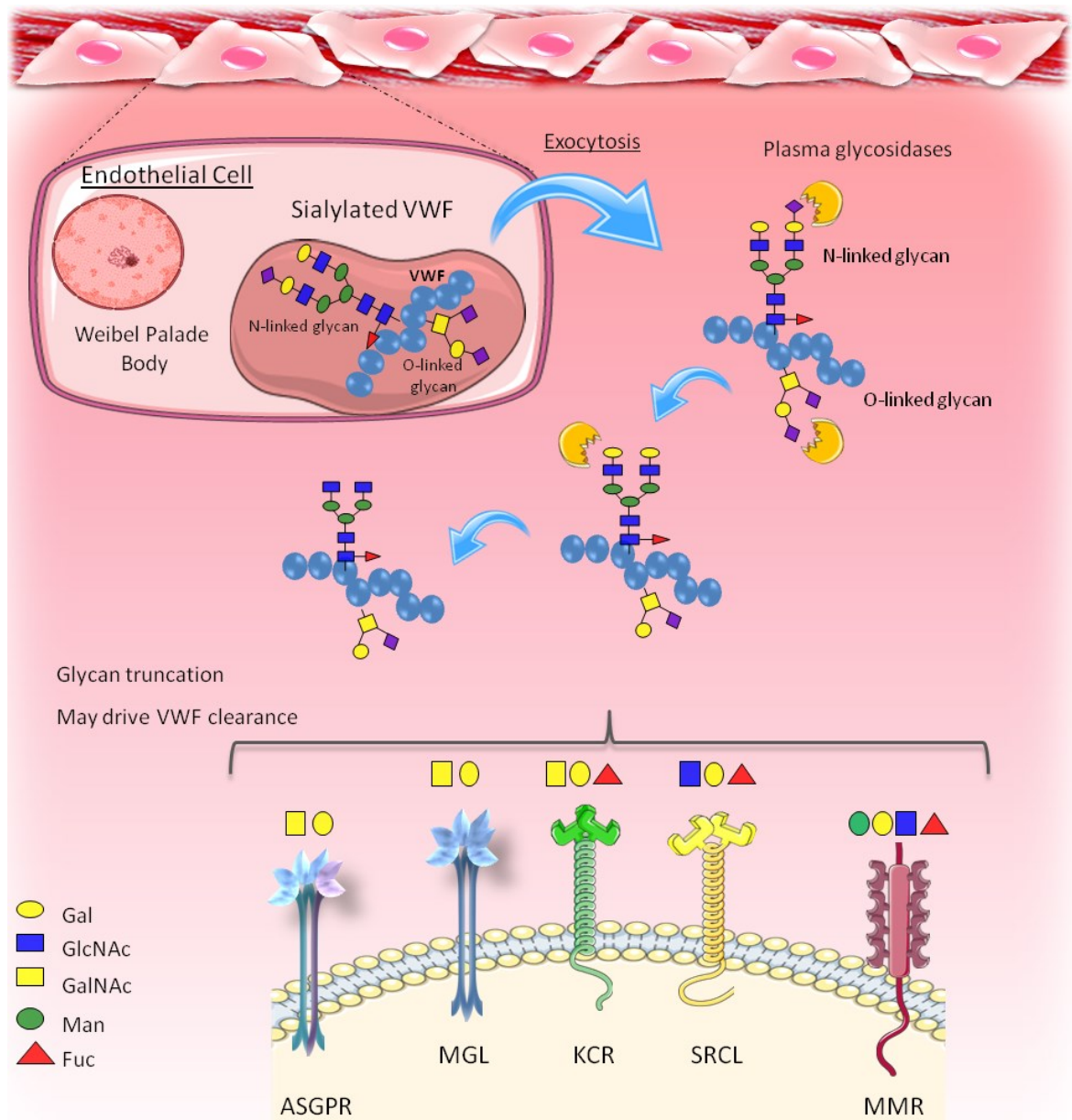


Figure 6.5: *In vivo* glycan truncation drives glycoprotein clearance.

Plasma neuraminidase induced N glycan truncation may represent a novel mechanism regulating the physiological clearance of VWF through a number of lectin receptors

6.2.1. Inhibition of endogenous neuraminidase activity results in changes in VWF sialylation

To investigate whether endogenous plasma neuraminidases modulate circulating VWF sialylation *in vivo*, wild type C57/Bl6J mice were treated with OP or PBS. After 24 hours, plasma samples were collected, and lectin binding analyses were performed. Interestingly, mice treated with OP exhibited significantly higher SNA binding (α 2-6 linked sialic acid) compared to PBS treated controls ($p < 0.05$) (Figure 6.6 A). In addition, OP treated mice also exhibited significantly increased MAA II binding (α 2-3 linked sialic acid) compared to controls ($p < 0.05$) (Figure 6.6 B). These data indicate that inhibition of endogenous plasma neuraminidases is associated with progressive loss of both of α 2-6 and α 2-3 linked sialic acid moieties from mVWF glycans. In keeping with this hypothesis, mice treated with OP also exhibited significantly reduced binding to RCA I (β 1-4 linked Gal) ($p < 0.05$) (Figure 6.6 C), demonstrating a reduced exposure of sub terminal Gal.

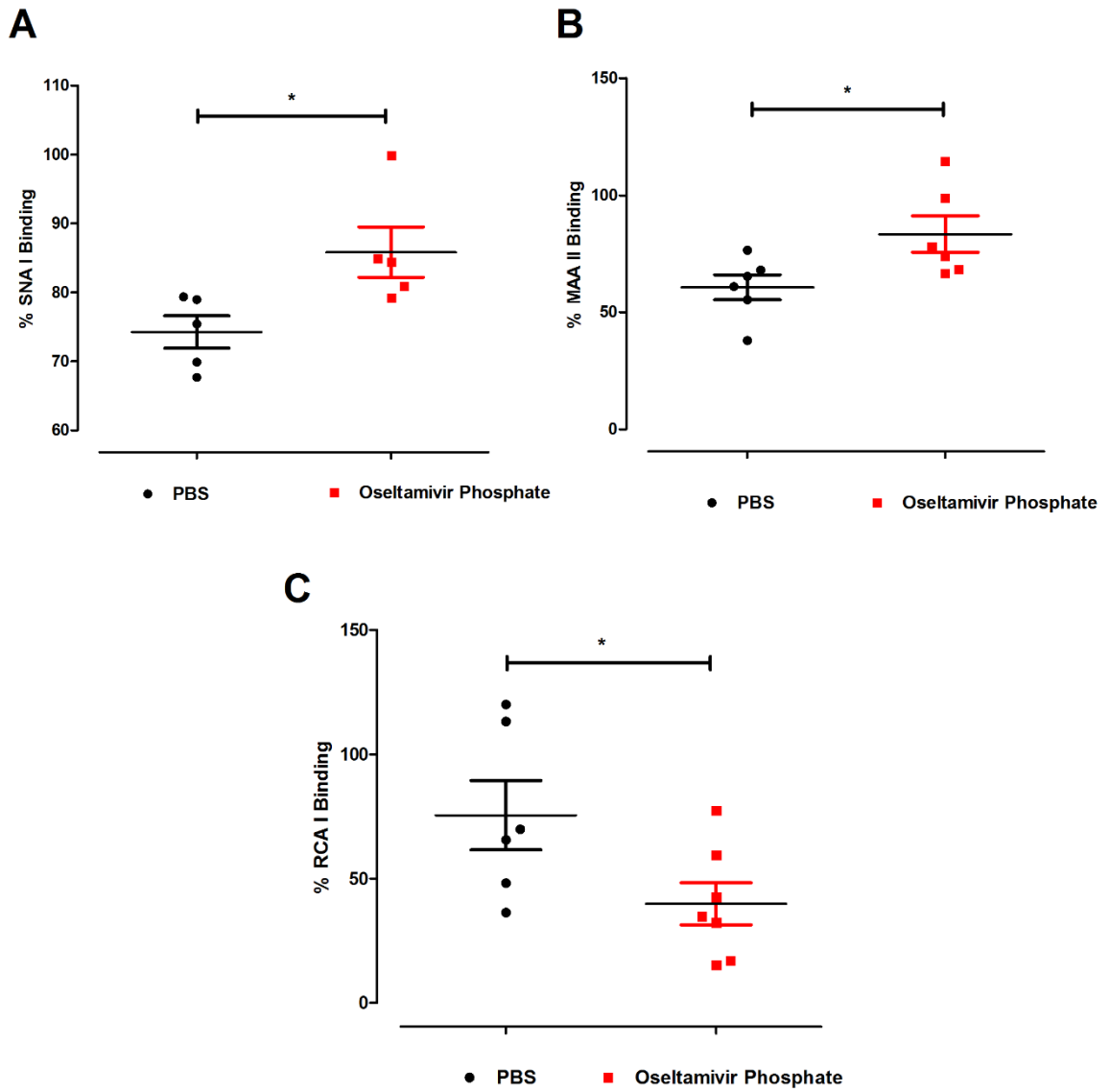


Figure 6.6: Murine VWF glycan profile following treatment with neuraminidase inhibitor

24 hours treatment with OP results in an increase in (A) SNA I and (B) MAA II, and a decrease in (C) RCA I plant lectin binding (*; $p < 0.05$).

6.2.2. Murine plasma VWF:Ag is significantly increased upon OP treatment

As demonstrated in Figure 6.6 VWF sialylation is lost in circulation through the action of endogenous neuraminidases. To further investigate the impact of this desialylation process we assessed plasma VWF:Ag levels in OP treated compared to PBS treated controls.

Interestingly, we observed that treatment with OP resulted in a small but significant increase in endogenous murine VWF:Ag levels compared to PBS treated controls (2.1 ± 0.4 U/mL versus 0.94 ± 0.2 U/mL respectively, $p < 0.05$) (Figure 6.7).

These data indicate that VWF glycan remodelling plays a role in regulating plasma VWF:Ag levels, likely through exposure of sub-terminal galactose and subsequent clearance through lectin receptors such as ASGPR and MGL.

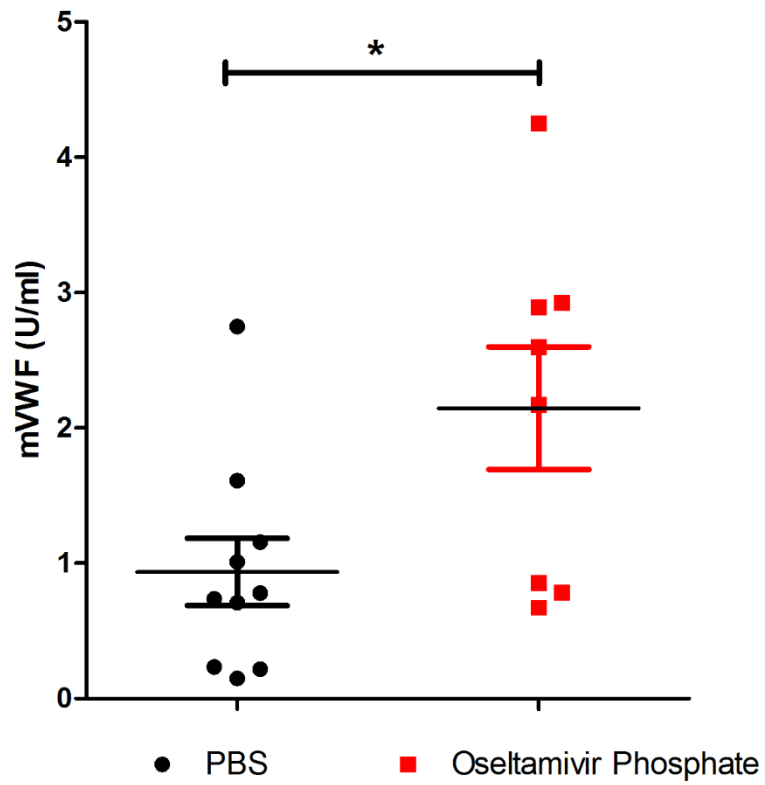


Figure 6.7: Murine plasma VWF:Ag levels following *in vivo* neuraminidase inhibitor treatment

Treatment with OP results in a significant 1.2-fold increase in plasma VWF:Ag levels compared to PBS treated controls (*; $p < 0.05$).

6.2.3. Plasma neuraminidases modulate pd-VWF glycosylation

Having demonstrated that plasma neuraminidases can modulate murine VWF sialylation and plasma VWF:Ag levels in wild type mice (Figures 6.6 and 6.7), we next sought to investigate the impact of these neuraminidases on injected human pd-VWF.

Human pd-VWF was infused in *VWF*^{-/-} mice and VWF sialylation status assessed using lectin binding assays 2 hours post infusion. For these studies, pd-VWF diluted in VWF deficient plasma was used as control.

We observed a significant reduction in SNA binding to pd-VWF 120 minutes post infusion, indicative of a loss of α 2-6 linked sialic acid from pd-VWF in murine circulation (Figure 6.8 A). Interestingly, we observed no change in MAA II binding, which suggests that endogenous neuraminidases may have a preference for α 2-6 over α 2-3 sialic acid linkages on human pd-VWF (Figure 6.8 B). Finally, in accordance with the decrease in α 2-6 linked sialic acid, we observed an increase in RCA binding consistent with increased Gal exposure (Figure 6.8 C).

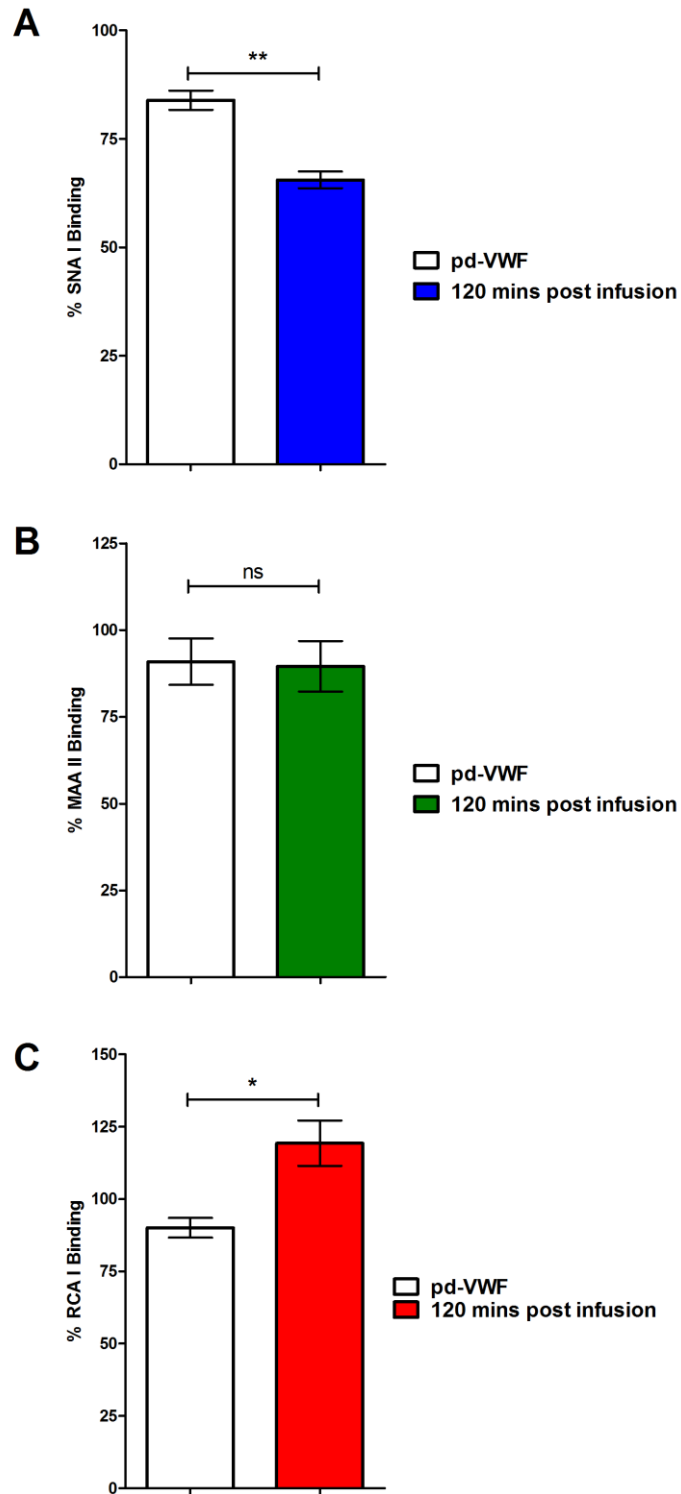


Figure 6.8: In vivo neuraminidase alters the sialylation of human pd-VWF in *VWF*^{-/-} mice.

(A) SNA (B) MAA II and (C) RCA lectin binding demonstrated that pd-VWF sialylation is lost *in vivo*.

6.2.4. Pre-treatment with Oseltamivir Phosphate extends pd-VWF half-life

As demonstrated in Figure 6.8, once injected into *VWF*^{-/-} mice, α 2-6 linked sialic is partially lost from human pd-VWF. To investigate whether this process contributes to the clearance of pd-VWF, clearance studies were repeated in the presence OP or PBS control. OP is a pan-neuraminidase inhibitor which suppresses the activity of multiple plasma neuraminidases (including Neu1 and Neu3) *in vivo*.

Interestingly, we observed that inhibition of endogenous murine neuraminidase activity with OP resulted in a significant increase in pd-VWF mean residence time in *VWF*^{-/-} mice (MRT: 58.7 ± 2.8 mins versus 40.2 ± 1.3 mins respectively) (Figure 6.9). These data indicate that endogenous neuraminidases contribute to the clearance of pd-VWF *in vivo*.

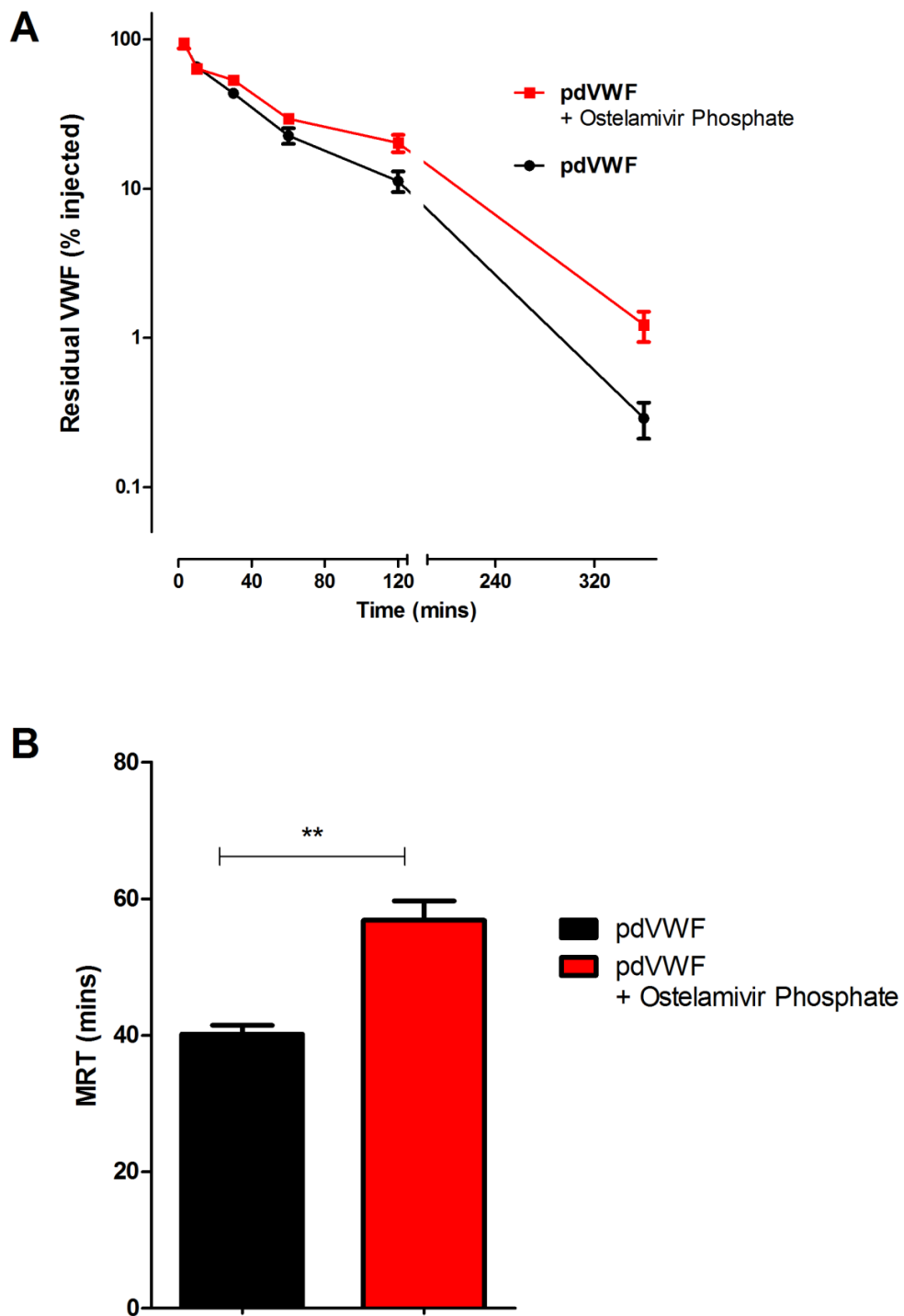


Figure 6.9: Neuraminidase inhibition extends pd-VWF clearance in *VWF*^{-/-} mice.

(A) Clearance of pd-VWF is significantly different upon treatment with OP. (B) MRT is significantly greater in OP treated mice (**; $p < 0.01$).

6.3. Sialylation of commercial recombinant VWF (Vondendi[®], Takeda)

A new commercial recombinant VWF product has recently been licensed for the treatment of patients with VWD. This product is expressed in Chinese hamster ovary (CHO) cells and thus exhibits a different glycosylation profile compared to pd-VWF. In particular, ABO(H) blood group determinants are not expressed on Vonvendi. Interestingly, early studies reported that this recombinant VWF product had an increased level of sialylation compared to pd-VWF concentrates (Turecek *et al*, 2009).

Given these differences in glycosylation, it is interesting to note that data from phase 2 and phase 3 studies have suggested that the half-life of infused rVWF (19.6 hours) is markedly longer than that of pd-VWF (range 12.8-15.8 hours) (Mannucci *et al*, 2013; Gill *et al*, 2015). As such, we sought to analyse the glycosylation profile of Vonvendi VWF and to further investigate the role of recombinant VWF sialylation on its circulatory clearance.

6.3.1. Vonvendi lectin panel

The sialylation profile of rVWF (Vonvendi®) was studied in comparison to (i) pd-VWF concentrate (Fandhi®), and (ii) HEK293T produced recombinant VWF (HEK-VWF) using a number of plant lectin binding assays. Unsurprisingly as it is derived from plasma, pd-VWF derived from Fandhi® concentrate exhibited a lectin binding profile similar to that of pooled reference plasma (SNA I; 97.3%, MAA II; 89.7%, WGA; 79%, RCA I; 97.4% compared to reference plasma) (Figure 6.10).

While early reports indicated that Vonvendi exhibits increased sialylation, we observed a significant decrease in α 2-6 linked sialic acid, evidenced by a reduction in SNA I binding for Vonvendi compared to Fandhi ($24.2 \pm 1.5\%$ versus $97.33 \pm 9.9\%$) (Figure 6.10). In contrast, we observed an equal amount of α 2-3 linked sialic expression on Fandhi and Vonvendi (MAA II binding; $89.7 \pm 4.6\%$ versus $96.23 \pm 11\%$) (Figure 6.10). Interestingly, we observed increased WGA binding for Vonvendi compared to Fandhi ($112.8\% \pm 7.4\%$ versus $76.2 \pm 7.9\%$) (Figure 6.10). These data are of particular interest as WGA has previously been shown to bind sialic acid in an α 2-8 linkage, perhaps indicating that Vonvendi VWF has a higher proportion of α 2-8 linked disialosyl motifs. HEK-VWF exhibited significantly reduced terminal sialylation (SNA I; 53.3%, MAA II; 17.7%, WGA; 35.1%, compared to reference plasma). Interestingly, despite a reduction in terminal sialylation, we observed no significant increase in sub-terminal Gal exposure as evidenced by RCA I binding (96.4% compared to reference plasma). These data may indicate that in addition to a reduction in terminal sialylation, HEK-VWF may also exhibit reduced sub-terminal Gal expression.

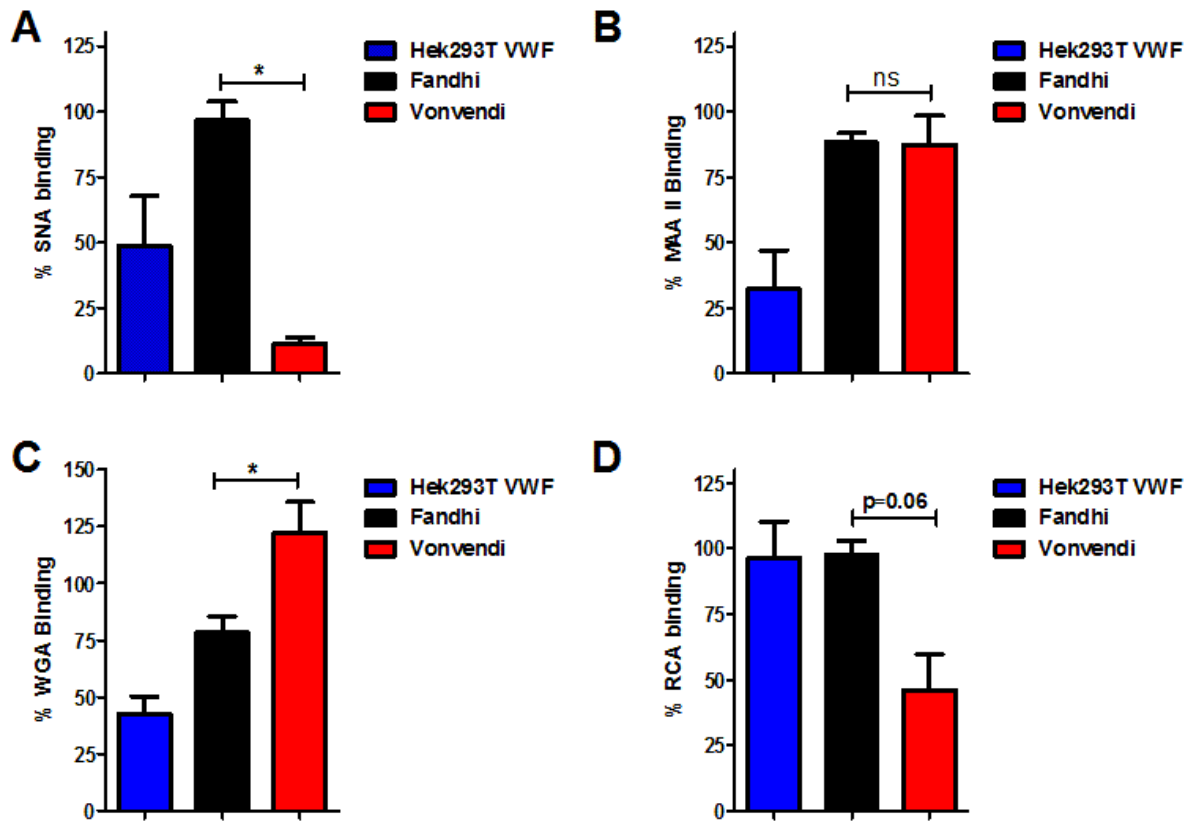


Figure 6.10: Vonvendi lectin binding profile

Binding of HEK293T produced VWF, Fandhi and Vonvendi to (A) SNA I, (B) MAA II, (C) WGA and (D) RCA I in comparison to reference plasma was assessed.

6.3.2. Vonvendi half-life is not significantly prolonged in *VWF*^{-/-} mice compared to pd-VWF

Given the critical role terminal sialylation plays in modulating VWF clearance, we sought to assess the circulatory half-life of each VWF concentrate in *VWF*^{-/-} mice.

Human studies have suggested that the half-life of rVWF (Vonvendi®) exhibits a markedly longer half-life *in vivo* compared to pd-VWF concentrates. Interestingly, we observed that the clearance of Vonvendi® in *VWF* deficient mice was not significantly prolonged compared to that of pd-VWF (Fandhi®) (Figure 6.11 A) (Half-life; 39.6 ± 2.6 versus 39.8 ± 6.2 mins respectively) (Figure 6.11 B).

Finally, given the low level of terminal sialylation detected on VWF produced in HEK293T cells, it is unsurprising that HEK-VWF exhibited relatively rapid clearance *in vivo* (Half-life; 14.3 ± 2.6 mins) (Figure 6.11).

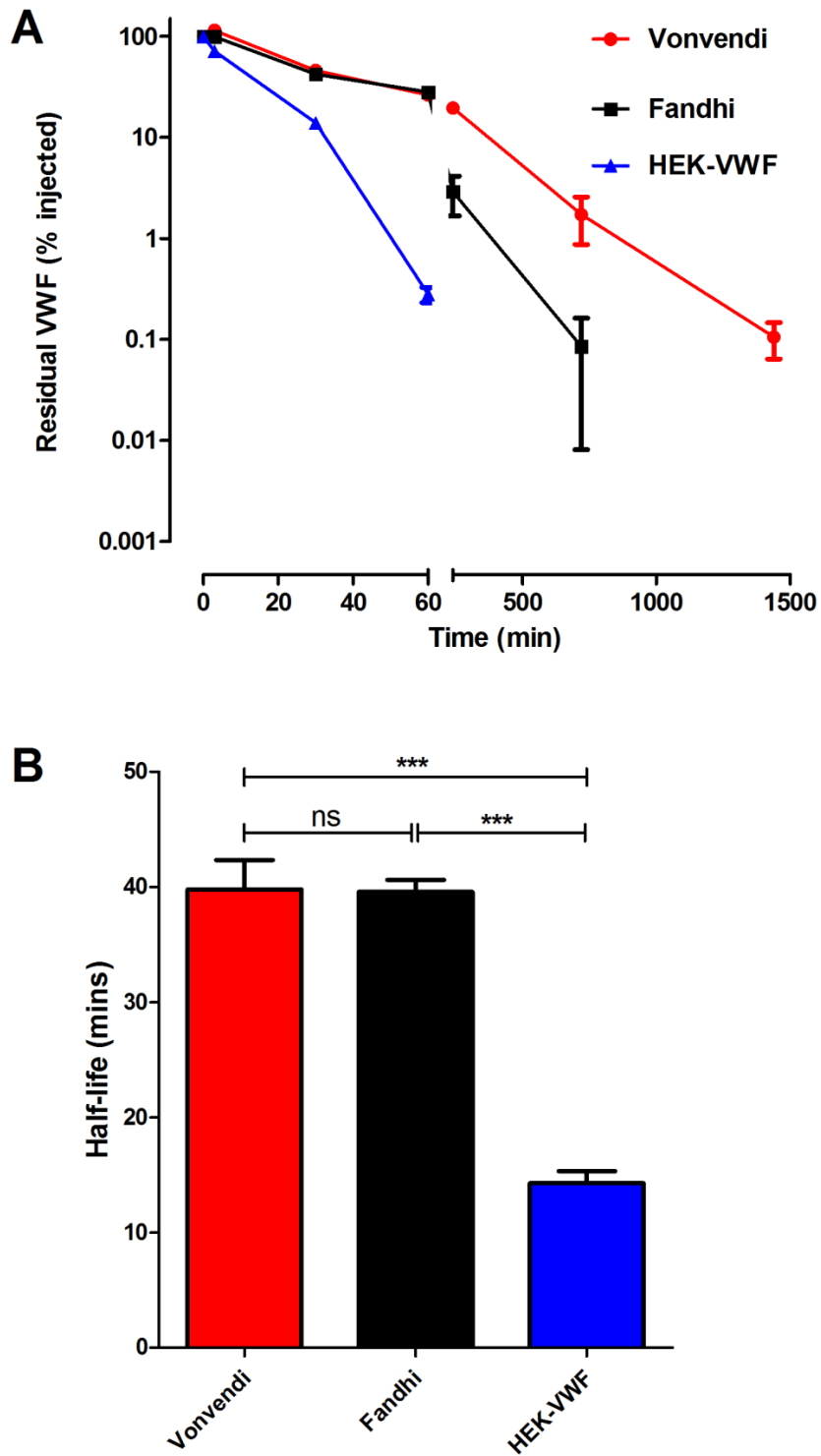


Figure 6.11: Clearance of Vonvendi, Fandhi and HEK-VWF in $VWF^{-/-}$ mice.

(A) Vonvendi and Fandhi exhibited a similar clearance pattern in $VWF^{-/-}$ mice. (B) HEK-VWF is cleared rapidly by comparison (***, $p < 0.001$).

6.4. Discussion

6.4.1. *MMR* deficiency results in increased plasma VWF:Ag levels

As part of this body of work, we investigated MMR as another putative VWF clearance receptor due to its cellular distribution and affinity for GlcNAc, Man and Fuc moieties. In support of our hypothesis, we observed that mice deficient in *MMR* exhibit significantly increased plasma murine VWF:Ag levels (Figure 6.2). Interestingly however, as demonstrated in Figure 6.3, the physiological clearance of endogenous murine VWF is not significantly different in *MMR*^{-/-} mice compared to wild type controls. Moreover, we observed that pd-VWF does not bind to immobilised MMR in a solid phase binding assay (Figure 6.4).

Consequently, the biological mechanisms responsible for the increased VWF:Ag levels observed in *MMR*^{-/-} mice remains undefined. Further studies will be required to investigate these mechanisms. Of note, a recent study by Kang *et al* demonstrated that disruption of the lysosomal enzyme α -galactosidase A results in a significant increase in VWF secretion in Fabry's disease (Kang *et al*, 2019). This study demonstrates that changes in VWF glycosylation can also impact upon VWF synthesis and secretion within ECs. This phenomenon which has long been demonstrated to influence secretion of Factor VIII (Zhang *et al*, 2005).

6.4.2. Endogenous neuraminidases regulate VWF clearance *in vivo*.

A recent study by Yang *et al* established an intrinsic method of glycoprotein turnover through the action of endogenous neuraminidases. We sought to investigate whether this mechanism of protein aging may play a role in modulating the physiological clearance of VWF. Interestingly we demonstrated that inhibition of endogenous neuraminidase activity through OP treatment resulted in a significant increase in VWF sialylation, and a concordant decrease in exposure of sub-terminal galactose (Figure 6.6). These data are consistent with the concept that sialylation is progressively lost in circulation through the action of endogenous neuraminidases. In addition, we observed that treatment with OP resulted a significant increase in plasma VWF:Ag levels (Figure 6.7). Moreover, we showed that upon infusion into *VWF*^{-/-} mice, pd-VWF undergoes desialylation and exposure of sub-terminal galactose (Figure 6.8). Furthermore, we demonstrated that clearance of pd-VWF was significantly prolonged following OP treatment (Figure 6.9).

Together these data provide novel insights into the mechanism underpinning the physiological clearance of VWF. In addition, these data may facilitate the development of novel approaches for the treatment of VWD. Desialylation of VWF and platelets have been shown to contribute to the coagulopathy observed in sepsis. As such further studies are required to investigate a putative therapeutic benefit of neuraminidase inhibition in this clinical setting.

6.4.3. Vonvendi exhibits different sialylation compared to pd-VWF concentrates.

Recent studies have investigated the glycosylation and sialylation of VWF in a number of different therapeutic concentrates (Riddell *et al*, 2019). In keeping with their observations, we demonstrated that pd-VWF concentrate (Fandhi) exhibits a similar lectin binding profile to that of reference plasma (Figure 6.10). Interestingly, we observed that HEK-VWF has a significantly reduced level of terminal sialylation, likely contributing to its enhanced circulatory clearance (Figure 6.11). Vonvendi is expressed in Chinese hamster ovary (CHO) cells and consequently expresses differences in glycosylation and sialylation compared to pd-VWF. In particular, it has been demonstrated that sialylation levels are increased (Turecek *et al*, 2009). Interestingly however, while we observed no change in α 2-3 linked sialylation, we observed a significant reduction in α 2-6 linked sialic acid on Vonvendi compared to Fandhi (Figure 6.10). In contrast, we observed a significant increase in WGA binding, indicating a potential increase in α 2-8 linked disialosyl motifs.

Given these differences in glycosylation, it is interesting that data from the phase 1 and phase 3 studies suggest that the half-life of infused rVWF (19.6 hours) is considerably longer than that of pd-VWF (range 12.8-15.8 hours) (Mannucci *et al*, 2013; Gill *et al*, 2015). In *VWF*^{-/-} mice we observed only a partial extension of VWF half-life for Vonvendi compared to Fandhi. Additional studies will be required to confirm these observations and to further investigate the mechanism underpinning the prolonged half-life Vonvendi exhibits in man.

7. Overall conclusions

VWD is the commonest inherited bleeding disorder in man, affecting up to 1% of the general population. Critically, enhanced circulatory clearance has been implicated in the pathophysiology of VWD. Nevertheless, the molecular mechanisms underpinning VWF clearance remain to be fully elucidated. The work presented in this thesis highlights the essential role of VWF glycosylation in modulating VWF clearance.

7.1. Additional non-ASGPR galactose sensitive receptors modulate the clearance of hyposialylated VWF

Previous studies have demonstrated that terminal sialic acid and galactose residues mediate the rate of VWF clearance through ASGPR. Our data support the previous conclusion of Grewal *et al* that ASGPR can mediate VWF clearance, as both pd-VWF and hyposialylated glycoforms thereof bind ASGPR1 *in vitro*, and *Asgr1*^{-/-} mice exhibit increased plasma VWF:Ag levels. Our findings affirm that desialylation of VWF results in enhanced circulatory clearance of VWF. Moreover, despite accounting for only 20% of total sialic acid on VWF, these data highlight a critical role for O-linked terminal sialylation in modulating VWF clearance.

Nevertheless, it is important to note that while pre-infusion of ASOR significantly attenuated the enhanced clearance of hyposialylated VWF in *VWF*^{-/-} mice, the enhanced clearance of hyposialylated VWF persists in a dual *VWF*^{-/-}/*Asgr1*^{-/-} mouse model. Moreover, pre-infusion with ASOR in *VWF*^{-/-}/*Asgr1*^{-/-} dual knockout mouse still results in attenuation of the enhanced clearance of hyposialylated VWF. Collectively, these findings demonstrate that novel ASGPR-independent pathways contribute to the

enhanced clearance of hyposialylated VWF. Given that quantitative variations in N- and O-linked sialylation have been described in specific patient cohorts, these findings are of direct clinical importance.

7.2. A novel role for the Macrophage Galactose Lectin in modulating VWF clearance

In vivo clearance experiments in dual $VWF^{-/-}/Asgr1^{-/-}$ mice demonstrated enhanced clearance of hyposialylated VWF, even in the absence of ASGPR. Moreover, our findings demonstrate that hyposialylated VWF co-localises with hepatic macrophages in mice sufficient and deficient in ASGPR. Furthermore, macrophage depletion attenuated the enhanced clearance of hyposialylated VWF *in vivo*. These results confirm that macrophages play a critical role in modulating the enhanced clearance of hyposialylated VWF.

MGL is a C-type lectin that binds to glycoproteins expressing terminal Gal/GalNAc residues. Interestingly, dose-dependent binding of human VWF to purified recombinant human MGL was confirmed using surface plasmon resonance. Moreover, pd-VWF was shown to co-localise with MGL on the surface of macrophages. In addition, plasma VWF:Ag levels were significantly elevated in $MGL1^{-/-}$ mice compared to controls. Importantly, the markedly enhanced clearance of hyposialylated VWF in $VWF^{-/-}/Asgr1^{-/-}$ mice was significantly attenuated in the presence of an anti-MGL inhibitory antibody. Moreover, clearance of endogenous murine VWF and infused human pd-VWF was demonstrated to be mediated by MGL. Collectively, these findings identify MGL as a

novel macrophage receptor for VWF that significantly contributes to the clearance of both wild-type and hyposialylated VWF (Ward *et al*, 2018).

7.3. O-linked glycans modulate MGL mediated clearance of VWF.

Having established MGL as a novel clearance receptor for VWF, we sought to further define the molecular mechanisms underpinning the interaction between VWF and MGL. We observed that α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF demonstrated enhanced binding to MGL compared to pdVWF. Interestingly, additional removal of α 2-6 linked sialic acid from the N glycans did not further promote binding to MGL. Furthermore, we demonstrated that the enhanced clearance of α 2-3 Neu-VWF is mediated by MGL alone. Conversely, we observed that both MGL and ASGPR contribute to the clearance of α 2-3,6,8,9 Neu-VWF.

Although PNGase removal of the N glycans from VWF did not affect MGL binding, treatment with O-glycosidase significantly attenuated binding. These data demonstrate that O-linked sialylation plays a critical role in protecting VWF from MGL mediated clearance. The mature VWF monomer contains 10 O-linked glycans, eight of which flank the A1 domain in two clusters of four glycans. Cluster variant studies demonstrated that loss of both O-linked glycan clusters attenuated MGL binding significantly. O linked glycan structures are known to play important roles in maintaining glycoprotein conformation. To further define the mechanism through which these O glycan structures influence MGL mediated clearance, binding of a truncated A1A2A3 fragment and isolated recombinant A1, A2 and A3 domains were examined. Interestingly, MGL-binding to full length rVWF and A1A2A3 were both significantly increased in the

presence of ristocetin. Although no binding of isolated A2 or A3 domains to MGL was observed, A1 domain binding to MGL was seen both in the presence and absence of ristocetin.

In conclusion, these findings define a novel role for O linked sialylation in protecting VWF from MGL mediated clearance. Our data further demonstrate that the cluster of O linked glycans around the A1 domain play a specific role in regulating VWF-MGL interaction, and that conformation of A1A2A3 may be important in determining accessibility of these glycans for the clearance receptor. Further studies will be required to determine whether abnormalities in these O-glycan determinants may be important in the pathophysiology of VWD, particularly in patients with type 1C VWD who lack *VWF* gene coding mutations.

7.4. Macrophage Mannose Receptor deficiency modulates VWF:Ag levels through as of yet unknown mechanisms

MMR is a C-type lectin with affinity for glycoproteins containing GlcNAc, Man and Fuc moieties. Given its lectin binding profile and cellular distribution (LSEC and macrophage) we identified the MMR as a putative VWF clearance receptor. Critically, we observed that mice deficient in *MMR* exhibit significantly increased plasma VWF:Ag levels. However, we demonstrated that the clearance of endogenous murine VWF was unchanged in *MMR*^{-/-} mice compared to wild type controls. Furthermore, we demonstrated that pd-VWF does not bind MMR *in vitro*. More studies are required to elucidate the molecular mechanisms underpinning the increased plasma VWF:Ag levels

observed in *MMR*^{-/-} mice. In particular, a key focus on determining whether decreased clearance or increased synthesis/secretion of VWF is the key underlying factor.

7.5. Endogenous neuraminidases contribute to the physiological clearance of VWF

In keeping with observations by Yang et al, we demonstrated that neuraminidases present in murine plasma modulate VWF sialylation *in vivo*. Critically, upon inhibition of endogenous neuraminidase activity, we were able to halt this process resulting in both an increase in plasma VWF:Ag and a prolongation of VWF half-life.

Collectively, these findings demonstrate an intrinsic pathway of VWF aging and turnover, through which circulating VWF is subjected to the activity of endogenous neuraminidases. Thus as VWF ages in plasma, it becomes progressively more hyposialylated, and becomes a target for MGL and/or ASGPR mediated clearance. While more studies are required, these findings are interesting with respect to therapeutic interventions to increase plasma VWF:Ag levels through inhibition of endogenous neuraminidase activity. Furthermore, desialylation of VWF and subsequent enhanced clearance has been demonstrated to contribute to the pathophysiology of sepsis.

7.6. Differential sialylation of Vonvendi may account for its prolonged half-life *in vivo*.

The first recombinant VWF replacement therapy; (Vonvendi) has recently been approved for therapeutic use. In addition to the elimination of the adverse risk of plasma-derived products, it has been reported that Vonvendi exhibits a prolonged half-life compared to plasma-derived VWF concentrates. Given the critical role of VWF glycosylation in modulating *in vivo* clearance, it is important to note the recombinantly produced proteins exhibit differential glycosylation patterns compared to plasma-derived concentrates. Indeed, we observed a significant difference in the lectin binding profile of Vonvendi compared to Fandhi. Paradoxically, despite prolonged half-life, we observed a significant decrease in α 2-6 linked sialic acid on Vonvendi compared to Fandhi. Interestingly however, we observed a marked increase in WGA binding, which may reflect an increase in α 2-8 linked sialylation on Vonvendi compared to pd-VWF. While more studies are required to further define the sialylation pattern of Vonvendi VWF, these data indicate poly-sialylation of VWF may provide an exciting opportunity for VWF glyco-engineering.

8. Future directions

8.1. Investigation into the hierarchy of lectin receptors in modulating VWF clearance

- As illustrated in this thesis a number of lectin receptors have been demonstrated to regulate VWF clearance. However, the relative importance of these receptors and their proportionate contribution to VWF plasma VWF:Ag levels remains elusive.
- *In vitro* solid phase binding assays and SPR will be utilised to assess relative affinity of VWF and glycoforms thereof to a panel of lectin receptors. In addition, cell-based competition binding assays will be utilised to assess VWF lectin receptor binding *in vitro*. Moreover, wild type and clearance receptor knockout mice will be used for *ex vivo* cell culture of murine macrophages and LSECs to investigate the role of specific receptors and cell type in modulating VWF binding.
- Besides the *Asgr1*^{-/-}, *MGL1*^{-/-} and *MMR*^{-/-} animals used in this study additional lectin knockout murine models such as *MGL2*^{-/-} and *MGL1*^{-/-}/*MGL2*^{-/-} dual knockout mice will be developed to further assess the contribution of these lectin receptors in modulating VWF clearance.

8.2. Further studies into the role of endogenous neuraminidases in modulating VWF clearance in physiological and pathological setting

- The findings presented in this thesis have described a physiological mechanism of VWF clearance through the action of endogenous neuraminidase activity. Neuraminidase inhibitor studies will be performed in a number of lectin receptor knockout mice to further understand the molecular mechanisms underpinning this clearance of VWF.
- Up to 40% of patients with VWD have no causative mutation in the *VWF* gene suggesting that other factors, including glycosylation, may play critical roles in modulating plasma VWF levels. As such, increased activity of plasma neuraminidases, and/or VWF susceptibility to the action of plasma neuraminidases will be investigated in a VWD patient cohort.
- In addition, the feasibility of utilising neuraminidase inhibition to increase VWF:Ag levels in patients with mild VWF deficiency will be further investigated.
- Finally, in addition to VWF, platelet counts, and intrinsic/extrinsic coagulation factor levels will be measured pre/post OP treatment to assess whether endogenous neuraminidase activity can regulate the clearance of platelets or other coagulation proteins.

8.3. Elucidating whether MGL can modulate FVIII clearance and immunogenicity in a VWF dependant or independent manner

- FVIII clearance has long been demonstrated to be VWF dependant, as such MGL mediated VWF clearance likely influences FVIII levels. Interestingly, 25% of FVIII is cleared from circulation in a VWF independent manner. Given that FVIII and VWF have a relatively similar glycosylation profile, MGL represents a putative candidate receptor for regulating FVIII clearance in both a VWF dependant and independent manner.
- Initially, *in vitro* solid phase and cell based binding assays will assess the whether FVIII can bind to MGL. In addition, a number of FVIII-glycoforms will be assessed for MGL binding to further understand the molecular mechanisms underpinning FVIII-MGL interaction.
- FVIII activity will be measured in *MGL1*^{-/-}, *MGL2*^{-/-} and *MGL1*^{-/-}/*MGL2*^{-/-} to assess the contribution of MGL to mediating FVIII clearance in a VWF dependant manner. Moreover, *VWF*^{-/-} will be crossbred with *MGL1*^{-/-}/*MGL2*^{-/-} to assess whether MGL can modulate FVIII levels in a VWF independent manner.
- Moreover, MGL has been demonstrated to modulate antigen presentation to CD4+ T cells. To investigate whether MGL can mediate FVIII neutralising antibody formation, wild type mice will be treated with recombinant human FVIII (4 weekly doses), subsequently the plasma will be assessed for the presence of anti-human FVIII antibodies 4 weeks after exposure. These studies can be repeated in *VWF*^{-/-}/*MGL1*^{-/-}/*MGL2*^{-/-} mice to assess whether this process is VWF dependant or independent.

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Appendix I
Abstracts leading to Oral presentations

Haematology Association of Ireland Annual meeting, 2016

O-linked sialylation on human VWF plays a role in modulating *in vivo* clearance through novel macrophage pathways.

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Introduction

Von Willebrand Factor (VWF) is a plasma sialoglycoprotein which plays an essential role in haemostasis. While much is known about the biosynthesis, structure and function of VWF, the mechanisms regarding the regulation of VWF clearance are poorly understood. Nonetheless, increased VWF clearance is important in the pathophysiology of Von Willebrand Disease (VWD). A role for VWF glycan moieties in contributing to the clearance of VWF has been demonstrated by the Asialoglycoprotein receptor (ASGPR); this hepatic receptor has been shown to be involved in the clearance of VWF in mice. Interestingly, GWAS studies have not identified an association between human plasma VWF levels and the ASGPR locus. Given the importance of glycan determinants in defining VWF plasma levels, we sought to investigate the contribution of the ASGPR in the clearance of human VWF.

Materials and Methods

VWF was purified from human plasma by gel filtration and subsequently treated with exoglycosidases for removal of α 2-3 or α 2-3,6,8,9 linked sialic acid creating two VWF-glycoforms; α 2-3Neu-VWF and Neu-VWF. To overcome the broad specificity of ASGPR antagonists, VWF^{-/-} and ASGPR1^{-/-} mice were crossed to create a double knockout line (VWF^{-/-}/ASGPR1^{-/-}), in which the clearance of VWF was assessed.

Results

Human VWF was cleared in a biphasic manner in VWF^{-/-}/ASGPR1^{-/-} mice, with an initial rapid phase, followed by a slow secondary phase. Despite a reportedly increased half-

life of murine VWF in ASGPR1^{-/-} mice, there was no difference in the clearance of human pdVWF in VWF^{-/-} and VWF/ASGPR1^{-/-} mice (MRT; 69.51±15.3mins and 67.2±20.8mins). Clearance of VWF glycoforms α2-3Neu-VWF and Neu-VWF was markedly enhanced in VWF^{-/-} mice (MRT 5.18±0.6 and 11.72±1.2). Work previously published from our research group has shown that O-linked sialic acid on pdVWF is predominantly α2-3 linked. Interestingly, these data suggest that while O-linked sialic acid account for only 20% of total VWF sialic acid, it plays an important role in modulating VWF clearance.

The enhanced VWF clearance associated with loss of sialic acid can be attenuated by pre-infusion of ASOR, thus we hypothesised that asialo-VWF variants were cleared by ASGPR. Surprisingly the absence of the ASGPR in VWF^{-/-}/ASGPR1^{-/-} mice failed to inhibit the rapid clearance for both α2-3Neu-VWF and Neu-VWF. Furthermore, pre-infusion of ASOR in these mice resulted in attenuated clearance of pdVWF, α2-3Neu VWF and Neu-VWF. These findings suggest that ASOR may inhibit VWF clearance in an ASGPR-independent manner. Moreover, these findings point to a role for additional galactose-sensitive receptors in the clearance of VWF.

Recent studies have implicated a role for macrophages in VWF clearance. To assess the contribution of macrophages in the clearance of asialo-VWF mice were treated with liposome-encapsulated clodronate. Interestingly, macrophage depletion in VWF^{-/-}/ASGPR1^{-/-} mice resulted in significantly prolonged survival of α2-3Neu-VWF and Neu-VWF.

Conclusion

Collectively these novel data suggest that asialo-VWF clearance is mediated through a macrophage-dependent pathway. In conclusion, these observations demonstrate that VWF glycans play a critical role in modulating *in vivo* clearance through both ASGPR-dependent and -independent pathways. Qualitative and quantitative variation in VWF glycosylation represents a key regulator of VWF clearance, and as such is likely to be of direct pathophysiological significance.

International Society of Thrombosis and Haemostasis congress, 2017

A novel macrophage-mediated pathway regulates enhanced clearance of hyposialylated von Willebrand factor *in vivo*.

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Background

von Willebrand Factor (VWF) is a plasma sialoglycoprotein which plays a critical role in haemostasis. While the biosynthesis and function of VWF are well characterized, the mechanisms underlying VWF clearance remain poorly understood. However, increased clearance is important in the aetiology of von Willebrand disease.

Aims

To define the mechanisms involved in the enhanced *in vivo* clearance of hyposialylated VWF.

Methods

The asialoglycoprotein receptor (ASGPR) plays a key role in clearing hyposialylated VWF. To investigate if other receptors and/or cell types may also be important, VWF^{-/-} and Asgr1^{-/-} mice were crossed creating a novel dual VWF^{-/-}/Asgr1^{-/-} knockout model. Human VWF (pdVWF) was modified using specific neuraminidases creating two glycoforms; α 2-3NeuVWF and α 2-3,6,8,9NeuVWF. *In vivo* clearance of these glycoforms was studied in VWF^{-/-}/Asgr1^{-/-} mice.

Results

Although previous studies described a critical role for ASGPR in regulating clearance of hyposialylated VWF, we observed markedly enhanced clearance of both α 2-3NeuVWF and α 2-3,6,8,9NeuVWF in VWF^{-/-}/Asgr1^{-/-} mice ($T_{1/2}$ = 8.2 \pm 0.6 and 3.2 \pm 0.4 vs. 50.6 \pm 2 mins for pdVWF, respectively). Importantly, the enhanced clearance of hyposialylated

VWF was not significantly attenuated in the presence or absence of ASGPR. In contrast however, clearance of hyposialylated VWF in VWF^{-/-}/Asgr1^{-/-} mice was reduced in the presence of asialosomucoid (p<0.05). Furthermore, immunohistochemistry demonstrated localization of asialo-VWF within hepatic macrophages. Finally, in vivo macrophage depletion with liposomal clodronate also significantly attenuated the enhanced clearance of hyposialylated VWF in VWF^{-/-}/ASGPR1^{-/-} mice (p<0.05).

Conclusion

Collectively, these findings demonstrate that a novel macrophage-dependent pathway is a critical modulator of the enhanced clearance of hyposialylated VWF. Given that quantitative variations in N- and O-linked sialylation has been described in specific patient cohorts, these findings are of direct clinical importance.

Haematology Association of Ireland Annual meeting, 2017

The Macrophage Galactose Lectin (MGL) modulates the clearance of von Willebrand Factor *in vivo*.

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Introduction

Von Willebrand Factor (VWF) is a plasma sialoglycoprotein which plays an essential role in maintaining normal haemostasis. While the structure, function and synthesis of VWF is well characterised, the molecular mechanisms underpinning VWF clearance remain poorly understood. Nonetheless, increase clearance of VWF is important in the pathophysiology of von Willebrand Disease (VWD). VWF is heavily glycosylated, with glycan moieties comprising 20% of VWF monomeric mass. Previous studies have shown that glycosylation is a regulator of VWF half-life. Furthermore, variations in VWF sialylation have been identified in VWD patient cohorts. In this study we sought to investigate the role of the Asialoglycoprotein receptor (ASGPR) in regulating VWF clearance and whether additional lectin receptors may modulate VWF circulatory half-life.

Materials and Methods

Human-VWF (pdVWF) was modified using specific neuraminidases creating two glycoforms; α 2-3NeuVWF and α 2-3,6,8,9NeuVWF. *In vivo* clearance of these glycoforms was studied in *VWF^{-/-}/Asgr1^{-/-}* mice. To assess VWF-MGL binding *in vitro*, surface plasmon resonance studies and solid phase binding assays were performed. Finally, specific clearance studies were performed after inhibition of MGL using a commercial anti-mouse MGL1/2 antibody.

Results

Studies in our laboratory have shown that the enhanced clearance rates observed for $\alpha 2$ -3Neu-VWF and $\alpha 2$ -3,6,8,9Neu-VWF, were not significantly different in the presence or absence of the ASGPR. These findings suggested that previously unrecognized ASGPR-independent pathways contribute to the enhanced clearance of hyposialylated-VWF *in vivo*. Interestingly, *in vivo* macrophage depletion with liposomal clodronate significantly attenuated the clearance of hyposialylated-VWF variants. Furthermore, *in vitro* binding studies demonstrated significantly enhanced binding of hyposialylated-VWF to differentiated THP1-macrophages ($p=0.0305$), suggesting macrophages may regulate hyposialylated-VWF clearance.

Macrophage Galactose Lectin (MGL) is a C-type lectin receptor with affinity for galactose/N-Acetylgalactosamine, expressed on macrophages and dendritic cells. Interestingly, we observed dose-dependent binding of wild-type human pd-VWF to purified recombinant MGL using surface plasmon resonance ($K_d(\text{app}) = 18.4 \pm 3.3 \text{ mg/ml}$). Furthermore, in solid phase binding assays we have shown that $\alpha 2$ -3Neu-VWF and $\alpha 2$ -3,6,8,9Neu-VWF bound MGL with significantly higher affinity than pdVWF ($142\% \pm 13.09$ and $152\% \pm 18.16$ Vs $100\% \pm 11.8$ respectively). Interestingly, the markedly enhanced clearance of both $\alpha 2$ -3Neu-VWF and $\alpha 2$ -3,6,8,9Neu-VWF in $VWF^{-/-}Asgr1^{-/-}$ mice was significantly attenuated in the presence of an mMGL1/2 inhibitory antibody, suggesting a novel role for MGL in regulating macrophage-mediated clearance of hyposialylated VWF. Furthermore, plasma VWF:Ag levels were significantly elevated in $MGL1^{-/-}$ mice compared to wild type controls ($152.6\% \pm 15.7$ vs $100\% \pm 16.99$; $p < 0.05$). Finally, clearance of pd-VWF in $VWF^{-/-}Asgr1^{-/-}$ mice was attenuated in the presence of mMGL1/2 inhibitory antibody, suggesting that MGL-mediated clearance is important even in the presence of ASGPR.

Conclusion

Our findings demonstrate that in the absence of the ASGPR, the enhanced clearance of hyposialylated-VWF remains. Moreover, we identify Macrophage Galactose Lectin (MGL) as a novel macrophage receptor which plays a critical role in modulating VWF clearance. Collectively, these findings reveal MGL as a novel macrophage lectin receptor

for VWF that significantly contributes to the clearance of both wild-type and hyposialylated-VWF. Given that quantitative variations in N- and O-linked sialylation have been described in specific VWD patient cohorts, these findings are of direct translational importance.

60th American society of Haematology Annual meeting, 2018

Defining the molecular mechanisms through which the Macrophage Galactose Lectin (MGL) receptor regulates von Willebrand factor clearance

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Introduction

Although the biological mechanisms underpinning VWF clearance remain poorly understood, accumulating data has shown that increased clearance is a common pathophysiology in type 1 VWD patients. Moreover, enhanced clearance has been implicated in the aetiology of types 2 and 3 VWD. Previous studies have shown that loss of terminal sialic acid from VWF glycans results in enhanced clearance. Furthermore, ST3Gal-IV knockout mice exhibit reduced plasma VWF levels due to rapid *in vivo* clearance. Initial studies suggested that clearance of hyposialylated VWF was mediated via the asialoglycoprotein receptor (ASGPR). However, we recently reported a novel role for the Macrophage Galactose Lectin (MGL) in regulating clearance of VWF (Ward *et al*, Blood 2018). In this study, we sought to elucidate the molecular mechanisms through which MGL interacts with human VWF.

Methods

Recombinant VWF variants, truncated A1A2A3 and isolated A1, A2 and A3 domains were expressed in HEK293T cells. Human plasma derived (pdVWF) was purified from commercial VWF concentrates as before. VWF glycoforms were generated by digestion with exoglycosidases. VWF-MGL interaction was assessed using plate-binding ELISA assays. *In vivo* clearance of VWF variants was assessed in both *Asgr1^{+/+}VWF^{-/-}* and *Asgr1^{-/-}VWF^{-/-}* mice in the presence/absence of anti-MGL blocking antibodies.

Results

Previous studies have shown 80% of total sialic acid on VWF is expressed on N glycans in α 2-6 linkage. In contrast, the other 20% of VWF sialylation is α 2-3 linked on O linked glycans. Removal O linked sialylation (with α 2-3 neuraminidase) was sufficient to increase VWF clearance in $VWF^{-/-}$ and $Asgr1^{-/-}VWF^{-/-}$ mice (half life = 9.0 ± 1 mins and 8.3 ± 2 mins). Interestingly, *in vivo* clearance of α 2-3 Neu VWF in $VWF^{-/-}$ and $Asgr1^{-/-}VWF^{-/-}$ mice was almost as rapid as that of asialo-VWF (digested with α 2-3,6,8,9 neuraminidase). Critically, the increased clearance of α 2-3 Neu VWF in both $VWF^{-/-}$ and $Asgr1^{-/-}VWF^{-/-}$ is attenuated in the presence of MGL blocking antibodies such that, at 120mins residual α 2-3 Neu VWF is 10%, compared to 7% for that of pdVWF. Plate-binding studies confirmed that α 2-3 Neu VWF and α 2-3,6,8,9 Neu VWF demonstrated enhanced binding to MGL compared to pdVWF (155% and 134% versus 100%; $p = 0.017$ and 0.006). In keeping with *in vivo* clearance data, removal of α 2-6 linked sialic acid from the N glycans did not further promote binding to MGL. Finally, although PNGase removal of VWF N glycan did not affect MGL binding, treatment with O-glycosidase significantly attenuated binding. Altogether, these data demonstrate that O-linked sialylation plays a critical role in protecting VWF from MGL mediated clearance.

The mature VWF monomer contains 10 O-linked glycans, with eight clustered around the A1 domain. To further investigate the role of these glycans in regulating MGL interaction, site directed mutagenesis was used to generate VWF molecules lacking either O-linked cluster 1 (T1248, T1255, T1256, T1263) or O-linked cluster 2 (T1468, T1477, S1486, T1487) respectively. Binding of Δ cluster 1 VWF to MGL was similar to that of wild-type VWF. In contrast, binding of the Δ cluster 2 VWF variant to MGL was markedly reduced (93 % vs 34%; $p = 0.0037$). O linked glycan structures are known to play important roles in maintaining glycoprotein conformation. To further define the mechanism through which these O glycan structures influence MGL mediated clearance, binding of a truncated A1A2A3 fragment and isolated recombinant A1, A2 and A3 domains were examined. Interestingly, MGL-binding to full length rVWF and A1A2A3 were both significantly increased in the presence of ristocetin. Although no binding of isolated A2 or A3 domains to MGL was observed, A1 domain binding to MGL was seen both in the presence and absence of ristocetin.

Conclusions

In conclusion, these findings define a novel role for O linked sialylation in protecting VWF from MGL mediated clearance. Our data further demonstrate that the cluster of O linked glycans located at the C-terminal end of the A1 domain play a specific role in regulating VWF clearance via MGL, and that conformation of A1A2A3 may be important in determining accessibility of these glycans for the clearance receptor. Further studies will be required to determine whether abnormalities in these O-glycan determinants may be important in the pathophysiology of VWD, particularly in patients with type 1C VWD who lack *VWF* gene coding mutations.

Abstracts leading to poster presentations

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Defining the role of the Asialoglycoprotein Receptor in the Clearance of von Willebrand Factor *in vivo*.

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Introduction

Von Willebrand Factor (VWF) is a sialoglycoprotein which plays an essential role in haemostasis. While the biosynthesis and function of VWF is characterised, the mechanism of VWF clearance is poorly understood. Nonetheless, increased VWF clearance is important in the aetiology of Von Willebrand Disease (VWD). The Asialoglycoprotein (ASGPR) has been demonstrated to regulate VWF clearance in mice; however, GWAS studies have not identified an association between human VWF levels and ASGPR.

Methods

VWF was treated with exoglycosidases for removal of sialic acid creating two glycoforms; α 2-3Neu-VWF and Neu-VWF. To overcome the broad specificity of ASGPR antagonists, double knockout ($VWF^{-/-}ASGPR1^{-/-}$) mice were bred.

Results & Conclusion

Despite a reportedly increased half-life of mVWF in $ASGPR1^{-/-}$ mice, there was no difference in the clearance of VWF in $ASGPR1^{+/+}$ and $ASGPR1^{-/-}$ mice. Surprisingly, the absence of ASGPR fails to inhibit the rapid clearance for asialo-VWF variants in $VWF^{-/-}/ASGPR1^{-/-}$ mice. Furthermore, infusion of ASOR in $VWF^{-/-}/ASGPR1^{-/-}$ mice results in attenuated clearance of VWF, α 2-3Neu-VWF and Neu-VWF. These findings point to additional receptors in the clearance of VWF.

To assess the contribution of macrophages in the clearance of VWF mice were treated with clodronate. Interestingly macrophage depletion in VWF^{-/-}/ASGPR1^{-/-} mice results in significantly prolonged survival of α 2-3Neu-VWF and Neu-VWF.

These data suggest that asialo VWF clearance is mediated through a macrophage dependent pathway. In conclusion, these observations demonstrate that VWF glycans play a role in modulating clearance through ASGPR-independent pathways. Variation in VWF glycosylation is a regulator of VWF clearance, and thus is likely to be of pathophysiological significance.

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Defining the molecular mechanisms through which the Macrophage Galactose Lectin (MGL) receptor regulates von Willebrand factor clearance

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Introduction

Von Willebrand Factor (VWF) is a plasma sialoglycoprotein which plays an essential role in maintaining haemostasis. While the structure, function and synthesis of VWF is well characterised, the molecular mechanisms underpinning VWF clearance remain elusive. Nonetheless, increase clearance of VWF is important in the pathophysiology of von Willebrand Disease (VWD). Loss of sialic acid from VWF glycans results in enhanced clearance, initial studies suggested this was mediated via the asialoglycoprotein receptor (ASGPR). However, we recently reported a novel role for the Macrophage Galactose Lectin (MGL) in regulating clearance of VWF (Ward *et al*, Blood 2018). In this study, we sought to elucidate the molecular mechanisms through which MGL interacts with human VWF.

Methods

VWF variants were expressed in HEK293T cells. Human VWF (pdVWF) was purified from commercial concentrates and VWF glycoforms were generated by digestion with exoglycosidases. VWF-MGL interaction was assessed using plate-binding ELISA assays. *In vivo* clearance of VWF was assessed in both *Asgr1*^{+/+}*VWF*^{-/-} and *Asgr1*^{-/-}*VWF*^{-/-} mice in the presence/absence of anti-MGL blocking antibodies.

Results

Previous studies have shown 80% of total sialic acid on VWF is expressed on N-glycans in α 2-6 linkage. In contrast, the other 20% of VWF sialylation is α 2-3 linked on O-linked glycans. Removal O-linked sialylation increases VWF clearance in *Asgr1^{+/+}VWF^{-/-}* and *Asgr1^{-/-}VWF^{-/-}* mice (half-life = 9.0 ± 1 and 8.3 ± 2 mins). Interestingly, *in vivo* clearance of α 2-3-Neu-VWF in *Asgr1^{+/+}VWF^{-/-}* and *Asgr1^{-/-}VWF^{-/-}* mice was almost as rapid as asialo-VWF. Critically, the increased clearance of α 2-3-Neu-VWF in both *Asgr1^{+/+}VWF^{-/-}* and *Asgr1^{-/-}VWF^{-/-}* is attenuated in the presence of MGL blocking antibodies such that, at 120mins residual α 2-3 Neu VWF is 10%, compared to 7% for that of pdVWF. Plate-binding studies confirmed that α 2-3-Neu-VWF and asialo-VWF demonstrated enhanced binding to MGL compared to pdVWF (155% and 134% versus 100%; $p=0.017$ and 0.006). Interestingly, removal of N-glycans didn't affect MGL binding however, removal of O-glycans significantly attenuated binding. These data demonstrate O-linked sialylation plays a critical role in protecting VWF from MGL mediated clearance.

Mature VWF contains 10 O-linked glycans, with eight clustered around the A1 domain. Site directed mutagenesis was used to generate VWF molecules lacking either O-linked cluster 1 or O-linked cluster 2. Binding of Δ cluster1-VWF to MGL was similar to that of wild-type, however, binding of the Δ cluster2-VWF to MGL was markedly reduced (93%vs34%; $p=0.0037$). Finally, binding of a truncated A1A2A3 fragment and isolated A1, A2 and A3 domains were examined. Interestingly, MGL-binding to full length rVWF and A1A2A3 was not significantly different, indicating domains downstream of A1A2A3 are not required for MGL-binding. Furthermore, while no binding of A2 or A3 domains to MGL was observed, A1 domain binding to MGL was seen.

Conclusions

These findings define a novel role for O-linked sialylation in protecting VWF from MGL mediated clearance. Our data further demonstrate that the cluster of O-linked glycans located at the C-terminal end of the A1 domain play a specific role in regulating VWF clearance via MGL, and that conformation of A1A2A3 may be important in determining accessibility of these glycans for the clearance receptor.

Identification of murine Macrophage Galactose Lectin 2 as a novel VWF clearance receptor sheds light on differential clearance pathways for hyposialylated VWF variants.

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Background:

Macrophage Galactose Lectin (MGL) has been identified to regulate VWF clearance (Ward, Blood 2018). Interestingly, two murine MGL homologs exist; murine-MGL1 and murine-MGL2. Moreover, the binding profile of murine-MGL2 is more homologous to human-MGL, suggesting contribution of murine-MGL1 and murine-MGL2 in regulating VWF clearance.

Aims:

To determine whether murine-MGL2 regulates VWF clearance *in-vivo*. To assess the roles of murine-MGL1/2 and the Asialoglycoprotein-Receptor in hyposialylated-VWF clearance.

Methods:

Binding of plasma-derived-VWF, α 2-3-Neu-VWF (O-sialylation removed) and α 2-3,6,8,9-Neu-VWF (N- and O-sialylation removed) to murine-MGL1 and murine-MGL2 was assessed *in-vitro*. VWF clearance *in-vivo* was assessed in the presence/absence of MGL1/2 blocking antibodies.

Results:

PdVWF binds murine-MGL1 *in-vitro* in a dose-dependent manner, interestingly, pdVWF also binds murine-MGL2. Importantly, the binding of pdVWF to murine-MGL2 is 1.9-fold greater than murine-MGL1 (p=0.0317). Furthermore, exposure of sub-terminal galactose significantly enhanced binding of α 2-3-Neu-VWF and α 2-3,6,8,9-Neu-VWF to murine-MGL2 *in-vitro*.

Murine-VWF half-life is prolonged in *MGL1*^{-/-} mice compared to wild-type (1.1±0.1hrs vs 1.915±0.15hrs, p<0.05). Blocking of murine-MGL2 in *MGL1*^{-/-} results in further half-life

extension (1.915 ± 0.15 hrs vs 2.5 ± 0.2 hrs, $p=0.028$), identifying mMGL2 as a novel murine-VWF clearance receptor. Critically, combined inhibition of murine-MGL1/2 results in a significant increase in murine-VWF half-life compared to wild-type (1.53 ± 0.16 hrs vs 3.74 ± 0.2 hrs, $p=0.0004$).

Inhibition of murine-MGL1/2 provides correction of the rapid clearance of $\alpha 2$ -3-Neu-VWF to that of pdVWF (40.2 ± 1.3 mins vs 45.7 ± 2.2 mins, $p=0.076$), suggesting MGL alone regulates $\alpha 2$ -3-Neu-VWF clearance.

In $VWF^{-/-}$ mice, partial extension of $\alpha 2$ -3,6,8,9-Neu-VWF half-life is observed upon murine-MGL1/2 inhibition. However, in $VWF^{-/-}/Asgr1^{-/-}$ murine-MGL1/2 inhibition results in correction of the rapid clearance rate, suggesting both MGL and the Asialoglycoprotein-receptor mediate clearance of $\alpha 2$ -3,6,8,9-Neu-VWF.

Conclusions:

Identification of murine-MGL2 sheds further light on the contribution of MGL to VWF clearance. Inhibition of murine-MGL1/2 results in a 2.4-fold increase in VWF half-life, suggesting a key role for MGL in VWF clearance. Furthermore, these data demonstrate differential clearance pathways for O-sialylation removed versus N/O-sialylation removed VWF.

Appendix II: publications

THROMBOSIS AND HEMOSTASIS

N-linked glycans within the A2 domain of von Willebrand factor modulate macrophage-mediated clearance

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Key Points

- The A1 domain of VWF contains a cryptic binding site that plays a key role in regulating macrophage binding and clearance.
- The N-linked glycans presented at N1515 and N1574 within the A2 domain of VWF modulate macrophage-mediated clearance.

Enhanced von Willebrand factor (VWF) clearance is important in the etiology of von Willebrand disease. However, the molecular mechanisms underlying VWF clearance remain poorly understood. In this study, we investigated the role of VWF domains and specific glycan moieties in regulating *in vivo* clearance. Our findings demonstrate that the A1 domain of VWF contains a receptor-recognition site that plays a key role in regulating the interaction of VWF with macrophages. In A1-A2-A3 and full-length VWF, this macrophage-binding site is cryptic but becomes exposed following exposure to shear or ristocetin. Previous studies have demonstrated that the N-linked glycans within the A2 domain play an important role in modulating susceptibility to ADAMTS13 proteolysis. We further demonstrate that these glycans presented at N1515 and N1574 also play a critical role in protecting VWF against macrophage binding and clearance. Indeed, loss of the N-glycan at N1515 resulted in markedly enhanced VWF clearance that was significantly faster than that observed with any previously described VWF mutations. In addition, A1-A2-A3 fragments containing the N1515Q or N1574Q substitutions also demonstrated significantly enhanced clearance. Importantly, clodronate-induced macrophage depletion significantly attenuated the increased clearance observed with N1515Q and N1574Q in both full-length VWF and A1-A2-A3. Finally, we further demonstrate that loss of these N-linked glycans does not enhance clearance in VWF in the presence of a structurally constrained A2 domain. Collectively, these novel findings support the hypothesis that conformation of the VWF A domains plays a critical role in modulating macrophage-mediated clearance of VWF *in vivo*. (*Blood*. 2016;128(15):1959-1968)

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Introduction

Von Willebrand factor (VWF) is a large multimeric sialoglycoprotein that plays 2 key roles in normal hemostasis.^{1,2} First, it mediates recruitment of platelets following injury to the vascular endothelium. Second, VWF also functions as a carrier molecule for factor VIII. *In vivo* expression of VWF occurs only within endothelial cells (ECs)³ and megakaryocytes,⁴ where VWF is initially synthesized as a monomer composed of a series of repeating domains in the order D'-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK. VWF synthesized within ECs undergoes constitutive secretion into the plasma. Prior to this secretion, VWF undergoes complex posttranslational modification within ECs, including significant N- and O-linked glycosylation.^{2,5,6}

The N- and O-linked glycans of human VWF have both been characterized and demonstrate significant heterogeneity.^{7,8}

Monosialylated or disialylated biantennary complex-type chains constitute the most common N-linked glycans expressed on VWF.^{6,7,9-11} Although the O-linked glycans of VWF also demonstrate marked heterogeneity, disialyl core 1 structures account for ~70% of the total population.^{8,12} Importantly, although the majority of its glycans are capped by negatively charged sialic acid,^{7,13} VWF is unusual in that a minority of both N-linked and O-linked carbohydrate chains express terminal ABO(H) blood group determinants.^{7,8,11}

Although substantial progress has been achieved in understanding the biosynthesis, structure, and functions of VWF, the biological mechanism(s) responsible for modulating VWF clearance from the plasma remain poorly understood.¹⁴ Nevertheless, accumulating data have shown that enhanced VWF clearance plays an important role in the

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etiology of both type 1 and type 2 VWD.¹⁵⁻²⁰ Recent evidence further suggests that hepatic and splenic macrophages may play key roles in mediating VWF clearance.²¹⁻²⁶ For example, differentiated primary human macrophages can bind and endocytose purified VWF *in vitro*.²¹ Furthermore, macrophage depletion significantly prolonged the *in vivo* survival of VWF infused into VWF^{-/-} mice.^{21,24,25} In addition, the macrophage lipoprotein receptor (LRP1) has been shown to bind to VWF in a shear-dependent manner.²³ Critically however, the specific regions of the VWF glycoprotein involved in modulating macrophage interactions remain unclear.

More than 30 different VWF point mutations have already been associated with enhanced clearance in patients with VWD.^{15,27,28} Intriguingly, the majority of VWF amino acid substitutions associated with enhanced clearance are clustered within the A1 domain.¹⁴ Emerging evidence suggests that at least some of these VWF mutations result in enhanced macrophage-mediated clearance *in vivo*. For example, Wohner et al recently showed that specific type 2B VWD variants (R1306Q and V1316M within the A1 domain) result in increased VWF clearance that is predominantly modulated through the macrophage LRP1 receptor.²⁶ Nevertheless, the molecular mechanism through which so many different amino acid substitutions clustered within the A1 domain of VWF result in enhanced clearance remains poorly understood.

Variation in VWF glycosylation profile has also been shown to significantly influence the clearance rate.²⁹⁻³⁴ For example, terminal ABO(H) blood group determinants significantly modulate *in vivo* clearance.³⁵ Consequently, plasma VWF levels are significantly lower in blood group O individuals than in non-O individuals.^{5,36} The asialoglycoprotein or Ashwell receptor (ASGPR) is a C-type lectin predominantly expressed on hepatocytes and is composed of 2 transmembrane protein subunits (Asgr-1 and Asgr-2).³⁷ Importantly, a role for Asgr1 in modulating VWF clearance has recently been described.³⁴ In addition, a number of other carbohydrate receptors, including galectin-1 (Gal-1), galectin-3 (Gal-3), Siglec-5, and CLEC4M, have also been shown to bind VWF.³⁸⁻⁴⁰ Furthermore, genome-wide association studies have reported associations between some of these receptors and plasma levels of the VWF-factor VIII complex.⁴¹ Nevertheless, given the complexity and heterogeneity of the N- and O-linked glycans expressed on VWF, the biological mechanisms through which specific VWF carbohydrate structures serve to regulate *in vivo* clearance remain poorly defined. In this study, we have used a series of *in vitro* and *in vivo* methodologies to investigate the importance of specific domains and glycans in modulating VWF clearance. Our findings demonstrate that N-linked glycans at N1515 and N1574 within the A2 domain of VWF play a critical role in protecting VWF against macrophage-mediated clearance.

Materials and methods

Expression and purification of recombinant VWF

The expression vector pcDNA-VWF encoding full-length recombinant VWF (rVWF) has previously been described.⁴² A DNA fragment containing VWFA1A2A3 (residues 1260-1874) was inserted into expression vector pEXPR-IBA 42 (IBA) via *NheI* and *PmeI* restriction sites. The same strategy was used for expressing VWFA1 (residues 1239-1472), VWFA2 (residues 1473-1668), and VWFA3 (residues 1671-1878). Human full-length rVWF, A1A2A3-VWF A1-VWF, A2-VWF, and A3-VWF were transiently expressed in HEK293T cells. Conditioned serum-free medium was harvested and concentrated as before.²⁴ A1-VWF, A2-VWF, A3-VWF, and A1A2A3-VWF variants were further purified via nickel affinity chromatography.

Site-directed mutagenesis of VWF was carried out to introduce point mutations at N1515 and N1574 within both full-length and truncated A1A2A3-VWF. In keeping with previous studies defining the biological significance of these glycans,⁴²⁻⁴⁴ each asparagine residue was mutated to glutamine (N1515Q and N1574Q) to eliminate the N-linked glycans at these positions. Mutations were verified by DNA sequencing to ensure the absence of any other randomly introduced mutations. Similarly, mutagenesis of full-length VWF was carried out to introduce point mutations N1493C and C1670S. These mutations result in the creation of a homologous disulfide bond in the A2 domain of VWF between the cysteine at 1493 and the native cysteine at 1669. This cysteine clamp mutation has previously been described to prevent A2 domain unfolding and render VWF insensitive to ADAMTS13 cleavage.^{45,46}

In vitro modification of VWF glycan structures

The N-linked glycan profile of rVWF and A1A2A3-VWF was modified using a specific exoglycosidase peptide N glycosidase F (PNGase F; New England Biolabs). VWF glycan digestions were carried out overnight under non-denaturing conditions at 37°C as previously reported.⁴² After digestion, residual VWF glycan expression was quantified using specific lectin enzyme-linked immunosorbent assays (ELISAs) as described previously.^{47,48}

Human RAP expression and purification

Low-density lipoprotein receptor-related protein-associated protein 1 (RAP) acts as a molecular chaperone by inhibiting ligand binding to LRP1, as well as other members of this receptor family. Human RAP complementary DNA coding Tyr35 to Leu357 (UNIPROTKB-P30533) was inserted into Novagen pET28a (+) bacterial expression vector (Novagen, Nottingham, UK) via *BamHI* and *Sall* restriction sites. Recombinant RAP protein was refolded from *Escherichia coli* inclusion bodies as described previously.⁴⁹

THP-1 binding assay

THP-1 cells were seeded on microwell plates (Nunc; Fisher Scientific) at a density of 5×10^6 cells/mL. For differentiation, media was supplemented with 20 ng/mL PMA (Sigma-Aldrich). After 72 hours, fresh growth medium was added to the cells, which were then rested for an additional 4 days before use.⁵⁰ VWF-THP-1 binding was performed at 4°C to prevent endocytosis. Full-length VWF or truncated A1A2A3-VWF variants were diluted in ice-cold serum-free growth medium incubated with the cells for 1 hour on ice. For analysis, the nuclei were stained with Hoechst 33342 (Thermo Fisher). Full-length bound VWF was detected using anti-human VWF (Dako) followed by Alexa Fluor 488 donkey anti-rabbit immunoglobulin G (IgG) (Life Technologies). Truncated A1A2A3-VWF variants were detected by Penta-His Alexa Fluor 488 conjugate (QIAGEN). THP-1 surface-bound VWF was quantified using the fluorescence microscopy IN Cell Analyzer 1000 (GE Healthcare). Eight fields of view were imaged per well at a magnification of $\times 20$. Image analysis was carried out using high throughput IN Cell 1000 Image Analysis Software (GE Healthcare). Data were graphed as percentage fluorescently labeled VWF per cell relative to maximal VWF binding (mean \pm standard error of the mean [SEM]).

VWF uptake by differentiated THP-1 cells was assessed using confocal microscopy. In brief, cells were seeded onto glass chamber sides and differentiated using PMA as above. Subsequently cells were incubated with VWF or glycoforms thereof in the presence of ristocetin (1 mg/mL) for 30 minutes at either 4°C or 37°C to assess binding and internalization, respectively. Cells were fixed with 4% paraformaldehyde for 20 minutes, in some cases permeabilized using 0.1% Triton X, and then incubated with 4,6-diamidino-2-phenylindole; polyclonal mouse anti-EEA1 (early endosomal antigen-1) (BD Biosciences); and/or polyclonal rabbit anti-VWF for 45 minutes. After washing, slides were stained with Alexa Fluor 594-conjugated anti-mouse IgG (Invitrogen) and Alexa Fluor 488-conjugated anti-rabbit IgG (Invitrogen, UK) for 15 minutes. Images were visualized using LSM700 (Carl Zeiss) Confocal Microscope, 63 \times plan-apochromat lens. Images were analyzed using ImageJ and Adobe Illustrator CC2015.3.

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

VWF^{-/-} mice were obtained from The Jackson Laboratory (Sacramento, CA). All animal experiments were approved by the Animal Research Ethics Committee, Trinity College Dublin, and were performed in compliance with the Irish Medicines Board regulations. VWF clearance studies were performed using mice between 6 and 10 weeks of age. In brief, VWF^{-/-} mice were injected intravenously with 30 nM (37.5 U/kg) of VWF or glycoforms thereof. At sequential time points, blood was collected into heparin-coated micro containers. Three to five mice per time point were used. Residual plasma VWF:antigen levels were determined by ELISA. In vivo macrophage depletion was performed as previously described,^{24,51} using IV infusion of clodronate liposomes (100 μ l/10 g body weight). Control mice were injected with PBS liposomes. To examine the role of the LRP1 clearance receptor, mice were administered 200 μ M LRP1-antagonist RAP 1 minute prior to injection of VWF.

Data presentation and statistical analysis

All experimental data and statistical analysis were performed using the GraphPad Prism program. Data are expressed as mean \pm SEM. To assess statistical differences for 2 data sets, data were analyzed using the Student unpaired 2-tailed *t* test. For multiple comparisons, data were analyzed using a one-way analysis of variance with post hoc Dunnett's test. For all statistical tests, *P* < .05 was considered significant.

Results

The A domains of VWF modulate macrophage-mediated clearance

Lenting et al previously reported that a truncated A1-A2-A3 VWF fragment expressed in BHK cells demonstrated a similar clearance pattern to that of full-length rVWF and postulated that a receptor-recognition site may be present within the A1-A3 region.⁵² To further study this hypothesis, we first investigated the clearance of rVWF and A1-A2-A3 that was expressed in a different cell line (HEK293T). Following tail vein injection in VWF^{-/-} mice, A1-A2-A3 (HEK) clearance was also similar to that of full-length VWF (HEK) (Figure 1A). Recent studies have shown that hepatic macrophages contribute to the clearance of full-length VWF.²¹⁻²⁶ To determine whether macrophages modulate A1-A2-A3 clearance in vivo, clearance studies were repeated following clodronate-induced macrophage depletion. In keeping with previous studies, clearance of full-length rVWF was significantly reduced following macrophage depletion (Figure 1B). Interestingly, in vivo clearance of A1-A2-A3 was also significantly attenuated following macrophage depletion (Figure 1B).

To further investigate a putative role for the A domains in regulating VWF clearance by macrophages, VWF binding to PMA-differentiated THP-1 cells in vitro was studied using HiContent image analysis. In preliminary studies, we observed that full-length plasma-derived VWF (pd-VWF) and rVWF both bound to THP-1 macrophages (supplemental Figure 1, available on the *Blood* Web site). In keeping with a role for the A domains in modulating macrophage interaction, full-length VWF binding was significantly enhanced in the presence of ristocetin (1 mg/mL) (supplemental Figure 1). Moreover, dose-dependent binding of the truncated A1-A2-A3 fragment to THP-1 macrophages in vitro was also observed (Figure 1C). Finally, the relative importance of the individual A domains within the A1-A2-A3 construct in determining macrophage binding was investigated. Significant in vitro binding of the A1 domain to THP-1 macrophages was observed (Figure 1D). However, in contrast, no significant binding was seen for

the isolated A2 or A3 domains. Importantly, the macrophage binding of the isolated A1 domain was also significantly greater than that of the combined A1-A2-A3 fragment. Collectively, these data support the hypothesis that the A domains of VWF contain a receptor-recognition site important in regulating the interaction of VWF with macrophages and suggest that the VWF A1 domain plays a particular critical role in determining macrophage binding.

In previous studies, we observed that PNGase F digestion of human pd-VWF to remove the N-linked glycans resulted in markedly increased clearance. Similarly, clearance of rVWF was also significantly increased \sim 2.5-fold following PNGase digestion (*P* < .05) (Figure 1E). Given the important role of A1-A2-A3 in regulating VWF clearance by macrophages, it is interesting that only 2 N-linked glycan sites are located in this region (at N1515 and N1574 within the A2 domain).^{7,42} To determine whether these N-linked glycans within A2 play a role in modulating VWF interaction with macrophages, binding of A1-A2-A3 before and after PNGase F digestion was investigated. Following PNGase treatment, in vitro binding of A1-A2-A3 to THP-1 macrophages was markedly increased (Figure 1F), suggesting that these N-linked glycans within A2 play a novel role in regulating macrophage interaction.

N-linked glycans at N1515 and N1574 are critical determinants of VWF clearance

Recent mass spectrometry analysis has demonstrated that both of the N-linked glycans at N1515 and N1574 within A2 are occupied by large complex carbohydrate structures (Figure 2A).⁷ To further investigate the role of these glycans in modulating macrophage interaction, the asparagine residues at N1515 and N1574 were individually mutated to glutamine residues (VWF-N1515Q and VWF-N1574Q) and expressed in HEK293T cells. In accordance with previous studies,⁴² VWF-N1515Q and VWF-N1574Q both displayed multimer distribution and collagen binding activities similar to wild-type (WT) VWF (data not shown). Following tail vein injection, in vivo clearance of VWF-N1515Q and VWF-N1574Q was markedly enhanced in VWF mice compared with WT-VWF (Figure 2B). Interestingly, clearance of VWF-N1515Q was faster than that of any other previously described VWF point mutations, including VWF-R1205H (data not shown; *P* < .05).²⁴ In keeping with previous studies, infusion of VWF-N1515Q and VWF-N1574Q had no significant effect on murine platelet counts (data not shown).⁴² In addition, SDS-PAGE of murine plasma samples under reducing conditions demonstrated no significant evidence of in vivo proteolysis of either VWF-N1515Q or VWF-N1574Q.

To investigate the molecular mechanism responsible for the enhanced VWF clearance associated with the loss of A2 domain N-linked glycans, we further characterized the effects of introducing the VWF-N1515Q and VWF-N1574Q substitutions into the A2 domain of the A1-A2-A3 fragment (A1-A2-A3-N1515Q and A1-A2-A3-N1574Q). In keeping with their effect in reducing the survival of full-length rVWF, both mutations also resulted in significantly enhanced clearance of the A1-A2-A3 fragment (Figure 2C). Cumulatively, these novel findings demonstrate that both of the N-linked glycans located at N1515 and N1574 within the A2 domain of VWF play critical roles in regulating clearance in vivo. Furthermore, the increased clearance associated with loss of N-linked glycan structures at either N1515 or N1574 is modulated through local effects within the A1-A2-A3 region.

N-linked glycans within A2 regulate enhanced clearance

Besides the 2 N-linked glycans located within the A2 domain, another 10 N-linked glycans are present on the VWF monomer

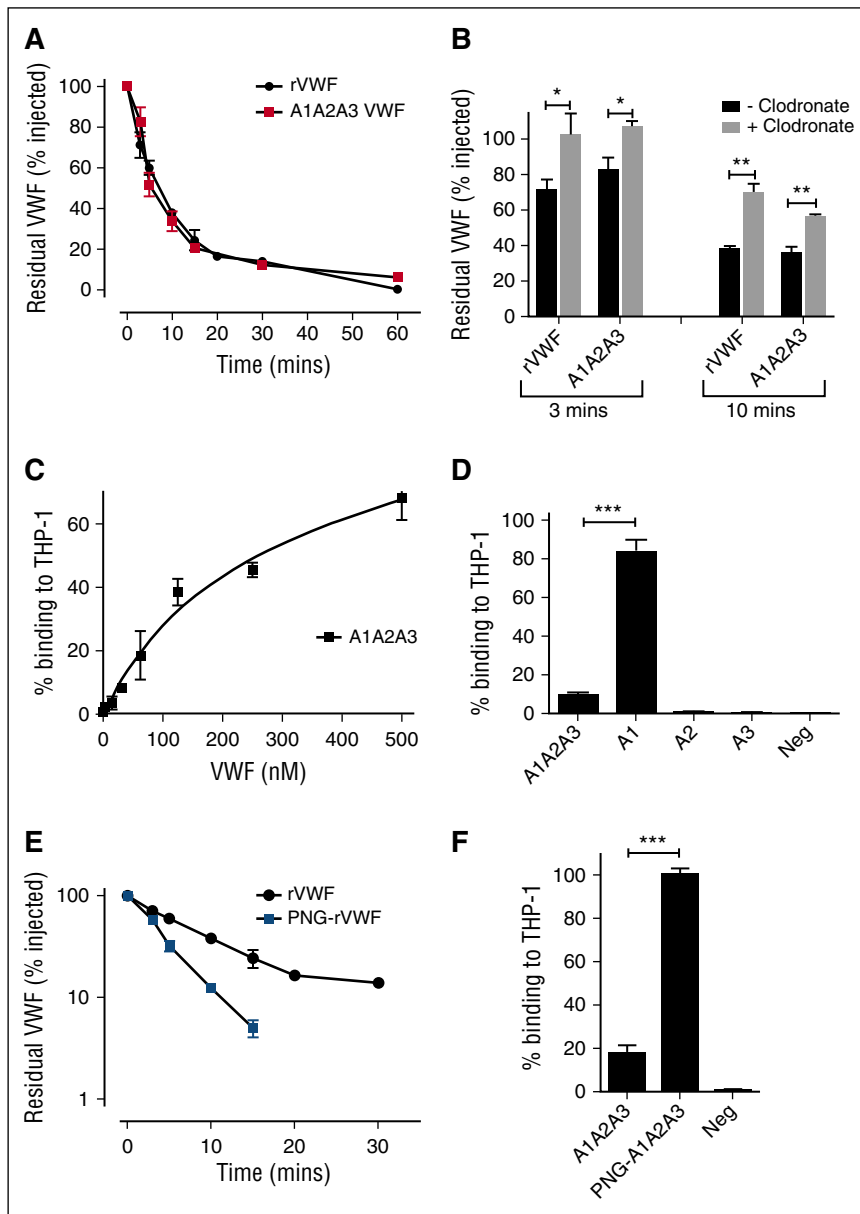


Figure 1. The A domains of VWF modulate macrophage-mediated clearance. (A) The in vivo clearance of a monomeric A1-A2-A3 VWF fragment in $VWF^{-/-}$ mice was compared with that of full-length rVWF. At each time point, the residual circulating VWF concentration was determined by VWF:antigen ELISA. All results are plotted as percentage residual VWF levels relative to the amount injected. Data are presented as mean \pm SEM. In some cases, the SEM cannot be seen due to its small size. Mean residence times for full-length and A1-A2-A3 were 11.3 ± 0.6 and 10.0 ± 0.61 minutes, respectively. (B) To study the role of macrophages in modulating clearance of A1-A2-A3 and full-length rVWF, in vivo clearance studies were repeated in $VWF^{-/-}$ mice 24 hours following clodronate-induced macrophage depletion. Blood was collected at 3- and 10-minute time points, and residual VWF quantified by ELISA. (C) The in vitro binding of A1-A2-A3 to macrophages was assessed using THP-1 macrophage cells as detailed in "Materials and methods." (D) Individual A-domain proteins A1, A2, and A3 were examined for binding to THP-1 macrophages. Significant binding was observed for the A1 domain compared with the A2 and A3 domains ($*P < .05$, $**P < .01$, and $***P < .001$, respectively; negative control is no VWF). (E) To investigate the role of VWF carbohydrate determinants in modulating VWF clearance, rVWF was treated with PNGase F (PNG-rVWF). N-linked glycan removal was confirmed using a specific lectin ELISA. In vivo survival was then measured in $VWF^{-/-}$ mice as before. Results are plotted as percentage residual VWF:antigen levels relative to the amount injected. Data are represented as mean \pm SEM. (F) To assess a potential role for VWF N-linked glycans in the A domains in regulating macrophage binding, A1-A2-A3 was treated with PNGase to remove the N-linked glycans in the A2 domain at N1515 and N1574 (PNG-A1A2A3). The ability of PNG-A1A2A3 to bind to THP-1 macrophages in the presence of ristocetin was then compared with WT A1-A2-A3 using HiContent image analysis as before. Data are graphed as percentage binding relative to maximal (mean \pm SEM) ($****P < .001$).

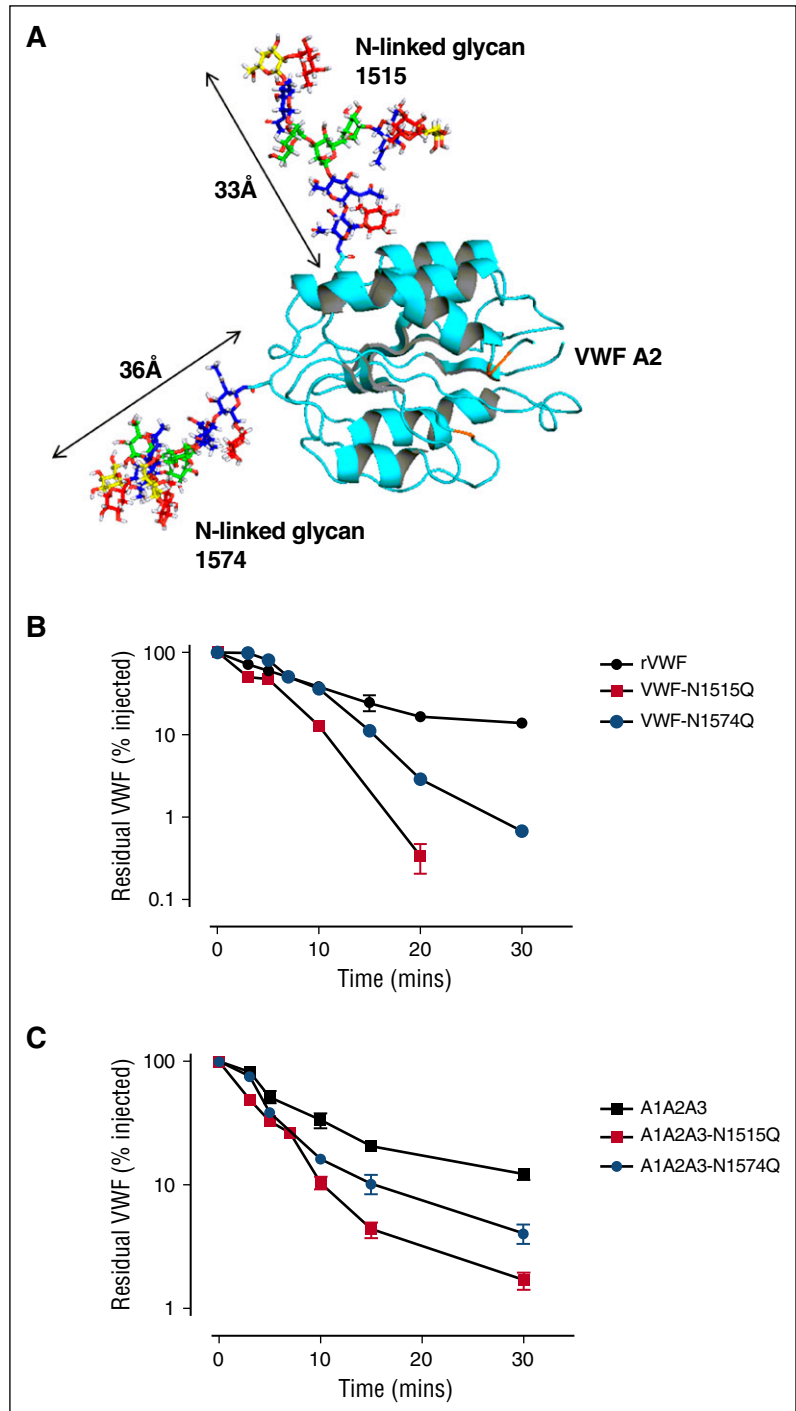
(Figure 3A).^{7,43} To determine whether these additional N-linked glycans may also be important in regulating VWF clearance, we expressed a rVWF variant from which the A2 domain was deleted ($\Delta A2$ -VWF) (Figures 3A-B). Interestingly, in vivo clearance of $\Delta A2$ -VWF was not accelerated following PNGase F digestion (Figure 3C), suggesting that the N-linked glycans within the A2 domain play a critical role in modulating the enhanced clearance of PNG-VWF.

Accelerated clearance of VWF N1515Q and VWF N1574Q is mediated by macrophages

To investigate whether VWF A2 domain glycan expression influences macrophage-mediated clearance, studies were repeated in $VWF^{-/-}$ mice following clodronate administration. We found that the increased clearance phenotypes of both VWF-N1515Q and VWF-N1574Q were significantly attenuated following clodronate-induced macrophage depletion (Figure 4A). Similarly, the enhanced clearance of both A1-A2-A3-N1515Q and A1-A2-A3-N1574Q

was also significantly reduced following macrophage depletion (Figure 4B). In keeping with previous reports,^{21,22} confocal microscopy studies performed following incubation at 4°C demonstrated that VWF-WT, VWF-N1515Q, and VWF-N1574Q all bound to differentiated THP-1 cells in vitro (supplemental Figure 2). When incubation studies were performed at 37°C, confocal studies showed colocalization of VWF-WT, VWF-N1515Q, and VWF-N1574Q with early endosomes (supplemental Figure 2), demonstrating that both VWF-N1515Q and VWF-N1574Q are taken up by macrophages. To further study the molecular mechanism through which the N-linked glycans expressed at N1515 and N1574 influence macrophage-dependent clearance, we used HiContent image analysis to compare the in vitro binding of A1-A2-A3-N1515Q, A1-A2-A3-N1574Q, and WT A1-A2-A3 to THP-1 cells. WT A1-A2-A3 demonstrated minimal macrophage binding (Figure 5). However, this binding was significantly enhanced in the presence of ristocetin (1 mg/mL). Importantly, following PNGase digestion to remove the 2 N-linked glycans at

Figure 2. N-linked glycans at N1515 and N1574 are critical determinants of VWF clearance in vivo. (A) A model of the VWF A2 domain was prepared as previously described.⁶⁶ Mass spectrometry analysis of human pd-VWF has provided extensive information regarding the N-glycome of VWF. Utilizing this information, a model of the VWF A2 domain with its associated glycans was constructed using Glycam Glycoprotein Builder software. N1515 and N1574 glycan structures were mapped onto the A2 domain crystal structure using this glycan modeling. This in silico analysis revealed that the complex glycans at N1515 and N1574 were both of significant size, spanning ~33 Å and ~36 Å in length, respectively. (B) To investigate a potential role for specific glycan sites in influencing VWF clearance, N1515 and N1574 in the A2 domain were targeted for removal by site-directed mutagenesis (VWF-N1515Q and VWF-N1574Q, respectively). In vivo clearance studies of these VWF glycan variants were performed as before and compared with WT rVWF. (C) Given that the glycans N1515 and N1574 reside within the A2 domain of VWF, we further sought to examine if these glycans could also influence the in vivo survival of an A1A2A3 VWF truncated fragment. To this end, site-directed mutagenesis was performed to eliminate the glycan at N1515 (A1A2A3-N1515Q) and N1574 (A1A2A3-N1574Q). Clearance examined in VWF^{-/-} mice as before. All results are plotted as percentage residual VWF:antigen levels relative to the amount injected. Data are presented as mean ± SEM.



N1515 and N1574, binding of A1-A2-A3 was dramatically increased, suggesting that the presence of these carbohydrate structures serves to prevent binding of A1-A2-A3 to macrophages. In keeping with this hypothesis, both A1-A2-A3-N1515Q and A1-A2-A3-N1574Q demonstrated significantly increased binding compared with WT A1-A2-A3 in either the presence or absence of ristocetin. Finally, PNGase treatment of both A1-A2-A3-N1515Q and A1-A2-A3-N1574Q served to further enhance macrophage binding, confirming that both N-linked glycans contribute to modulating macrophage interaction. Cumulatively, these in vivo and in vitro data confirm that the N-linked glycans

within the A2 domain of play important roles in regulating macrophage-dependent VWF clearance

Glycan structures at N1515 and N1574 in the A2 domain influence LRP1-mediated clearance

Recent studies have demonstrated that macrophage LRP1 plays an important role in regulating VWF clearance.^{23,26,53} In addition, LRP1 has been shown to bind to VWF in a shear-dependent manner. Importantly, Rastegarlarani et al previously demonstrated that the inhibitory effects of RAP on VWF clearance were predominantly modulated through macrophage LRP1 rather than LRP1

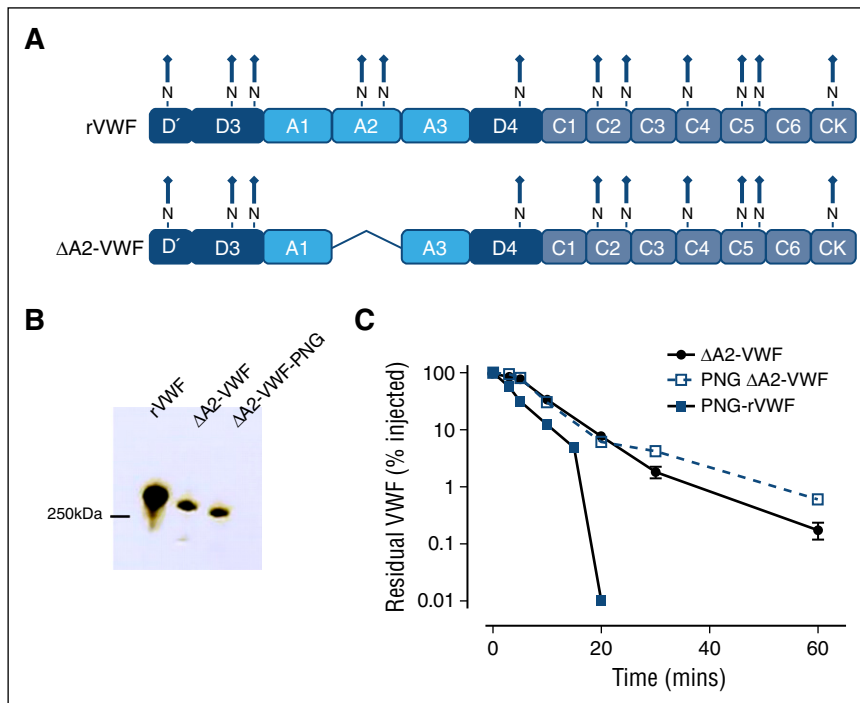


Figure 3. N-linked glycans within the VWF A2 domain regulate enhanced clearance. Our findings suggest that the N-linked glycan within A2 may have a specific role in modulating VWF clearance. To examine if glycans outside the A2 domain may also influence VWF survival, a fragment of VWF with the A2 domain deleted was constructed. (A) Consequently, this VWF variant (Δ A2-VWF) fails to express the N-linked glycans N1515 and N1574. (B) This VWF variant was subjected to PNGase F treatment (PNG Δ A2-VWF) to remove all remaining N-linked glycans. (C) Clearance was assessed in VWF^{-/-} mice as before. All results are plotted as percentage residual VWF:antigen levels relative to the amount injected. Data are presented as mean \pm SEM.

expressed in other cells or other macrophage lipoprotein receptors.²³ To further investigate whether VWF A2 domain glycans influence LRP1-mediated clearance in vivo, VWF-N1515Q and VWF-N1574Q clearance studies in VWF^{-/-} mice were repeated in the presence or absence of RAP. In keeping with previous reports, we confirmed that clearance of WT-VWF was significantly reduced in the presence of RAP (Figure 6). Interestingly, the increased clearance of VWF-N1515Q and VWF-N1574Q were also both significantly attenuated in the presence of RAP (Figure 6). These findings are consistent with those observed above following clodronate-induced macrophage depletion (Figure 4A-B) and suggest that the VWF N-linked glycans at N1515 and N1574 modulate macrophage-dependent clearance at least in part through an LRP1-mediated mechanism.

Removal of the N-linked glycan at N1515 does not enhance clearance in VWF with a structurally constrained A2 domain

Large complex N-linked glycans, such as those expressed in the VWF A2 domain, have been previously shown to have important effects on glycoprotein conformation.⁵⁴⁻⁵⁷ We hypothesized that loss of A2 domain N-linked glycans causes conformational changes that result in enhanced VWF clearance by macrophages. Interestingly, recent studies have described a structurally constrained VWF variant with an engineered long-range disulfide bond (Cys1493-Cys1669) within the A2 domain (Figure 7A).⁴⁵ To address our hypothesis, we therefore proceeded to express the N1515Q mutation in this cysteine-clamp VWF variant (VWF-CC-N1515). Insertion of the cysteine clamp alone in A2 (VWF-CC) had no significant effect upon A1-A2-A3 clearance compared with WT A1-A2-A3 (Figure 7B). Importantly however, the rapid in vivo clearance of A1-A2-A3 associated with loss of the N1515 glycan was ablated in the presence of this structurally constrained A2 domain (Figure 7B). Collectively, these data suggest that loss of the N-linked glycans in A2 trigger enhanced VWF clearance by macrophages through conformational changes.

Discussion

Although the biosynthesis, structure, and functional properties of VWF have been well characterized, the molecular mechanisms through which VWF is cleared remain poorly understood.^{2,14} However, accumulating data have demonstrated that macrophages play important roles in regulating VWF clearance in vivo,²¹⁻²⁶ and a number of putative macrophage receptors for VWF have been identified.^{23,26,38} Critically, however, the specific regions of the VWF glycoprotein involved in modulating interactions with these different macrophage receptors have not been determined. In this study, using a series of in vivo and in vitro methodologies, we demonstrate that the A1-A2-A3 domains of VWF contain a receptor-recognition site important in mediating VWF binding to macrophages in vitro and in regulating VWF clearance by macrophages in vivo. Furthermore, studies using isolated recombinant A domains demonstrated that the A1 domain plays a critical role in regulating macrophage binding. This observation is in keeping with previous studies demonstrating that full-length VWF binding to macrophages is significantly enhanced in the presence of ristocetin, botreocetin, or shear stress.^{22,23,26} Importantly, however, our novel data further demonstrate that the ability of the isolated A1 domain to interact with macrophages is markedly attenuated when the A1 domain is linked to the other A domains (A1-A2-A3 truncation), suggesting that the receptor binding site may not be accessible in normal globular VWF.

Previous reports have demonstrated that desialylation of human VWF results in a marked reduction in plasma half-life.^{29,32-34} In addition, although the molecular mechanisms involved were not elucidated, enhanced VWF clearance has also been associated with loss of specific O-linked glycans.^{30,58} In this paper, we demonstrate that complete loss of N-linked glycan expression following PNGase F digestion also results in markedly enhanced macrophage-mediated VWF clearance. Interestingly, recent studies have reported that N-linked glycan expression on factor X also plays a key role in

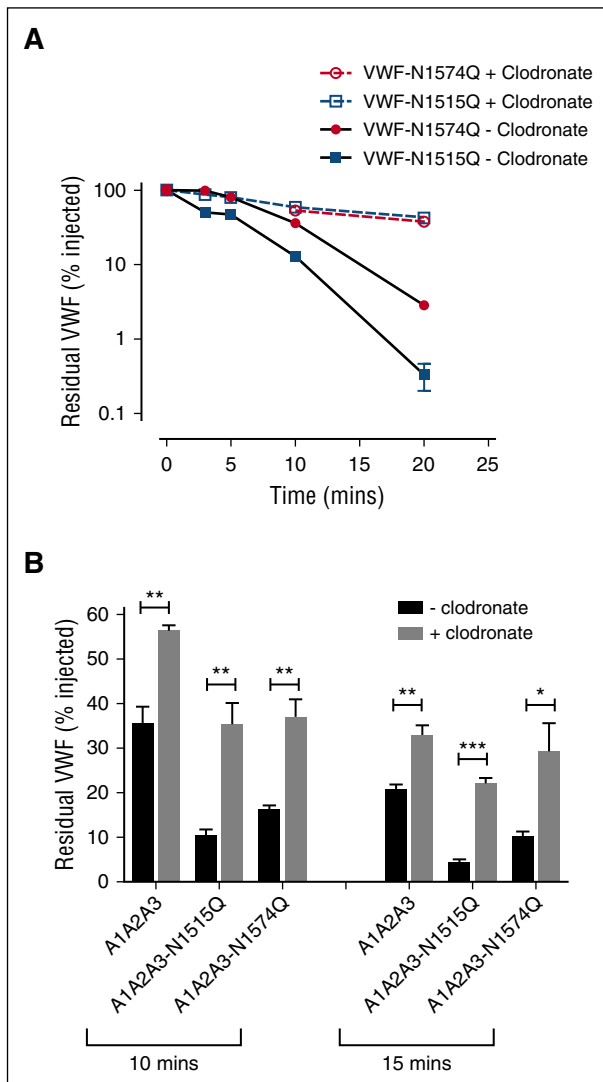


Figure 4. Accelerated clearance of VWF N1515Q and VWF N1574Q is mediated by macrophages. (A) In order to assess the potential contribution of macrophages in modulating the enhanced clearance of VWF glycan variants, clearance of VWF N1515Q and VWF N1574Q was repeated in VWF^{-/-} mice 24 hours after clodronate-induced macrophage depletion. (B) To determine whether macrophages play a role in regulating the reduced survival of A1A2A3-N1515Q and A1A2A3-N1574Q, in vivo clearance studies were also re-assessed in VWF^{-/-} mice following clodronate treatment. Data are graphed as percentage residual VWF relative to the amount injection (* $P < .05$, ** $P < .01$, and *** $P < .001$).

regulating interaction with macrophages.⁵⁹ Although the molecular mechanism through which N-linked glycan determinants regulate coagulation glycoprotein clearance remains unknown, the effect may be due to general properties of the complex sugar chains or instead may be attributable to particular carbohydrate structures located at specific N-linked sites.^{42,43} Given the role of the A1-A2-A3 domains in modulating the interaction of VWF with macrophages, we further investigated whether the 2 N-linked glycosylation sites located at N1515 and N1574, respectively, might play a particular role in regulating VWF clearance. Importantly, we observed that loss of N-linked glycans following site-directed mutagenesis at either N1515 or N1574 resulted in markedly enhanced clearance of full-length rVWF. Furthermore, introduction of the N1515Q or N1574Q substitutions into the A1-A2-A3 fragment also resulted in significantly enhanced clearance.

In addition, the enhanced clearance phenotypes observed with N1515Q and N1574Q in full-length VWF, and also in the truncated A1-A2-A3 fragment, were all significantly attenuated following clodronate-induced macrophage depletion. Altogether, these novel findings therefore demonstrate that the N-linked glycan structures within the A2 domain play an important role in protecting VWF against macrophage-mediated clearance in vivo. Moreover, the reduced survival of VWF observed following loss of the N-linked glycan structures in A2 is predominantly due to local effects within the A1-A2-A3 region triggering enhanced macrophage clearance.

Accumulating data suggest that the LRP1 receptor may play a key role in regulating macrophage binding and clearance of VWF.^{23,26} Furthermore, Wohner et al recently reported that the A1 domain of VWF (but not the isolated A2 or A3 domains) could bind to purified LRP1 in vitro.²⁶ Consequently, we investigated whether the LRP1 receptor may be involved in modulating the enhanced clearance of VWF-N1515Q and VWF-N1574Q. Interestingly, the reduced survival of both VWF-N1515Q and VWF-N1574Q was significantly attenuated in the presence of RAP. These findings suggest that the N-linked glycan expressed at N1515 and N1574 play a critical role in protecting VWF against LRP1-mediated macrophage clearance. Although the molecular mechanism(s) through which these N-linked glycans regulate LRP1-mediated clearance remain unclear, previous studies have demonstrated that expression of carbohydrate determinants can directly influence glycoprotein interactions through either charge-mediated mechanisms, or by modifying glycoprotein conformation.⁵⁴⁻⁵⁷ Consequently, we hypothesize that the protective effect of the large complex N-linked glycan structures in the A2 domain may be due to steric hindrance, with covering of cryptic LRP1 binding sites. Alternatively, and perhaps more likely, variation in A2 domain carbohydrate structures may cause conformational changes that result in enhanced LRP1-modulated clearance. This hypothesis is supported by the observation that removal of the N-linked glycans at N1515 no longer enhances VWF clearance in the presence of a structurally constrained A2 domain. Although our findings

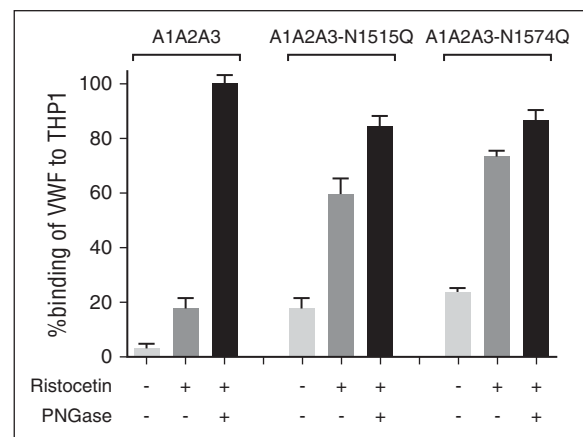


Figure 5. N-linked glycans N1515 and N1574 modulate in vitro binding of VWF to macrophages. To examine the biological mechanisms mediating the enhanced clearance of A1A2A3-N1515Q/N1574Q, we assessed binding to THP-1 macrophages in vitro. The binding of A1A2A3 VWF and the glycan variants A1A2A3-N1515Q and A1A2A3-N1574Q to THP-1 macrophages was examined in the presence or absence of 1mg/ml ristocetin. Additionally, all the A1A2A3 variants were subjected to PNGase treatment to remove both N-linked glycans (black columns) and THP-1 macrophage binding was measured. Data are graphed as percentage binding relative to maximal binding (mean \pm SEM).

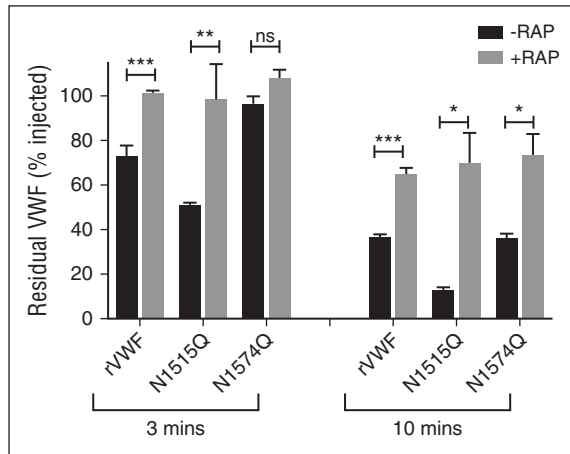


Figure 6. Glycan structures at N1515 and N1574 in the A2 domain influence LRP1-mediated clearance. Recent studies have shown that macrophage LRP1 plays an important role in regulating in vivo clearance of VWF. Moreover, RAP prolongs VWF survival in vivo predominantly by inhibiting this macrophage LRP1 mediated clearance. To investigate whether the effect of VWF glycans on macrophage-mediated clearance were modulated via LRP1, clearance studies for wild type rVWF and glycan variants N1515Q and N1574Q were repeated in VWF^{-/-} mice in the presence or absence of the LRP1 antagonist RAP. Blood was collected at 3 and 10 minutes after injection, and data are graphed as percentage residual VWF relative to the amount injected (**P* < .05, ***P* < .01, and ****P* < .001; ns, not significant).

demonstrate a critical role for LRP1 in modulating macrophage-mediated clearance of VWF glycoforms, it is important to consider that a number of other macrophage receptors can also bind VWF.^{38,39,60} Included among these macrophage receptors are the lectins ASGPR, Gal-1, and Gal-3. We observed that VWF binding to both galectins was significantly reduced following loss of N-linked glycans (data not shown). Moreover, ASGPR inhibition with asiolo-orosomucoid did not attenuate the enhanced in vivo clearance of VWF-N1515Q. Nevertheless, previous studies have shown that LRP1 can form heterologous functional complexes with a number of other macrophage receptors, including β 2-integrins.⁶⁰ Additional studies will be necessary to determine whether the N1515 and/or N1574 glycans influence VWF interactions with any of these other macrophage receptors in addition to LRP1.

Previous studies have demonstrated that VWF clearance occurs independently of ADAMTS13 proteolysis and is not influenced by VWF multimer size.^{52,61} Nonetheless, it is interesting that in addition to influencing VWF clearance by macrophages, the N-linked glycans expressed within the A2 domain of VWF have also been shown to modulate susceptibility to proteolysis by ADAMTS13.^{42,62} In particular, McKinnon et al showed that loss of the N-linked glycan at N1574 resulted in significantly enhanced VWF proteolysis by ADAMTS13.⁴² Furthermore, differential scanning fluorimetry has confirmed that glycosylation at N1574 plays an important role in stabilization of the A2 domain against unfolding.⁴⁴ However, in contrast to its marked effect upon clearance, loss of the VWF N1515 glycan did not significantly influence susceptibility to ADAMTS13 cleavage and did not have any significant effect on the thermostability of the A2 domain.^{42,44} Further studies will be necessary to define the biological mechanisms through which the carbohydrate structures at N1515 and N1574 regulate macrophage-mediated clearance. However, it is interesting that ABO(H) blood group determinants, which influence both VWF proteolysis by ADAMTS13 and VWF clearance,

are expressed on both of these complex N-linked glycans within the A2 domain of pd-VWF.^{7,42}

In the normal circulation, VWF adopts a globular conformation, such that the glycoprotein Ib α (GPIb α) binding site in the VWF A1 domain remains largely hidden.⁶³ However, exposure to mechanical shear stress results in unwinding of globular VWF. As part of this unfolding process, previous studies have described both uncoupling of the A1A2A3 tridomain cluster, as well as conformational changes within the individual A domains.^{64,65} Consequently the platelet binding site in A1 becomes exposed, and the ADAMTS13 cleavage site (Tyr1605-Met1606) that is buried within the A2 domain becomes accessible.^{64,66} Our data, together with those recently published from other groups,^{22,23,26} suggest that conformational changes in the VWF A domains lead to exposure of cryptic receptor binding sites within A1-A2-A3 that trigger macrophage clearance. Given the thrombotic potential of VWF in its active conformation, targeting of unfolded VWF for rapid macrophage-mediated clearance has biological plausibility. Our findings are also consistent with the hypothesis that specific mutations within the A1 domain result in enhanced clearance due to increased binding to both platelet Gp1b α and macrophage LRP1.⁶⁷ Further studies will be required to define the roles played by LRP1 and other macrophage receptors in modulating the enhanced clearance phenotypes associated with type 1C mutations located in other VWF domains. Interestingly however, preliminary data suggest that several other independent regions of VWF (including D'D3 and D4) are also able to bind to LRP1.¹⁴

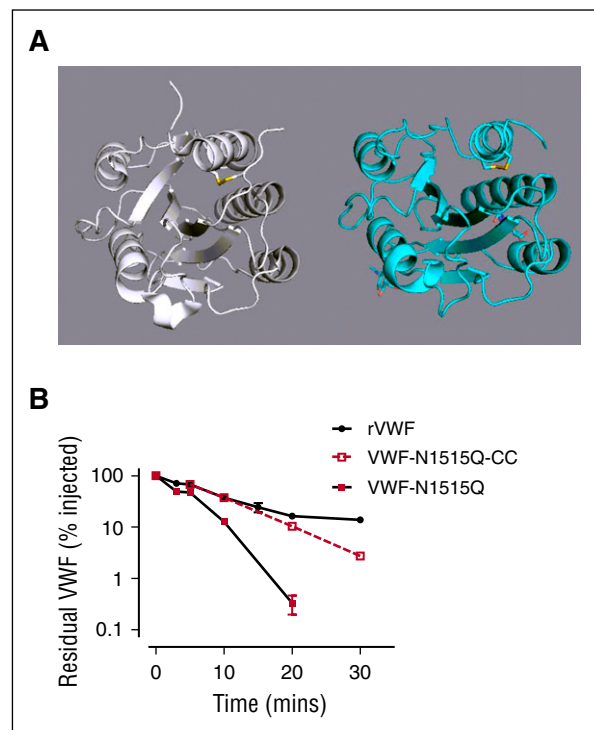


Figure 7. Removal of the N-linked glycans at N1515 does not enhance clearance in VWF with a structurally constrained A2 domain. (A) To examine a potential role for A2 domain conformation in modulating clearance VWF, a previously described cysteine-clamp mutation (N1493C/C1670S) was inserted into full-length rVWF (rVWF-CC) and VWF-N1515Q (VWF-N1515Q-CC). This mutation creates a structurally constrained A2 due to the presence of a long-range disulfide bridge, homologous to those present in the A1 and A3 domains. (B) Clearance was assessed in VWF^{-/-} mice. All results are plotted as percentage residual VWF:antigen levels relative to the amount injected. Data are presented as mean \pm SEM.

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Authorship

Contribution: A.C., J.M.O., S.A., G.B., S.W., C.D., T.M.B., and T.A.J.M. performed experiments; A.C., J.M.O., P.G.F., T.M.B.,

R.J.S.P., M.L., T.A.J.M., and J.S.O. designed the research and analyzed the data; and all authors were involved in writing and reviewing the paper.

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N-linked glycans within the A2 domain of von Willebrand factor modulate macrophage-mediated clearance

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ORIGINAL ARTICLE

N-linked glycan truncation causes enhanced clearance of plasma-derived von Willebrand factor

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Essentials

- von Willebrand factor (VWF) glycosylation plays a key role in modulating *in vivo* clearance.
- VWF glycoforms were used to examine the role of specific glycan moieties in regulating clearance.
- Reduction in sialylation resulted in enhanced VWF clearance through asialoglycoprotein receptor.
- Progressive VWF N-linked glycan trimming resulted in increased macrophage-mediated clearance.

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Summary. *Background:* Enhanced von Willebrand factor (VWF) clearance is important in the etiology of both type 1 and type 2 von Willebrand disease (VWD). In addition, previous studies have demonstrated that VWF glycans play a key role in regulating *in vivo* clearance. However, the molecular mechanisms underlying VWF clearance remain poorly understood. *Objective:* To define the molecular mechanisms through which VWF N-linked glycan structures influence *in vivo* clearance. *Methods:* By

use of a series of exoglycosidases, different plasma-derived VWF (pd-VWF) glycoforms were generated. *In vivo* clearance of these glycoforms was then assessed in VWF^{-/-} mice in the presence or absence of inhibitors of asialoglycoprotein receptor (ASGPR), or following clodronate-induced macrophage depletion. *Results:* Reduced amounts of N-linked and O-linked sialylation resulted in enhanced pd-VWF clearance modulated via ASGPR. In addition to this role of terminal sialylation, we further observed that progressive N-linked glycan trimming also resulted in markedly enhanced VWF clearance. Furthermore, these additional N-linked glycan effects on clearance were ASGPR-independent, and instead involved enhanced macrophage clearance that was mediated, at least in part, through LDL receptor-related protein 1. *Conclusion:* The carbohydrate determinants expressed on VWF regulate susceptibility to proteolysis by ADAMTS-13. In addition, our findings now further demonstrate that non-sialic acid carbohydrate determinants expressed on VWF also play an unexpectedly important role in modulating *in vivo* clearance through both hepatic ASGPR-dependent and macrophage-dependent pathways. In addition, these data further support the hypothesis that variation in VWF glycosylation may be important in the pathophysiology underlying type 1C VWD.

Keywords: glycosylation; macrophages; metabolic clearance rate; von Willebrand disease; von Willebrand factor.

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Introduction

In normal plasma, von Willebrand factor (VWF) circulates as a complex multimeric sialoglycoprotein [1,2]. VWF plays critical roles in normal hemostasis, by mediating platelet adhesion to exposed subendothelial tissues at

sites of vascular injury, and serving as a carrier molecule for procoagulant factor VIII [2,3]. *In vivo* expression of VWF occurs only in endothelial cells (ECs) [4] and megakaryocytes [5]. VWF synthesized in ECs is either secreted constitutively into the plasma, or stored in intracellular Weibel–Palade bodies. During its synthesis, VWF undergoes complex post-translational modifications, including significant glycosylation and multimerization [2,6]. This begins in the endoplasmic reticulum, where high-mannose (Man)-containing oligosaccharide chains are added to the N-linked sites of VWF monomers prior to dimerization [7]. Subsequently, in the Golgi, the N-linked glycans of VWF undergo sequential modification to generate complex-type carbohydrate structures, and O-linked glycosylation takes place [8]. Consequently, each VWF monomer contains 12 N-linked and 10 O-linked glycosylation sites, and these oligosaccharide chains together account for almost 20% of the final monomeric mass [9].

Recent mass spectrometry studies have characterized both the N-linked glycans and O-linked glycans of human plasma-derived VWF (pd-VWF) [10,11]. These complex carbohydrate structures on VWF show significant heterogeneity (Fig. 1). However, monosialylated or disialylated biantennary chains constitute the most common N-linked glycan (Fig. 1A,C) [9,11–13], whereas a disialyl core 1

structure is the most common O-linked glycan expressed on pd-VWF [10,14] (Fig. 1B,D). The carbohydrate chains of EC-derived VWF are unusual in that they also express terminal ABO(H) blood group determinants [13,15,16]. These ABO(H) determinants differ only with respect to a single sugar residue, and are expressed as terminal sugars on a minority of the N-linked (13%) and O-linked (1%) glycan chains of VWF [10,11]. The complex N-linked and O-linked carbohydrate structures of VWF are of direct physiologic relevance in that they influence several key aspects of VWF biology [17]. In particular, terminal sialic acid and ABO(H) determinants expressed on VWF glycans play a major role in modulating susceptibility to proteolysis by ADAMTS-13 [18–20].

The molecular mechanisms involved in regulating VWF clearance from the plasma remain poorly defined [21]. Nonetheless, recent studies have clearly demonstrated that enhanced VWF clearance plays an important role in the pathogenesis of von Willebrand disease (VWD) [21]. In particular, a number of single amino acid substitutions (including VWD-Vicenza R1205H) have been associated with enhanced clearance in patients with both type 1 and type 2 VWD [22–26]. Previous studies have demonstrated that VWF glycosylation also plays a major role in regulating *in vivo* clearance [27–30]. For example, Gallinaro *et al.* showed that ABO(H) blood group influences VWF

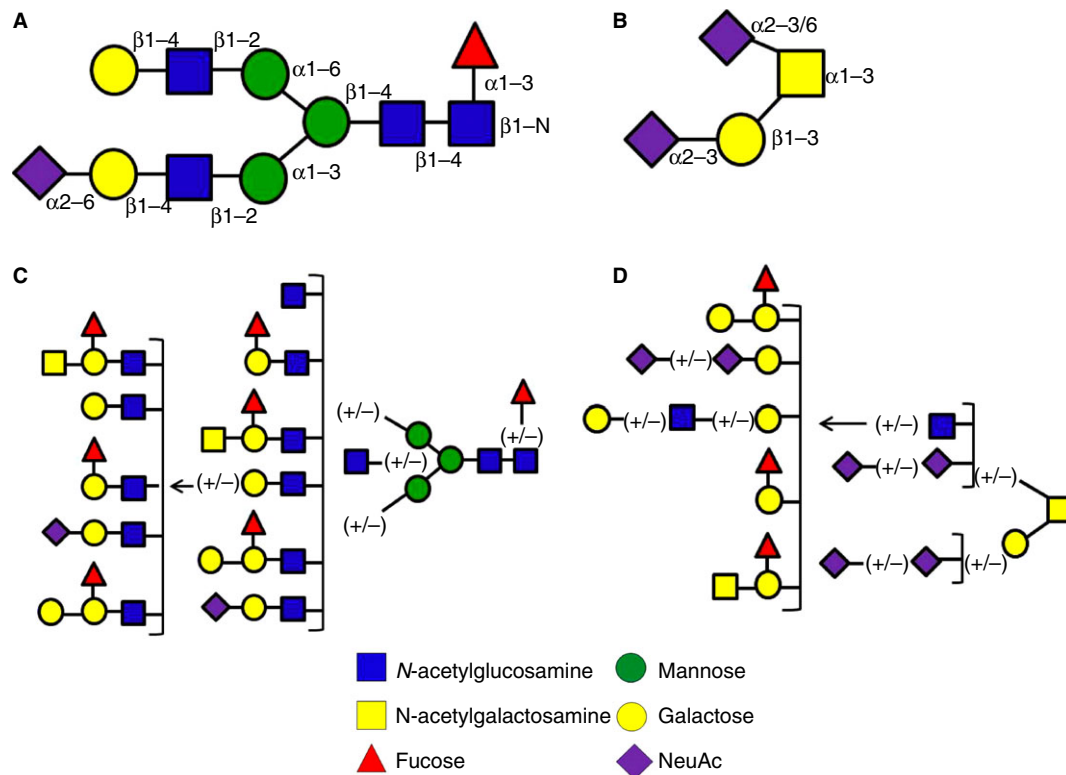


Fig. 1. N-linked and O-linked glycans expressed on human plasma-derived von Willebrand factor (pd-VWF) show marked heterogeneity. (A, B) The most common N-linked and O-linked glycan structures expressed on human pd-VWF. (C, D) Illustration of the marked degree of heterogeneity in the N-linked and O-linked glycans of von Willebrand factor. NeuAc, N-acetylneuraminic acid.

clearance [31]. Following DDAVP administration, VWF clearance was significantly faster in group O than in non-O individuals [32]. Interestingly, however, it remains unclear whether this ABO effect on VWF clearance is directly modulated via ABO(H) glycan determinants expressed on the N-linked glycans and/or O-linked glycans of VWF [33]. Enhanced VWF clearance in the RIIIS/J mouse has also been shown to result from abnormal EC glycosyltransferase expression [34]. All together, these data highlight the importance of VWF carbohydrate structures in modulating its clearance. Recent studies have identified a number of specific lectin receptors that can bind VWF. These include the asialoglycoprotein or Ashwell receptor (ASGPR) [35], galectin-1 [36], galectin-3 [36], Siglec-5 [37], and CLEC4M [38]. Critically, however, the molecular mechanisms through which VWF glycans modulate interaction with these lectins and/or other cellular receptors to regulate *in vivo* clearance have not been defined.

Recent studies have demonstrated that, in addition to the importance of desialylation, other changes in N-linked glycan structures also play critical roles in regulating the clearance of plasma glycoproteins. In particular, Yang *et al.* demonstrated that, as secreted glycoproteins age in the plasma, there is not only loss of sialic acid, but also progressive stepwise elimination of sugar moieties from the termini of their N-linked glycans [38]. The first step in this process is the loss of terminal α 2-6-linked sialic acid, with consequent exposure of subterminal Gal. However, because of the actions of endogenous glycosidases present in normal plasma, further progressive N-linked glycan remodeling then ensues, leading to the subsequent unmasking of *N*-acetylglucosamine (GlcNAc) and Man linkages (Fig. 1A) [39]. Using a series of VWF glycoforms, we sought to further investigate the role of specific glycan moieties expressed on pd-VWF in regulating its clearance *in vivo*. Our findings support the hypothesis that N-linked sialic acid protects VWF against clearance by ASGPR. Importantly, however, we further demonstrate that progressive loss of VWF N-linked glycans also causes markedly enhanced VWF clearance. Moreover, this increased clearance is ASGPR-independent, and is modulated at least in part via macrophage LDL receptor-related protein 1 (LRP1).

Materials and methods

Isolation and purification of human plasma and platelet VWF

pd-VWF was purified from the VWF-containing concentrate Haemate P (CSL Behring, Prussia, PA, USA), as previously described [20,40]. Haemate P suspension was applied to a Sepharose CL-2B XK 16/70 gel filtration column (GE Healthcare Life Sciences, Amersham, UK) pre-equilibrated with a buffer containing 20 mM Tris-HCl and

10 mM NaCl (pH 7.4). VWF was eluted with the same buffer. Eluate fractions were then assessed for VWF antigen (VWF:Ag), multimer distribution, and purity [15,20,40].

In vitro modification of VWF glycan structures

A series of specific exoglycosidases were used to modify the glycosylation profile of pd-VWF, including α 2-3,6,8,9-neuraminidase (from *Arthrobacter ureafaciens*; Calbiochem, Merck Millipore, Feltham, UK), β 1-4-galactosidase (from *Streptococcus pneumoniae*; Calbiochem, Merck Millipore), and peptide *N*-glycosidase F (PNGase F) (from *Flavobacterium meningosepticum*; New England Biolabs, Hitchin, UK). VWF glycan digestions were carried out overnight under non-denaturing conditions at 37 °C, as previously reported [16,20,40].

Quantitative analysis of VWF glycan expression

Following glycosidase digestion, changes in VWF carbohydrate expression were assessed with the use of specific lectin ELISAs. In brief, purified VWF diluted in phosphate-buffered saline (PBS) with Tween-20 (0.5%) was captured with deglycosylated polyclonal anti-VWF 1 : 250 (Dako, Glostrup, Denmark) onto microtiter wells. The anti-VWF antibody was deglycosylated by PNGase F (New England Biolabs) treatment, overnight at 37 °C. Non-specific binding was blocked with Protein-Free Blocking Buffer (Thermo Fisher Scientific, Dublin, UK). Various concentrations of pd-VWF were incubated for 2 h at 37 °C. Biotinylated lectins, i.e. those from *Sambucus nigra* (0.1 μ g mL⁻¹), *Maackia amurensis* (2.5 μ g mL⁻¹), and *Ricinus communis* (0.5 μ g mL⁻¹) (Vector Laboratories, Peterborough, UK), were incubated for 1 h. Lectin binding was detected with high-sensitivity streptavidin-horseradish peroxidase (HRP) (Pierce, Thermo Fisher Scientific, Dublin, UK), and subsequent incubation with the substrate 3,3',5,5'-tetramethylbenzidine (R&D Systems, Abingdon, UK). Absorbance was read at 450 nm, and lectin binding was expressed as a percentage of control unmodified pd-VWF. All ELISAs were repeated three times, and dilutions were measured per duplicate.

Human receptor-associated protein (RAP) expression and purification

Human RAP cDNA (UNIPROTKB-P30533) was inserted into Novagen pET28a(+) bacterial expression vector (Novagen, Nottingham, UK). Recombinant RAP protein was refolded from *Escherichia coli* inclusion bodies as described previously [41].

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

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

All animal experiments were approved by the Animal Research Ethics Committee, Trinity College Dublin, and were performed in compliance with the Irish Medicines Board regulations on mice between 6 weeks and 10 weeks of age. VWF^{-/-} mice were given 0.75 U of pd-VWF or glycoforms thereof, via lateral tail vein injection. Mice were anesthetized with 2.5% tribromoethanol (0.2 mL per 10 g of body weight), and blood was collected from a subclavicle incision into heparin-coated microcontainers at appropriate time points. Three to five mice per time point were used. Residual plasma VWF:Ag levels were determined at specific time points by ELISA. In preliminary experiments, we demonstrated that the polyclonal rabbit anti-human VWF antibody used in this ELISA was not influenced by VWF glycans. For macrophage depletion, mice were injected intravenously with clodronate liposomes (100 μ L per 10 g of body weight) 24 h prior to injection of VWF as previously described [42,43]. Control mice were injected with PBS liposomes. Additionally VWF clearance was performed in the presence of the antagonist asialo-orosomucoid (ASOR). Orosomucoid protein (Sigma Aldrich, Arklow, Wicklow, UK) was treated with 16 mU of α 2-3,6,8,9-neuraminidase, to produce ASOR. Mice were intravenously injected with 300 μ g of ASOR in 100 μ L of PBS 1 min prior to injection of VWF. Alternatively, mice were given 200 μ M LRP1 antagonist RAP 1 min prior to injection of VWF.

Tissue collection

VWF^{-/-} mice were injected intravenously with pd-VWF or pd-VWF-derived glycoforms. Control mice were injected with PBS. Ten minutes after infusion, mice were killed, tissues were perfused, and livers were collected into 10% buffered formalin. The fixed tissues were processed and embedded in paraffin wax. Four-micrometer tissue sections were cut, dewaxed, and incubated with peroxidase-conjugated polyclonal rabbit anti-human VWF (Dako). Chromogen detection was performed with diaminobenzidine (DAB) (Dako, Carpinteria, CA, USA) solution (0.5 mL of stock DAB in 4.5 mL of Tris buffer with 20 μ L of hydrogen peroxide). Slides were counterstained with hematoxylin, and examined by light microscopy.

In vitro LRP1 cluster IV binding studies

VWF binding to recombinant LRP1 cluster IV (rLRP1-cIV) was performed as previously described [44]. Briefly, human rLRP1-cIV (R&D Systems) was immobilized on a microtiter plate (Maxisorb; Nunc, Thermo Fisher, Dublin, Ireland) at 5 μ g mL⁻¹ overnight at 4 °C. Wells were blocked with Tris-buffered saline containing 0.1% Tween-20 and 2.5 mM CaCl₂ supplemented with 5% bovine serum albumin for 2 h at 37 °C. pd-VWF (10–0 μ g mL⁻¹) diluted in assay buffer was added to immobilized rLRP1-cIV in the presence and absence of ristocetin (1 mg mL⁻¹) or RAP

(10 μ g mL⁻¹), and incubated for 2 h at 37 °C. Bound VWF was probed with HRP-labeled anti-VWF antibody (Dako) for 1 h at 37 °C.

Data presentation and statistical analysis

All experimental data were analyzed with GRAPHPAD PRISM version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). Data were expressed as mean values \pm standard error of the mean. To assess statistical differences, data were analyzed with Student's unpaired two-tailed *t*-test. For all statistical tests, *P*-values of < 0.05 were considered to be significant. Clearance study data were fitted to monoexponential or biexponential equations, based on analysis of the Akaike information criterion. The slope and intercept of the equation of the line were used to calculate pharmacokinetic parameters, including mean residence time and half-life (*t*_{1/2}).

Results

VWF glycan expression following glycosidase digestion

To investigate the role of glycans in modulating pd-VWF clearance, VWF was treated with a series of exoglycosidases to generate specific VWF glycoforms (Fig. 2A). In previous studies, we and others have demonstrated that these glycosidases have no significant effect on pd-VWF multimer composition or collagen-binding activity [20,40,45]. Following each glycosidase digestion (with α 2-3,6,8,9-neuraminidase, β 1-4-galactosidase, and PNGase F, respectively), residual VWF glycan expression was assessed with a panel of lectin-binding ELISAs. Sialic acid expression on pd-VWF was characterized with *S. nigra* (specific affinity for α 2-6-linked sialic acid) and *M. amurensis* (specific affinity for α 2-3-linked sialic acid) lectin-binding ELISAs (Fig. 2B,C). VWF digested with α 2-3,6,8,9-neuraminidase (Neu-VWF) demonstrated a significant reduction in sialic acid expression as detected by both *S. nigra* and *M. amurensis* lectin-binding ELISAs. Importantly, VWF digested with PNGase F (PNG-VWF) also showed a marked reduction in total *S. nigra* binding, but no significant effect on *M. amurensis* binding. These findings are consistent with previous mass spectrometry and HPLC data suggesting that the terminal sialic acid on the N-linked glycans of pd-VWF is predominantly α 2-6-linked to penultimate galactose (Gal) residues [11,20]. Conversely, the O-linked sialic acid on pd-VWF may be either α 2-6-linked or α 2-3-linked [10,14]. Following pd-VWF digestion with neuraminidase, binding of *R. communis* to exposed terminal β -linked Gal residues in Neu-VWF was significantly increased (Fig. 2D). In order to further investigate the role of this subterminal Gal residue in regulating VWF clearance, pd-VWF was digested with α 2-3,6,8,9-neuraminidase followed by β 1-4-galactosidase to create NeuGal-VWF. Treatment with

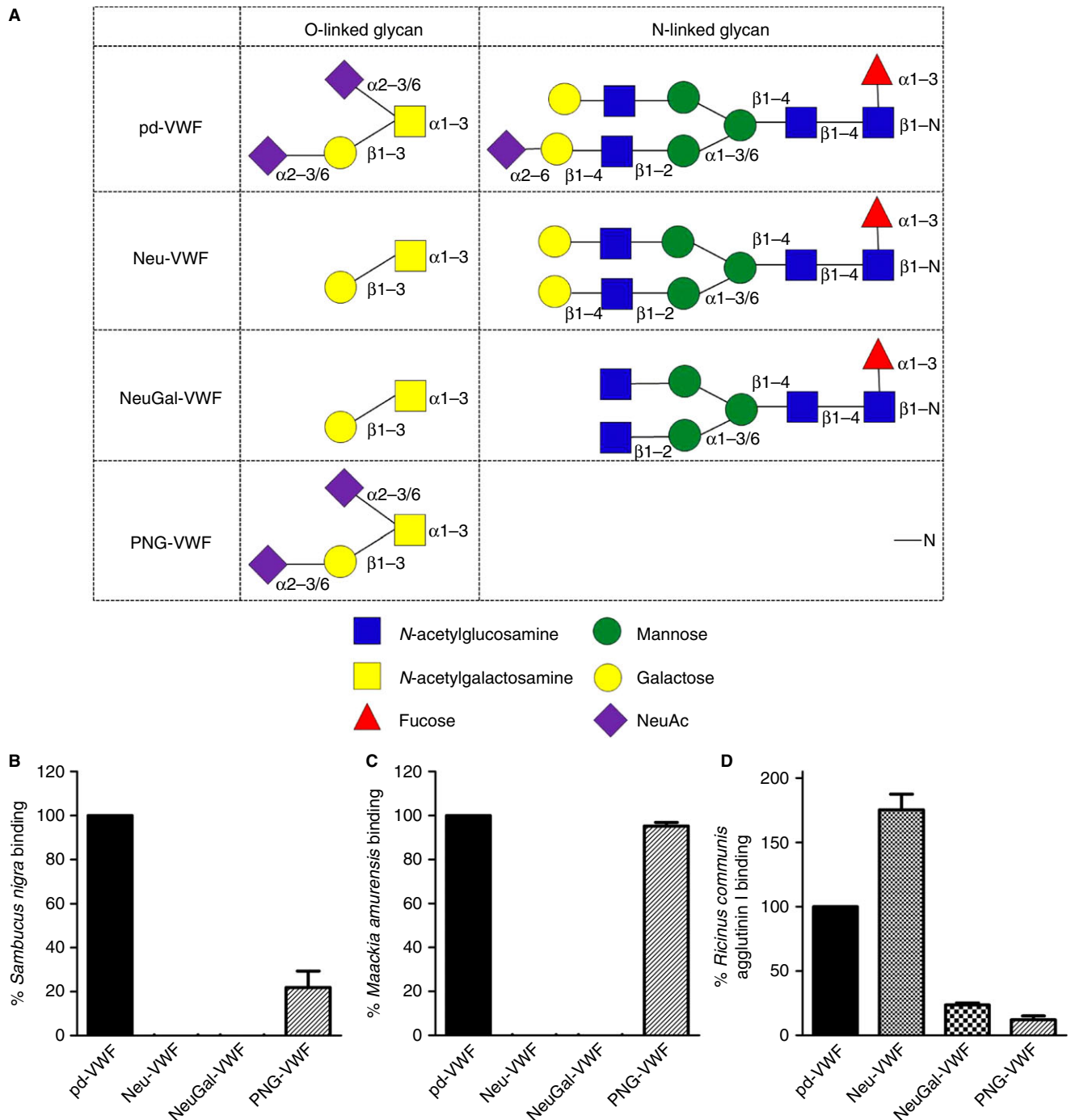


Fig. 2. N-linked and O-linked glycans expressed on human plasma-derived von Willebrand factor (pd-VWF) before and after exoglycosidase digestion. (A) Residual glycan structures expressed on von Willebrand factor (VWF) digested with α 2-3,6,8,9-neuraminidase (Neu-VWF), VWF digested with α 2-3,6,8,9-neuraminidase followed by β 1-4-galactosidase (NeuGal-VWF), and VWF digested with peptide N-glycosidase F (PNG-VWF). Treatment with α 2-3,6,8,9-neuraminidase results in the loss of capping sialic acid and exposure of subterminal galactose (Gal) on both the N-linked and O-linked glycans of pd-VWF. Sequential digestion with both α 2-3,6,8,9-neuraminidase and β 1-4-galactosidase leads to the exposure of N-acetylglucosamine at the termini of N-linked glycan chains. Finally, peptide N-glycosidase F treatment results in the loss of N-linked glycan structures, but does not influence O-linked carbohydrate determinants. (B, C) Following glycosidase digestions, residual expression of α 2-3,6-linked and residual expression of α 2-3-linked sialic acid were analyzed with modified *Sambucus nigra* (B) and *Maackia amurensis* (C) lectin ELISAs, respectively. (D) Similarly, expression of terminal β 1-4 Gal residues following each glycosidase digestion was assessed with *Ricinus communis* lectin ELISA. All ELISAs were performed in triplicate, and the results presented represent the mean values \pm standard error of the mean, unless otherwise stated. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$, respectively. NeuAc, N-acetylneuraminic acid.

β 1-4-galactosidase successfully removed the Gal residues exposed following neuraminidase digestion (Fig. 2D), leaving GlcNAc residues as terminal moieties on the N-linked glycans (Fig. 2A). Collectively, this series of *ex vivo* glycosidase digestions of pd-VWF was thus utilized to generate a series of VWF glycoforms with progressively reduced N-linked glycans (Fig. 2A).

Glycan expression on pd-VWF plays a critical role in regulating *in vivo* clearance

Following tail vein infusion in VWF^{-/-} mice, purified pd-VWF was cleared in a biphasic manner, characterized by a rapid initial phase ($t_{1/2} = 10 \pm 4.3$ min) and a slower secondary phase ($t_{1/2} = 48.1 \pm 10$ min) [33,46]. In keeping with previous reports [28,29], desialylation of pd-VWF with α 2-3,6,8,9-neuraminidase markedly enhanced VWF clearance (Fig. 3). Exposed Gal and GlcNAc residues following sialic acid removal from pd-VWF glycans can be recognized by ASGPR. To investigate whether other VWF carbohydrate determinants influence pd-VWF clearance, the effect of combined sialic acid and subterminal Gal removal from pd-VWF (creating NeuGal-VWF) was

studied. We observed that clearance of NeuGal-VWF was significantly enhanced as compared with pd-VWF ($t_{1/2} = 2.9 \pm 0.1$ min; $P < 0.05$) (Fig. 3). Furthermore, PNGase F digestion to remove the N-linked glycans of pdVWF also resulted in a markedly decreased plasma half-life ($t_{1/2} = 1.4 \pm 0.03$ min) (Fig. 3). Interestingly, the clearance of PNG-VWF was actually significantly faster than that of Neu-VWF at all time points ($P < 0.05$). In keeping with previous studies [45,46], infusion of Neu-VWF, NeuGal-VWF and PNG-VWF had no significant effect on murine platelet counts (data not shown). All together, these data are in keeping with previous studies in confirming the importance of VWF sialylation in determining its clearance *in vivo*. However, our data further demonstrate that other changes in N-linked glycan expression also play critical roles in regulating the plasma half-life of VWF.

The N-linked glycan of VWF regulates clearance through both ASGPR and additional macrophage-dependent pathways

In preliminary studies, we confirmed that *in vivo* clearance of pd-VWF was significantly inhibited in the presence of ASOR, and following clodronate-induced macrophage depletion (Fig. S1). To determine the role of the ASGPR in modulating the effects of VWF glycans on clearance, VWF glycoform clearance studies were repeated in the presence of ASOR. To investigate whether VWF glycan expression influences macrophage-mediated clearance, studies were also repeated in VWF^{-/-} mice following clodronate administration. In keeping with previous reports [29,35], we found that the increased clearance phenotype of Neu-VWF was significantly attenuated in the presence of ASOR (data not shown), confirming that ASGPR plays a critical role in modulating the enhanced clearance of hyposialylated VWF. Both ASOR and macrophage depletion had some effect in reversing the rapid clearance of NeuGal-VWF, which expresses terminal GlcNAc residues on its N-linked glycans (Fig. 4A). The ASOR effect probably reflects the fact that residual β 1-3-Gal remains on the O-linked glycans of VWF following β 1-4-galactosidase digestion and can still facilitate ASGPR-mediated clearance (Fig. 2A). In contrast to what was found for Neu-VWF and NeuGal-VWF, the markedly enhanced clearance of PNG-VWF was not significantly inhibited by ASOR (Fig. 4B). For example, no residual plasma PNG-VWF was detectable in either the presence or the absence of ASOR by 10 min following infusion. Although ASOR had no significant effect, macrophage depletion by liposomal clodronate significantly inhibited the enhanced clearance of PNG-VWF, such that ~30% of the infused PNG-VWF was still detectable after 10 min.

To further investigate the cellular basis underlying the enhanced clearance of pd-VWF following loss of N-linked glycans, VWF^{-/-} mice were injected with either PBS control, NeuGal-VWF, or PNG-VWF. Ten minutes following

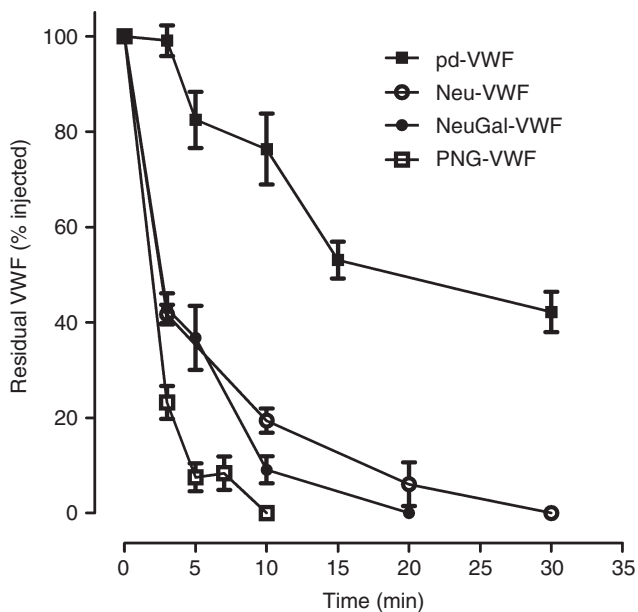


Fig. 3. Glycan expression on plasma-derived von Willebrand factor (pd-VWF) plays a critical role in regulating *in vivo* clearance. To investigate the role of von Willebrand factor (VWF) carbohydrate determinants in modulating VWF clearance, purified human pd-VWF was treated with either α 2-3,6,8,9-neuraminidase (giving Neu-VWF), a combination of α 2-3,6,8,9-neuraminidase and β 1-4-galactosidase (giving NeuGal-VWF), or peptide *N*-glycosidase F (giving PNG-VWF). *In vivo* clearance was then assessed in VWF^{-/-} mice, and compared with that of wild type pd-VWF. At each time point, the residual circulating VWF concentration was determined by VWF antigen (VWF:Ag) ELISA. All results are plotted as percentage residual VWF:Ag levels relative to the amount injected. Data are represented as mean \pm standard error of the mean (SEM). In some cases, the SEM cannot be seen, owing to its small size.

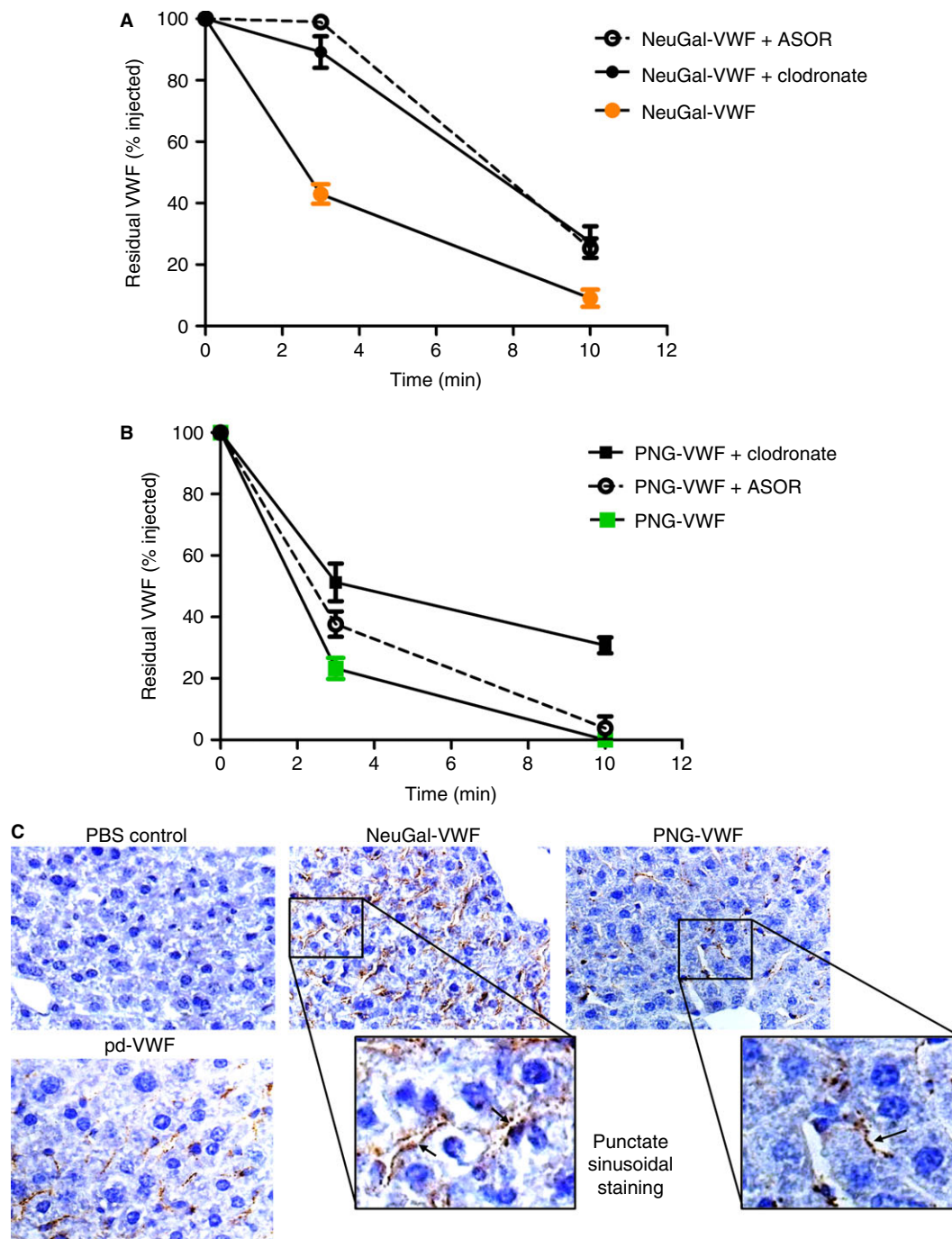


Fig. 4. The N-linked glycans of von Willebrand factor (VWF) regulate clearance through both asialoglycoprotein receptor (ASGPR) and additional macrophage-dependent pathways. ASGPR and macrophages have both been implicated in regulating the *in vivo* clearance of VWF. To study the roles of ASGPR and macrophage-mediated clearance in modulating the effects of VWF glycans on half-life, clearance studies were repeated in $VWF^{-/-}$ mice in the presence or absence of asialo-orosomucoid (ASOR) or following clodronate-induced macrophage depletion. (A) Both ASOR and macrophage depletion significantly attenuated the rapid clearance of VWF digested with $\alpha 2-3,6,8,9$ -neuraminidase followed by $\beta 1-4$ -galactosidase (NeuGal-VWF). (B) In contrast to what was found for NeuGal-VWF, ASOR did not significantly inhibit the markedly increased clearance of VWF digested with peptide *N*-glycosidase F (PNG-VWF). However, macrophage depletion by liposomal clodronate significantly attenuated the enhanced clearance of PNG-VWF. (C) To further investigate the cellular basis underlying the enhanced clearance of pd-VWF following loss of N-linked glycans, $VWF^{-/-}$ mice were injected with either phosphate-buffered saline (PBS) control or 300 nm pd-VWF, NeuGal-VWF or PNG-VWF diluted in 100 μ L of sterile-filtered PBS. Ten minutes following infusion, mice were killed, tissues were perfused, and livers were collected into 10% buffered formalin. The fixed tissues were then stained with peroxidase-conjugated rabbit anti-human VWF, as described in Materials and methods. Finally, slides were counterstained with hematoxylin (magnification: $\times 400$).

infusion, murine livers were collected, fixed, and stained with peroxidase-conjugated rabbit anti-human VWF. We observed no anti-VWF staining in liver sections of VWF^{-/-} mice after treatment with PBS (Fig. 4C). In contrast, liver sections from mice treated with NeuGal-VWF or PNG-VWF showed significant VWF staining along liver sinusoids, most likely Kupffer cells (Fig. 4C). Collectively, these data demonstrate that, in addition to VWF sialylation regulating ASGPR-mediated clearance, other N-linked glycan structures on pd-VWF also influence *in vivo* clearance through additional macrophage-dependent mechanisms.

Loss of VWF N-linked glycans causes enhanced LRP1-modulated clearance by macrophages

Recent studies have demonstrated that macrophage LRP1 binds to VWF in a shear-dependent manner, and is important in regulating VWF clearance *in vivo* [44,47,48]. To investigate whether VWF glycan expression influences this LRP1-mediated macrophage clearance pathway, VWF glycoform clearance studies in VWF^{-/-} mice were repeated in the presence or absence of RAP. Rastegarlarani *et al.* have previously demonstrated that the effects of RAP on VWF clearance are predominantly modulated through inhibition of macrophage LRP1 [48]. In keeping with previous reports, we confirmed that clearance of pd-VWF was significantly reduced in the presence of RAP (Fig. 5A) [48]. In contrast, the presence of RAP did not significantly influence the increased clearance of Neu-VWF (data not shown), consistent with the concept that this enhanced clearance is predominantly mediated through ASGPR. Interestingly, however, the increased clearance of NeuGal-VWF and, particularly, that of PNG-VWF were both significantly attenuated in the presence of RAP (Fig. 5B,C). These findings are consistent with what was observed following clodronate-induced macrophage depletion (Fig. 4A,B). Wohner *et al.* recently reported that VWF binds to cluster IV of LRP1 [48]. In keeping with this observation, we confirmed that wild-type pd-VWF bound to purified rLRP1-cIV *in vitro* (Fig. 6A). Furthermore, this binding was abolished in the presence of RAP (Fig. 6B). Importantly however, we observed significantly enhanced binding of both NeuGal-VWF and PNG-VWF to cluster IV (Fig. 6C). All together, these *in vitro* and *in vivo* data suggest that VWF N-linked glycans modulate macrophage-dependent clearance at least in part through an LRP1-mediated mechanism.

Discussion

The biological mechanisms underlying VWF clearance *in vivo* remain poorly characterized [21]. However, previous data from studies in both humans and murine models have collectively demonstrated that VWF carbohydrate structures play a critical role in regulating its clearance

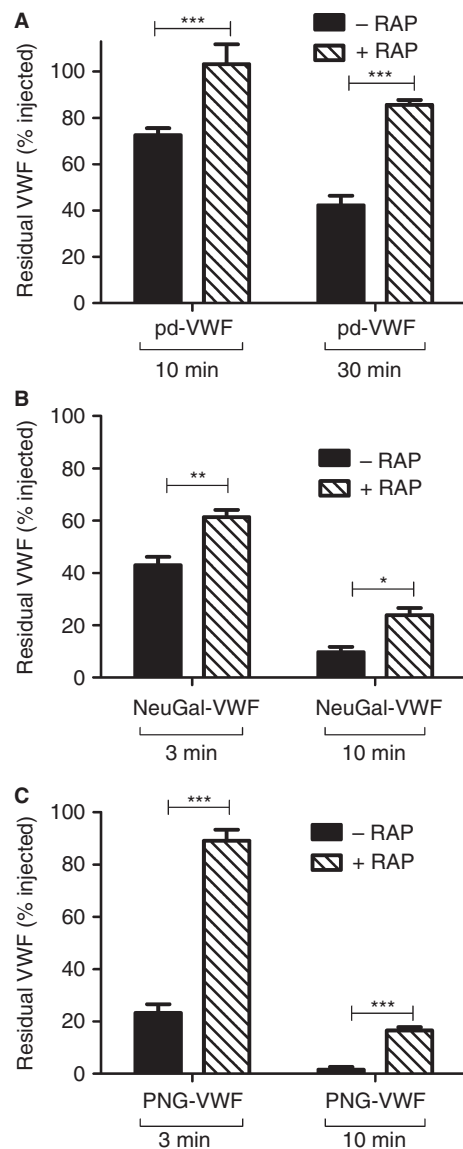


Fig. 5. Loss of von Willebrand factor (VWF) N-linked glycans causes enhanced LDL receptor-related protein 1 (LRP1)-modulated clearance by macrophages. Recent studies have shown that macrophage LRP1 plays an important role in regulating the *in vivo* clearance of VWF. Moreover, receptor-associated protein (RAP) prolongs VWF survival *in vivo* predominantly by inhibiting this macrophage LRP1-mediated clearance. To investigate whether the effects of VWF glycans on macrophage-mediated clearance were modulated via LRP1, *in vivo* clearance studies for wild type plasma-derived VWF (pd-VWF) (A), VWF digested with α 2-3,6,8,9-neuraminidase followed by β 1-4-galactosidase (NeuGal-VWF) (B) and VWF digested with peptide N-glycosidase F (PNG-VWF) (C) were repeated in VWF^{-/-} mice in the presence or absence of RAP.

[27–29,31,34,35]. Like many other plasma glycoproteins, the majority of the N-linked and O-linked glycan chains of human pd-VWF are capped by negatively charged sialic acid residues [10,11]. We have previously demonstrated that α 2-6-linked sialic acid expressed on the N-linked glycans of human pd-VWF plays an important role in regulating susceptibility to proteolysis by ADAMTS-13 [20,49].

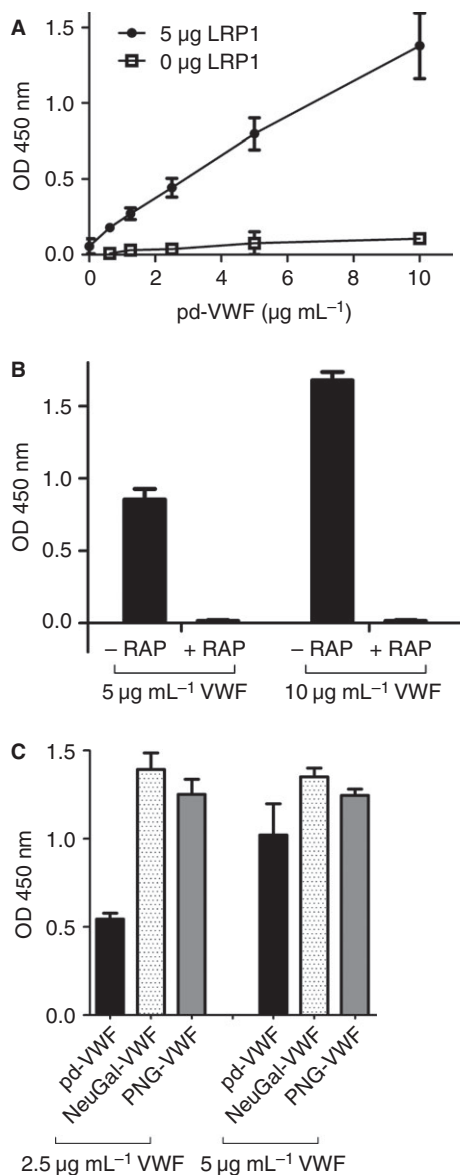


Fig. 6. Loss of von Willebrand factor (VWF) N-linked glycans causes enhanced LDL receptor-related protein 1 (LRP1) cluster IV binding *in vitro*. To directly investigate the role of VWF glycans in modulating binding to LRP1, purified human plasma-derived VWF (pd-VWF) was incubated on wells coated with recombinant human LRP1 cluster IV or control uncoated wells. (A) pd-VWF bound to human LRP1 cluster IV in a dose-dependent manner. (B) pd-VWF binding to LRP1 cluster IV was abolished in the presence of receptor-associated protein (RAP). (C) Binding of VWF digested with α 2-3,6,8,9-neuraminidase followed by β 1-4-galactosidase (NeuGal-VWF) and binding of VWF digested with peptide N-glycosidase F (PNG-VWF) to immobilized LRP1 cluster IV were significantly increased as compared with untreated pd-VWF. Data are represented as mean \pm standard error of the mean.

In keeping with previous studies, preliminary experiments in this study confirmed that combined removal of both N-linked and O-linked sialic acid following digestion with α 2-3,6,8,9-neuraminidase resulted in markedly increased VWF clearance in VWF^{-/-} mice [29]. Furthermore, this enhanced VWF clearance was significantly attenuated in the presence

of ASOR, supporting the hypothesis that ASGPR plays a major role in removing desialylated VWF from the circulation. Taken together, these findings therefore suggest that, during post-translational modification in ECs, the N-linked and O-linked oligosaccharide chains of VWF are sialylated in the Golgi prior to its secretion into the plasma. Following initial secretion, terminal α 2-6-linked sialic acid expressed predominantly on the N-linked glycans of VWF specifically enhance ADAMTS-13 cleavage at the endothelial surface, but also protect VWF against non-specific proteolysis by other plasma proteases. Finally, as the secreted VWF glycoprotein becomes progressively older in the plasma, these terminal sialic acid residues are lost, resulting in the exposure of subterminal Gal residues, which trigger rapid clearance of aged VWF by ASGPR.

Yang *et al.* recently showed that, in addition to the importance of desialylation, other changes in N-linked glycan structures also play critical roles in regulating the clearance of plasma glycoproteins [38]. Importantly, these data demonstrated that, as secreted glycoproteins age in the plasma, there is a progressive stepwise loss of terminal sugar moieties from N-linked glycan oligosaccharide chains. Given this important advance in our understanding of the evolution of N-linked glycan structures with glycoprotein aging, we sought to investigate the effect of progressive truncation of N-linked glycans on VWF clearance. Our novel findings demonstrate that, in addition to the importance of N-linked sialylation in modulating VWF clearance, progressive further trimming of the N-linked glycans of VWF also leads to markedly enhanced plasma clearance. In this context, it is interesting that PNG-VWF was actually cleared significantly faster than Neu-VWF. As expression of the ASGPR carbohydrate binding partners (Gal and GalNAc) is significantly reduced on PNG-VWF, our data further demonstrate that the enhanced clearance observed with loss of N-linked glycans from VWF must involve additional ASGPR-independent pathways.

Recent studies have demonstrated that hepatic and splenic macrophages bind to VWF *in vitro*, and are involved in regulating the clearance of VWF *in vivo* [33,42,44,47,48,50]. We observed that the enhanced clearance of both NeuGal-VWF and PNG-VWF was significantly attenuated following clodronate-induced macrophage depletion. The mechanisms underlying the enhanced macrophage-mediated clearance of VWF following N-linked glycan remodeling remain unclear. Interestingly however, Kurdi *et al.* recently reported that N-linked glycans on FX also play a role in modulating macrophage binding [51]. Moreover, previous studies have demonstrated that carbohydrate structures can influence protein-protein interactions through both conformational and charge-mediated mechanisms [52-55]. The hypothesis that progressive elimination of sugar moieties from VWF N-linked glycan termini can influence conformation is supported by previous work showing that, although Neu-VWF can induce platelet aggregation in the absence of

ristocetin, this platelet aggregation ability is significantly attenuated for NeuGal-VWF [56]. Furthermore, we previously demonstrated that, although Neu-VWF is resistant to ADAMTS-13 proteolysis, this ADAMTS-13-resistant phenotype is abolished in NeuGal-VWF [20].

A number of different macrophage receptors have been implicated in modulating VWF clearance, including LRP1 and β_2 -integrins [36,44,48]. In addition, recent studies have demonstrated that LRP1 binds to VWF in a shear-dependent manner [48]. Interestingly, VWF-LRP1 binding is significantly enhanced in the presence of ristocetin, or in the presence of type 2B VWD amino acid substitutions [44]. Our data also show that the enhanced macrophage-mediated clearance of VWF associated with progressive N-linked glycan trimming is mediated, at least in part, via LRP1. Further studies will be required to elucidate the molecular mechanism(s) through which the presence of complex N-linked glycans on VWF function to protect against macrophage-mediated and LRP1-mediated clearance.

In conclusion, our data demonstrate that the carbohydrate determinants expressed on VWF not only regulate susceptibility to proteolysis by ADAMTS-13, but also play a critical role in modulating *in vivo* clearance through both hepatic ASGPR-dependent and macrophage-dependent pathways. In addition, these findings further support the hypothesis that qualitative and quantitative variation in VWF glycosylation may be important in the pathophysiology of type 1C VWD. Further studies will be required to determine whether specific glycosylation at individual N-linked and/or O-linked sites in VWF are of particular importance in modulating clearance.

Addendum

J. M. O'Sullivan, S. Aguila, E. McRae, S. E. Ward, O. Rawley, A. Chion, T. M. Brophy, and L. Brady performed experiments. J. M. O'Sullivan, E. McRae, P. G. Fallon, T. M. Brophy, R. J. S. Preston, O. Sheils, A. Chion, 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, Boehringer Ingelheim, Leo Pharma, and Octapharma. He has also served on the advisory boards of Baxter, Bayer, Octapharma, CSL

Behring, Daiichi Sankyo, Boehringer Ingelheim, and Pfizer. He has also received research grant funding awards from Baxter, Bayer, Pfizer, and Novo Nordisk. The other authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Clearance of purified pd-VWF in VWF^{-/-} mice.

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Plasmin Cleaves Von Willebrand Factor at K1491-R1492 in the A1–A2 Linker Region in a Shear- and Glycan-Dependent Manner In Vitro

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Objective—Previous studies have demonstrated a role for plasmin in regulating plasma von Willebrand factor (VWF) multimer composition. Moreover, emerging data have shown that plasmin-induced cleavage of VWF is of particular importance in specific pathological states. Interestingly, plasmin has been successfully used as an alternative to ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type 1 motif) in a mouse model of thrombotic thrombocytopenic purpura. Consequently, elucidating the molecular mechanisms through which plasmin binds and cleaves VWF is not only of basic scientific interest but also of direct clinical importance. Our aim was to investigate factors that modulate the susceptibility of human VWF to proteolysis by plasmin.

Approach and Results—We have adapted the VWF vortex proteolysis assay to allow for time-dependent shear exposure studies. We show that globular VWF is resistant to plasmin cleavage under static conditions, but is readily cleaved by plasmin under shear. Although both plasmin and ADAMTS13 cleave VWF in a shear-dependent manner, plasmin does not cleave at the Tyr1605-Met1606 ADAMTS13 proteolytic site in the A2 domain. Rather under shear stress conditions, or in the presence of denaturants, such as urea or ristocetin, plasmin cleaves the K1491-R1492 peptide bond within the VWF A1–A2 linker region. Finally, we demonstrate that VWF susceptibility to plasmin proteolysis at K1491-R1492 is modulated by local N-linked glycan expression within A1A2A3, and specifically inhibited by heparin binding to the A1 domain.

Conclusions—Improved understanding of the plasmin–VWF interaction offers exciting opportunities to develop novel adjunctive therapies for the treatment of refractory thrombotic thrombocytopenic purpura.

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Key Words: glycosylation ■ heparin ■ plasmin ■ shear ■ von Willebrand factor

Von Willebrand factor (VWF) is a large plasma sialoglycoprotein that plays a critical role in primary hemostasis by mediating platelet adherence to exposed subendothelial collagen at sites of vascular injury.¹ In addition, VWF also acts as a carrier for procoagulant FVIII, protecting it against premature proteolytic degradation and clearance.² VWF is synthesized in endothelial cells and megakaryocytes initially as a monomer composed of a series of repeating domains (D'-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK).³ VWF monomers are assembled into dimers through the formation of C-terminal disulfide bonds in the endoplasmic reticulum.⁴ Subsequently in the Golgi, a further round of N-terminal disulfide bond formation converts VWF dimers into longer multimeric structures.^{5,6} Multimeric forms of up to 100- or 200-mers may be present in the circulation.⁷ Multimeric composition is a critical determinant of VWF functional activity as high molecular weight (HMW) VWF multimers bind to both collagen and

platelets with significantly increased affinities and are thus more efficient in inducing platelet aggregation.^{1,8}

In normal plasma, pathological accumulation of HMW VWF multimers is prevented by proteolysis of multimers with the plasma metalloprotease, ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type-1 repeats), which cleaves a single peptide bond (Tyr1605-Met1606) within the A2 domain of VWF.^{9,10} Inherited or acquired ADAMTS13 deficiency results in the accumulation of ultralarge VWF (UL-VWF) multimers in plasma.^{11,12} These UL-VWF can trigger the formation of pathological platelet aggregates, which obstruct the microvasculature, thereby causing thrombotic thrombocytopenia purpura (TTP).^{13,14} Congenital TTP has been associated with a series of different ADAMTS13 gene mutations.^{11,15} Interestingly, although inherited TTP often presents during infancy, significant variability in both age of onset and disease severity have been reported.^{16,17} Furthermore,

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Nonstandard Abbreviations and Acronyms

ADAMTS13	a disintegrin and metalloproteinase with thrombospondin type 1 motif
HMW	high molecular weight
TTP	thrombotic thrombocytopenic purpura
VWF	von Willebrand factor
WT	wild type

ADAMTS13 deficiency and circulating UL-VWF multimers can be observed in patients with inherited ADAMTS13 deficiency during the periods of clinical remission.^{14,18} Finally, complete deficiency of ADAMTS13 in transgenic mice models was not of itself sufficient to cause a TTP phenotype.^{19,20} Collectively, these findings suggest that the loss of ADAMTS13 may be necessary but not sufficient to induce clinical TTP, and support the hypothesis that additional factors beyond ADAMTS13 may contribute to TTP pathogenesis *in vivo*.^{14,21}

Interestingly, Tersteeg et al²² recently showed that significant activation of plasminogen to plasmin constitutes a common feature in patients during acute episodes of TTP. This observation is important because plasmin has previously been shown to proteolyze ADAMTS13.²³ However, *in vitro* and *in vivo* studies have confirmed that plasmin can also successfully degrade UL-VWF platelet aggregates.^{22,24} On the basis of these data, a possible role for plasmin as a novel therapy for patients with refractory TTP was proposed.²² In addition, Herbig and Diamond²⁵ have further demonstrated that abnormal VWF fibers formed under pathological flow conditions are resistant to ADAMTS13 proteolysis, but remain susceptible to cleavage by plasmin. All together, these emerging data suggest that plasmin-induced cleavage of VWF may be of both physiological and pharmacological significance. Critically, however, the molecular mechanisms underlying VWF proteolysis by plasmin remain poorly understood.

Previous studies have demonstrated that shear may enhance proteolysis of VWF by plasmin, as well as several other plasma proteases.^{22,24} In this study, we directly compared VWF proteolysis under static or shear conditions in the same assay and demonstrate that plasmin-mediated proteolysis of VWF is shear dependent. In addition, plasmin-mediated proteolysis of recombinant VWF truncated proteins was performed to localize a critical plasmin cleavage site to K1491-R1492 within the linker region between the A1 and A2 domains of VWF. Finally, we demonstrate that VWF susceptibility to plasmin proteolysis at K1491-R1492 is modulated by local N-linked glycan expression within A1A2A3 and is specifically inhibited by heparin binding to the A1 domain.

Materials and Methods

Materials and Methods are available in the [online-only Data Supplement](#).

Results**pd-VWF Is Cleaved by Plasmin in a Shear-Dependent Manner**

Recent studies have suggested that shear may influence the susceptibility of VWF to proteolysis by plasmin.^{22,24}

To investigate this hypothesis, we adapted the vortex-ADAMTS13 cleavage assay as previously described by the Long Zheng laboratory.^{26,27} After incubation of pd-VWF with purified plasmin under static conditions for 5 minutes, no significant VWF proteolysis was observed (Figure 1A; Figure I in the [online-only Data Supplement](#)). In contrast, however, in the presence of vortex shear, a significant and time-dependent reduction in HMW multimers was apparent (0 minutes versus 5 minutes; $P=0.005$; Figure 1A and 1B; Figure I in the [online-only Data Supplement](#)). Moreover, a corresponding increase in low molecular weight multimers was also observed (0 minutes versus 5 minutes; $P<0.0001$). As the VWF preparation used was plasma-derived, and other plasma proteases can cleave VWF,^{28,29} the vortex VWF proteolysis experiment was repeated in the absence of added plasmin. In the absence of plasmin, no significant reduction in HMW was observed despite 5 minutes vortex (0 minutes versus 5 minutes, $P=0.64$; Figure 1B). Furthermore, shear-related cleavage of VWF in this vortex assay was completely ablated in the presence of the known plasmin inhibitors aprotinin (0.5 $\mu\text{mol/L}$; $P<0.0001$) or ϵ -aminocaproic acid (50 mmol/L ; $P=0.006$), respectively (Figure 1C). To determine whether the shear-induced ADAMTS13 cleavage site within the A2 domain of VWF is also cleaved by plasmin, immunoblotting was performed after vortexing using an antibody that specifically recognizes the ADAMTS13 140kDa N-terminal A2-cleavage product.³⁰ Following pd-VWF exposure to ADAMTS13 under shear, we observed a progressive increase in this cleavage product (Figure 1D). In contrast, however, in the presence of plasmin, no such cleavage band was seen despite the progressive loss in HMW multimers (Figure 1D). All together, these findings demonstrate that plasmin cleaves VWF in a shear-dependent manner. Moreover, although ADAMTS13 also cleaves VWF in a shear-dependent fashion, the plasmin-cleavage site(s) in VWF is distinct to that of ADAMTS13.

VWF Conformation Modulates Susceptibility to Plasmin Proteolysis

To further investigate how VWF conformation influences susceptibility to plasmin proteolysis, we studied VWF cleavage in the presence of the denaturant urea. Under static conditions, full-length VWF proteolysis by plasmin was significantly enhanced in the presence of increasing concentrations of urea (low molecular weight VWF after 120 minutes: 0 versus 1.5 mol/L urea $P=0.027$; Figure 2A). Ristocetin binding to the A1 domain causes a conformational change that enhances VWF binding to platelet glycoprotein Iba.³¹ Moreover, ristocetin binding also significantly enhances VWF proteolysis by ADAMTS13.³¹ Similarly, we observed that plasmin-induced VWF proteolysis was also significantly increased in the presence of ristocetin (low molecular weight VWF after 120 minutes: 0 versus 1.5 mg/mL ristocetin $P=0.006$; Figure 2B; Figure II in the [online-only Data Supplement](#)). Previous studies have demonstrated that globular VWF is resistant to ADAMTS13 cleavage.³² Similarly, our findings demonstrate that multimeric VWF is also resistant to plasmin proteolysis unless exposed to shear stress or in the presence of unfolding agents.

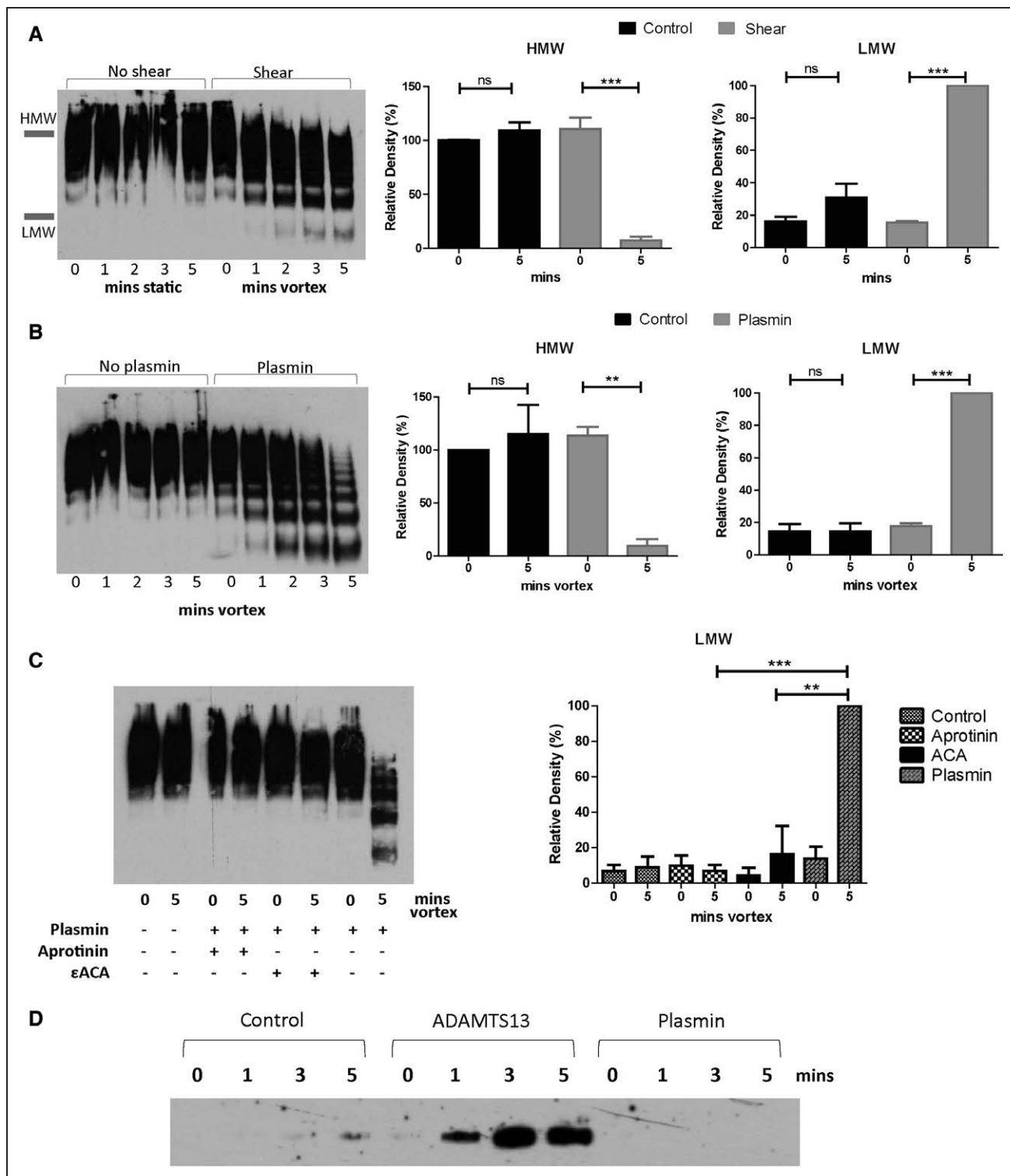


Figure 1. Human von Willebrand factor (VWF) is cleaved by plasmin in a shear-dependent manner. **A**, Purified human VWF (6 μg/mL) was incubated with plasmin (7.7 nmol/L) in the presence or absence of shear (vortexed at 2500 rpm). Samples are collected at 0, 1, 2, 3, and 5 minutes into tubes containing 15 μmol/L aprotinin. VWF proteolysis at each time point was analyzed by sodium dodecyl sulfate-agarose gel electrophoresis, immunoblotting, and densitometry as described in Materials and Methods section of this article. The area examined for densitometric analysis of high molecular weight (HMW) and low molecular weight (LWM) VWF is illustrated here and is applicable to all subsequent experiments. All experiments were performed in triplicate, and results described represent the means±SEM (***P*<0.001 and ****P*<0.0001 in comparison to control; ns, not significant). **B**, Purified pd-VWF was subjected to vortex-induced shear as before, in the presence or absence of added purified human plasmin (7.7 nmol/L). Samples were collected into aprotinin, and proteolysis assessed as above. **C**, Proteolysis of pd-VWF by plasmin under shear was repeated in the presence or absence of either aprotinin (0.5 μmol/L) or ε-aminocaproic acid (50 mmol/L). **D**, To investigate whether plasmin and ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type 1 motif) cleave VWF at the same site, pd-VWF was vortexed at 2500 rpm in the presence of either recombinant (*Continued*)

Figure 1. Continued human ADAMTS13 (3 nmol/L) or purified plasmin (7.7 nmol/L). Samples were collected at 0, 1, 3, and 5 minutes into either EDTA (50 mmol/L) for ADAMTS13-proteolysis studies, or aprotinin (15 μmol/L) for plasmin-proteolysis studies. All samples were then reduced with 100 mmol/L DTT (dithiothreitol), and analyzed by immunoblotting with a monoclonal antibody specific for the 140 kDa ADAMTS13-cleaved VWF product. ACA indicates aminocaproic acid.

Plasmin Cleaves VWF Within the A1–A2 Domains

Given the marked effect of ristocetin in enhancing VWF proteolysis by plasmin, we hypothesized that plasmin may cleave within the A1–A2–A3 domain region. To address this hypothesis, we examined plasmin-mediated proteolysis of recombinant VWF A domain truncated fragments (Figure 3A). In contrast to full-length VWF, recombinant A1A2A3-VWF was proteolyzed by plasmin under static conditions in the absence of any denaturants (Figure 3B). Moreover, this plasmin-mediated cleavage of A1A2A3-VWF was further enhanced in the presence of ristocetin (Figure 3C). Furthermore, although we observed plasmin-induced proteolysis of A1A2-VWF over time (Figure 3D), no significant cleavage of A2A3-VWF was seen (Figure 3D). Cumulatively, these data confirm a critical role for the A1 domain (aa1239–1494) in regulating VWF proteolysis by plasmin.

Plasmin Cleaves A1A2-VWF at K1491-R1492

As the A1A2 cleaved product is His-tagged at the C terminus, N-terminal sequencing of this product was performed to identify the site of proteolytic cleavage. RNSMV (Arg-Asn-Ser-Met-Val) were identified as the first five amino acids of the cleavage fragment. This sequence is only present in one location in human VWF, in the linker region between the A1 and A2 domains (¹⁴⁹²RNSMV¹⁴⁹⁶) (Figure 4A), and therefore suggests that plasmin cleaves between K1491 and R1492. Interestingly, both of these residues are highly conserved (Figure 4A). To confirm this putative plasmin cleavage site, K1491 was mutated to alanine in A1A2-VWF. In contrast to the plasmin-mediated proteolysis of wild-type (WT) A1A2, no proteolysis of A1A2-K1491A was observed over time (Figure 4B). Similarly, although WT D'D3A1A2A3-VWF was susceptible to plasmin cleavage, D'D3A1A2A3-K1491A/R1492A was resistant to plasmin proteolysis (Figure 4C).

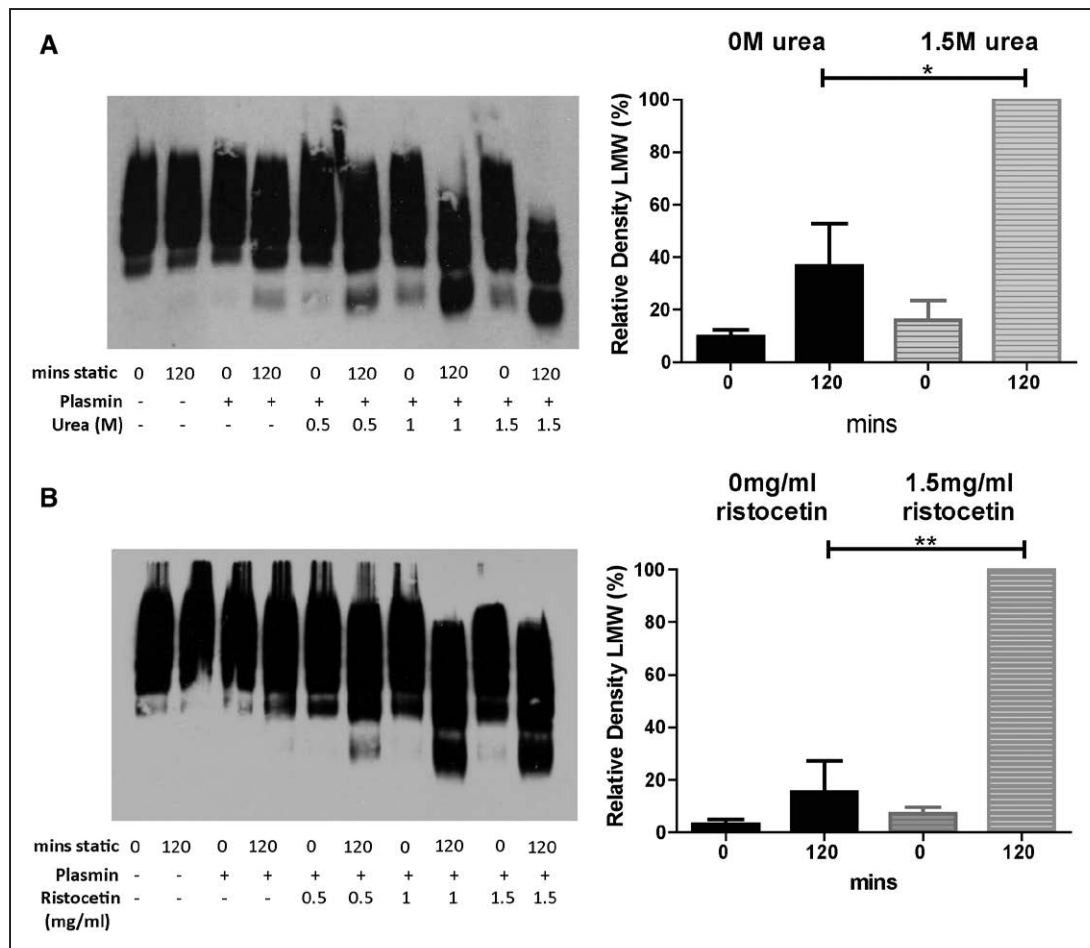


Figure 2. Von Willebrand factor (VWF) conformation modulates susceptibility to plasmin proteolysis. To investigate whether VWF conformation influences susceptibility to plasmin proteolysis, pd-VWF was incubated under static conditions with 7.7-nmol/L human plasmin in the presence of either (A) increasing concentrations of urea (0.5–1.5 mol/L) or (B) increasing concentrations of ristocetin (0.5–1.5 mg/mL). After incubation, samples were removed into aprotinin and rate of VWF multimer cleavage assessed by immunoblotting and densitometry. All experiments were performed in triplicate, and results described represent the means±SEM (**P*<0.05, ***P*<0.001).

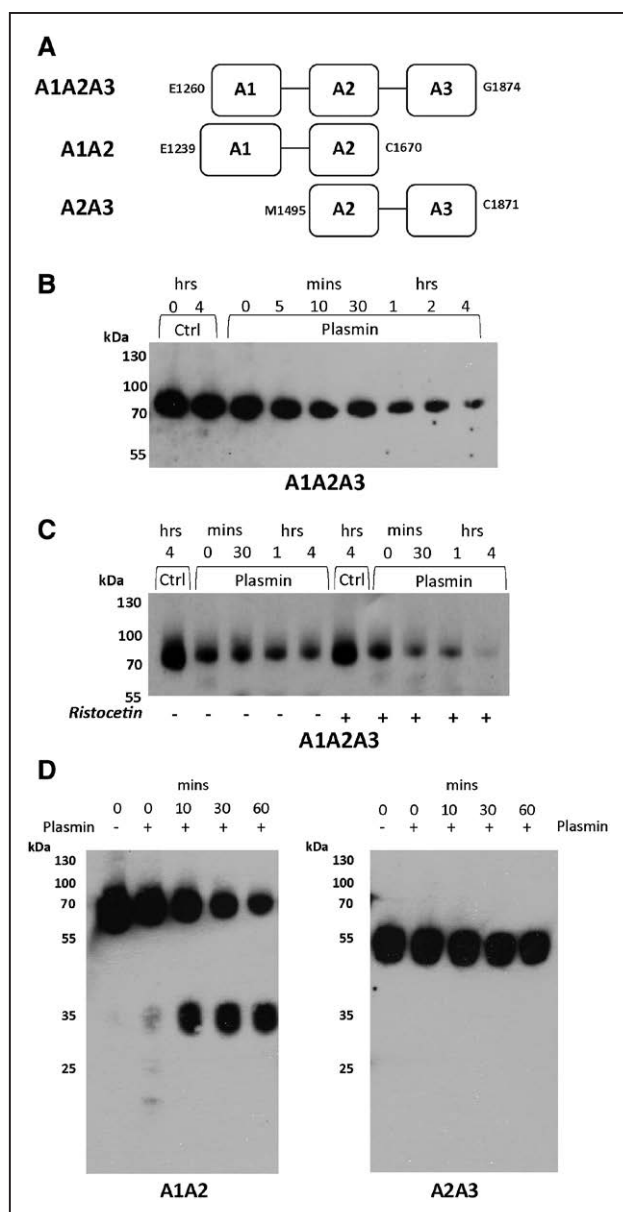


Figure 3. Plasmin cleaves within the A1–A2 domains of human von Willebrand factor (VWF). **A**, To investigate site(s) of plasmin-mediated proteolysis within VWF, a series of truncated VWF domain fragments were expressed and purified from HEK293T cells. **B**, Recombinant human A1A2A3-VWF was incubated with plasmin for up to 4 hours under static conditions. Samples were removed at specified time points for proteolysis analysis by immunoblotting as before. **C**, Recombinant A1A2A3-VWF was incubated with plasmin under static conditions in the absence or presence of ristocetin (1 mg/mL), and proteolysis analyzed on 4% to 12% Bis Tris acrylamide gels with immunoblotting. **D**, Recombinant human A1A2-VWF or A2A3-VWF were incubated with plasmin (12.8 nmol/L) for up to 1 hour. Samples were removed at each time point for analysis on 4% to 12% Bis Tris acrylamide gels with immunoblotting with anti-His horseradish peroxidase. WT indicates wild type.

Collectively, these findings demonstrate that plasmin cleaves VWF at the K1491–R1492 bond in the linker region between the A1 and A2 domains in this shear-sensitive region of VWF.

Heparin Inhibits Plasmin Cleavage of VWF

Several VWF-binding partners significantly enhance VWF susceptibility to ADAMTS13 proteolysis, including FVIII,³³

platelet glycoprotein Iba,³⁴ ristocetin,³⁵ and heparin.³⁴ Both FVIII and glycoprotein Iba are proteolyzed by plasmin.^{36,37} Consequently, we further investigated whether heparin binding might also promote plasmin proteolysis of full-length VWF. Interestingly, in contrast to the effect of heparin in enhancing VWF proteolysis by ADAMTS13, we observed a significant and concentration-dependent inhibition of plasmin-mediated proteolysis of pd-VWF under shear by heparin (Figure 5A). Similarly, proteolysis of the truncated A1A2-VWF fragment by plasmin under static conditions was also inhibited by heparin (Figure 5B). Tersteeg et al²² previously postulated that the lysine-rich ¹⁴⁰⁵KKKK¹⁴⁰⁸ region in the A1 domain may be important in modulating plasminogen interaction with VWF. Interestingly, previous studies have also reported a heparin-binding role for this region.³⁸ To elucidate the mechanism underlying the inhibitory effect of heparin on plasmin-mediated VWF proteolysis, we investigated plasminogen binding to A1A2-VWF in the presence of heparin using the same assay as described by Tersteeg et al²². In keeping with previous studies, immobilized recombinant A1A2-VWF bound to plasminogen in a saturable and concentration-dependent manner (Figure 5C). Moreover, this binding was inhibited by the soluble lysine analog ϵ -aminocaproic acid (data not shown). In the presence of heparin, binding of WT A1A2-VWF to plasminogen was markedly attenuated (Figure 5C). All together, these findings are consistent with the hypothesis that the lysine-rich ¹⁴⁰⁵KKKK¹⁴⁰⁸ region in the A1 domain of VWF plays critical roles in modulating interaction with both heparin and plasmin. Importantly, we further observed that binding of the A1A2-K1491A VWF variant to plasminogen was similar to that of WT A1A2-VWF, despite the loss of the plasmin cleavage site (Figure 5D). This observation confirms that the plasmin(ogen) binding and proteolytic sites on VWF are distinct.

Plasmin Cleaves VWF in a Glycan-Dependent but ABO-Independent Manner

Previous studies from our laboratory and others have demonstrated that VWF glycan determinants play a major role in modulating susceptibility to ADAMTS13 proteolysis.^{35,39,40} Given the role of N-linked glycans in modulating A2 domain conformation,⁴¹ we hypothesized that local N-linked glycans within A1A2A3 might also be important in modulating VWF proteolysis by plasmin. Only two N-linked glycans are expressed on A1A2A3-VWF at N1515 and N1574, respectively, within the A2 domain. Specific removal of these 2 N-linked glycans from A1A2A3-VWF by PNGase F digestion resulted in significantly increased susceptibility to plasmin proteolysis (4 hours; WT versus PNGase-treated, $P=0.013$; Figure 6A).

ABO(H) blood determinants expressed on both the N- and O-linked glycans of human pd-VWF have been shown to modulate the susceptibility of pd-VWF to ADAMTS13 cleavage.^{35,42} As ABO(H) determinants are not expressed on recombinant VWF,^{43,44} we used blood group-specific pd-VWF to investigate whether ABO also influences plasmin-mediated proteolysis of VWF. Despite the significant effect of ABO blood group in regulating VWF proteolysis by ADAMTS13, no significant difference in plasmin-mediated proteolysis was observed for group AB VWF compared with group O

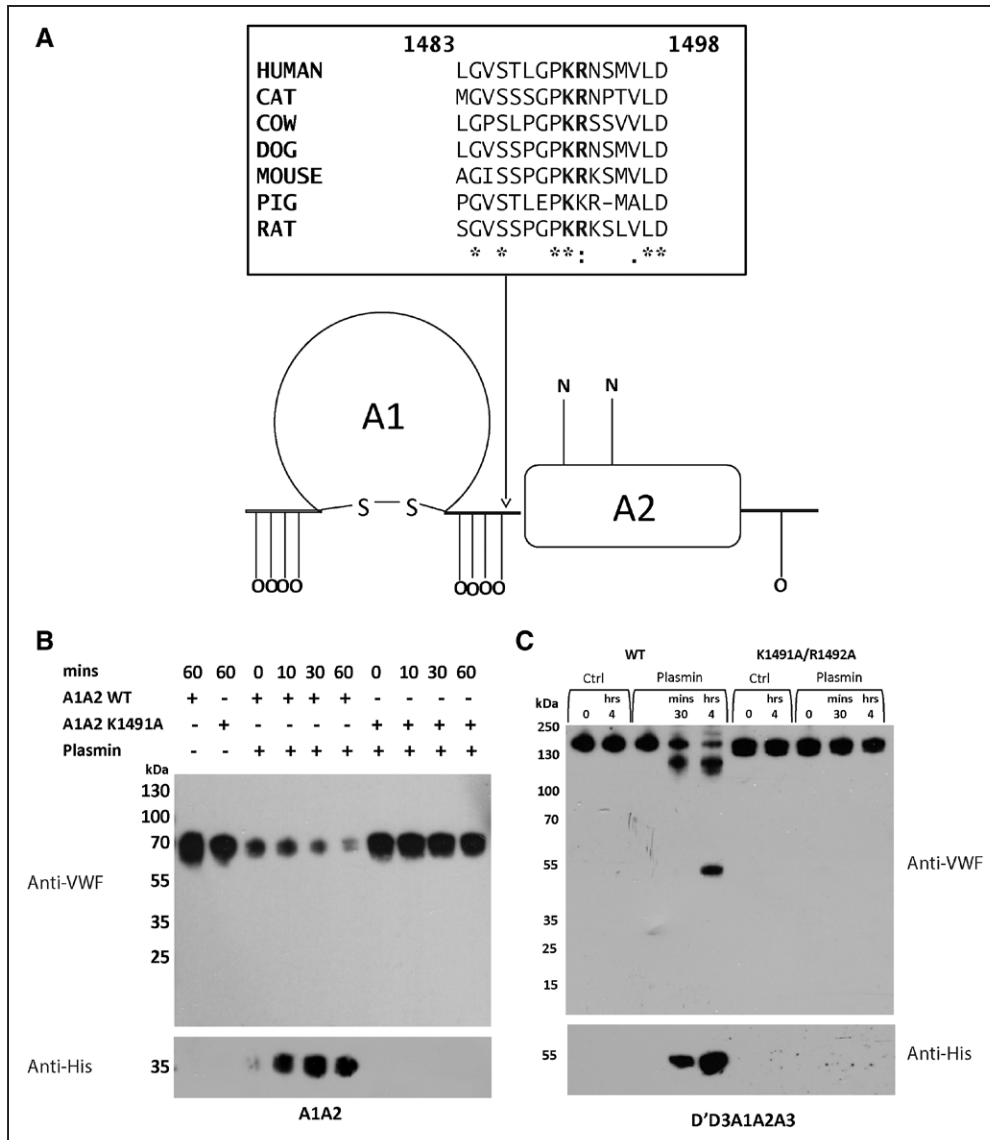


Figure 4. Plasmin cleaves A1A2-von Willebrand factor (VWF) at K1491-R1492. **A**, N-terminal sequencing of the C-terminally His-tagged product of plasmin-cleaved VWF identified RNSMV (Arg-Asn-Ser-Met-Val) as the first five amino acids of this protein band. This corresponds to a C-terminal region of the VWF A1 domain. The amino acid sequences of VWF protein from several species (human, feline, bovine, canine, murine, and porcine) were aligned using the T-Coffee multiple sequence alignment program. **B**, VWF A1A2 or A1A2 K1491A (10 μ g/mL) were incubated with plasmin for up to 1 hour. Samples were removed at each time point and proteolysis analyzed by immunoblotting with anti-VWF horseradish peroxidase (HRP) or anti-His HRP. **C**, VWF D'A3 or D'A3 K1491A/R1492A (10 μ g/mL) were incubated with plasmin for up to 4 hours. Samples were removed at each time point for analysis on 4% to 12% Bis Tris acrylamide gels with immunoblotting with anti-VWF HRP or anti-His HRP.

VWF (HMW 5 minutes AB versus O, $P=0.1979$; Figure 6C). Nevertheless, taken together, these data demonstrate that N- and O-glycans expressed within the A1A2A3 domains of VWF play important roles in modulating proteolysis by both plasmin and ADAMTS13.

Discussion

VWF multimer composition is a critical determinant of its hemostatic function.¹ The biological importance of regulating plasma VWF multimer distribution is illustrated by the fact that pathological accumulation of abnormal UL-VWF multimers is associated with thrombotic microangiopathy in TTP.^{45,46} Conversely, in patients with type 2A von Willebrand

disease, increased proteolysis results in loss of normal HMW VWF multimers, and confers a significant bleeding phenotype.⁴⁷ Under steady-state conditions, VWF multimer distribution in normal plasma is primarily regulated by ADAMTS13-mediated proteolysis. Nevertheless, other proteases can also cleave multimeric VWF in vitro.²¹ Moreover, accumulating recent evidence suggests that these proteases may have roles in regulating VWF multimer composition in vivo under specific conditions.^{22,48-52} For example, in a murine model of TTP, plasmin could regulate VWF multimer distribution in the absence of ADAMTS13.²² Several other pathological conditions have also been associated with significant reductions in plasma ADAMTS13 levels, including acute

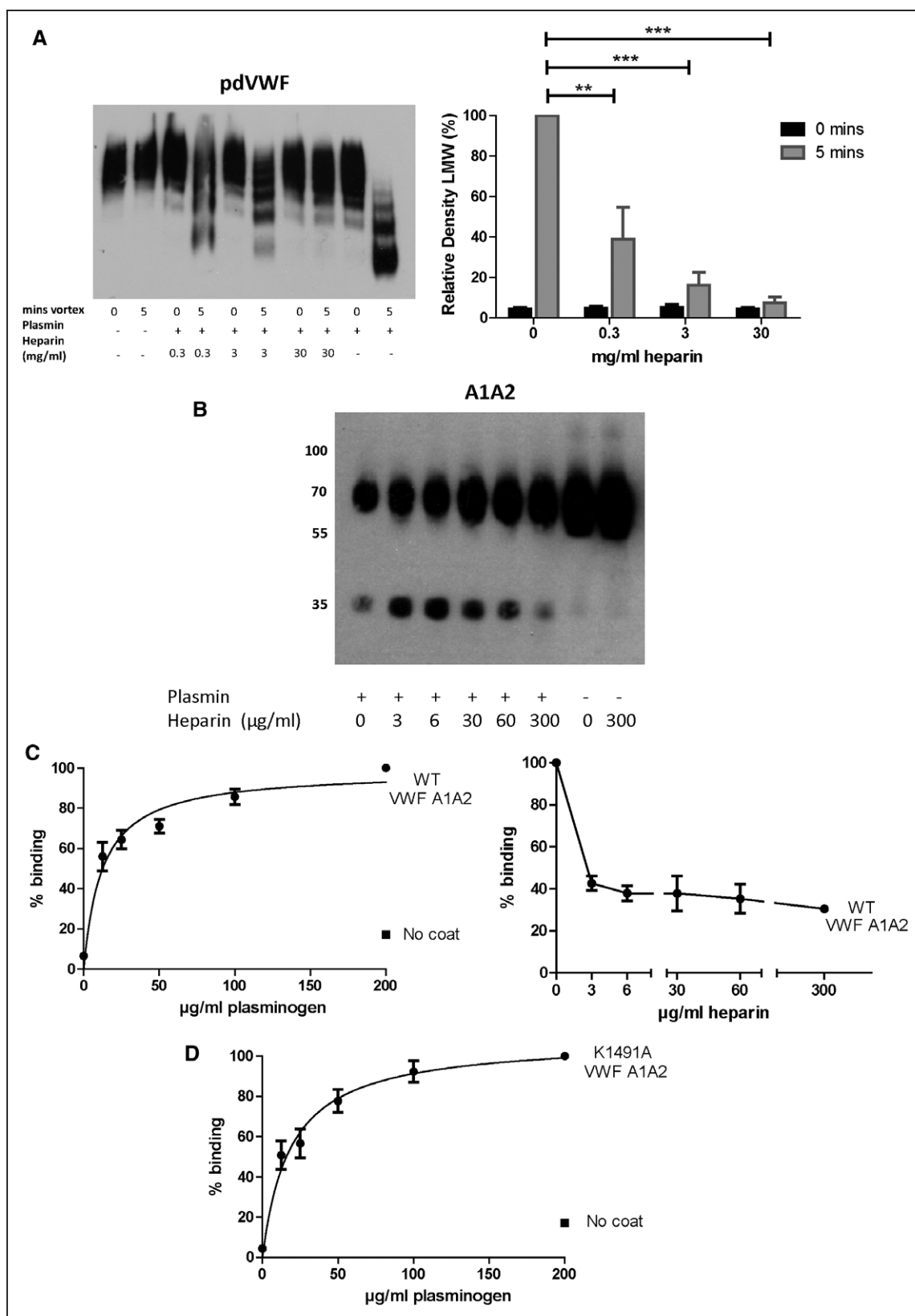


Figure 5. Heparin inhibits plasmin cleavage of von Willebrand factor (VWF). **A**, Full-length pd-VWF was vortexed at 2500 rpm in the absence or presence of plasmin or heparin (0.3–30 mg/mL). Samples are removed at 0 and 5 minutes for analysis on 1.8% sodium dodecyl sulfate-agarose gels with immunoblotting and densitometry. **B**, Recombinant A1A2-VWF (10 $\mu\text{g/mL}$) was incubated with plasmin in the presence of heparin (0–300 $\mu\text{g/mL}$) for 1 hour. Samples were removed at 1 hour for proteolysis analysis. **C**, Plate-binding of plasminogen (25–200 $\mu\text{g/mL}$) to immobilized recombinant human wild-type (WT) A1A2-VWF was detected by anti-plasminogen horseradish peroxidase (HRP). Binding experiments were repeated in the presence or absence of added heparin (0–300 $\mu\text{g/mL}$). **D**, Binding of plasminogen (25–200 $\mu\text{g/mL}$) to immobilized A1A2-K1491A (5 $\mu\text{g/mL}$) was detected by anti-plasminogen HRP as before.

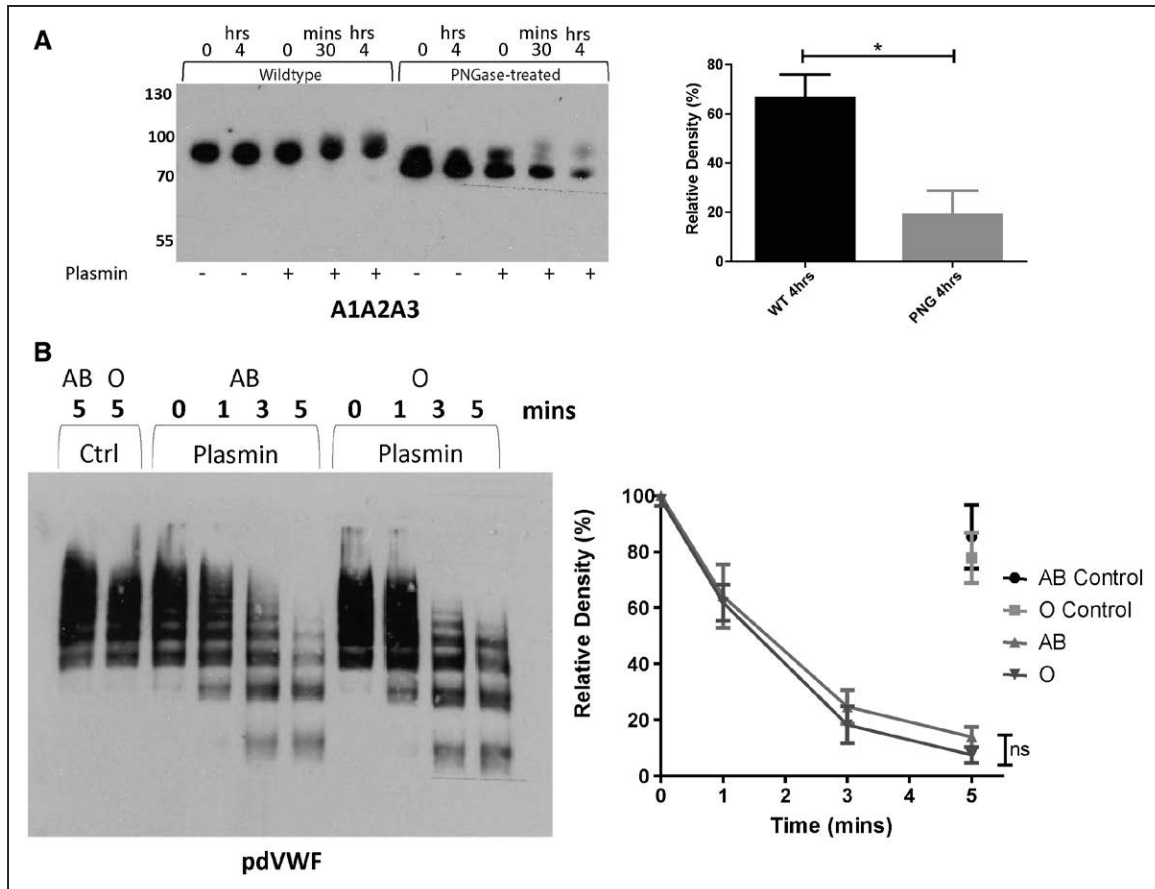


Figure 6. Plasmin cleaves von Willebrand factor (VWF) in a glycan-dependent but ABO-independent manner. **A**, N-linked glycans were removed from A1A2A3-VWF by digestion with PNGase F. Wild-type and PNGase F-treated A1A2A3-VWF (10 μ g/mL) were then incubated with plasmin for up to 4 hours. Samples were removed at each time point for analysis on 4% to 12% Bis Tris acrylamide gels with immunoblotting. **B**, Human pd-VWF was purified from pooled blood group AB or blood group O normal donors. The AB- and O-VWF preparations were then vortexed at 2500 rpm in the absence or presence of plasmin (7.7 nmol/L). Samples were removed at 0, 1, 3, and 5 minutes for analysis on 1.8% sodium dodecyl sulfate-agarose gels with immunoblotting. The loss of high molecular weight multimers over time was determined by densitometry.

liver injury,⁴⁸ cerebral malaria,⁵³ sickle cell disease,⁵⁴ and Dengue.⁵¹ Importantly, however, despite low or even undetectable ADAMTS13 activity levels, UL-VWF multimers are not observed in many individuals with these conditions.⁴⁹ The absence of UL-VWF in these different patient cohorts is likely to be attributable to different molecular mechanisms, including consumption in platelet-rich microthrombi. Nevertheless, recent reports^{21,22,48–52} have also postulated that VWF proteolysis through ADAMTS-13 independent pathways may be important in this context.

In the normal circulation, VWF adopts a globular conformation.⁵⁵ Shear stress triggers unwinding of globular VWF and conformational changes within the A domains,³² exposing the glycoprotein Iba-binding site in the A1 domain and the ADAMTS13 cleavage site in the A2 domain.^{56,57} In this study, we directly compare plasmin-mediated proteolysis of VWF under both static and shear conditions in a controlled environment and show that human pd-VWF is relatively resistant to proteolysis by 7.7 nmol/L plasmin under static conditions. Previous studies reporting proteolysis of VWF by plasmin under static conditions have used significantly higher plasmin concentrations.^{24,58} In contrast, however, our

data show that HMW VWF is rapidly cleaved by 7.7 nmol/L plasmin under exposure to shear stress, which would parallel conditions present in stenosed arteries *in vivo*. Local plasmin concentrations of \approx 10 nmol/L at sites of vascular injury, and $>$ 100 nmol/L after systemic thrombolytic therapy, have been estimated.^{24,59} Although both plasmin and ADAMTS13 cleave VWF in a shear-dependent manner, our findings demonstrate that plasmin does not cleave VWF at the ADAMTS13 site (Tyr 1605-Met 1606 in A2).

Using a series of recombinant VWF truncated proteins, we have localized a plasmin cleavage site in VWF. Plasmin specifically cleaves VWF at the K1491-R1492 peptide bond that is located in the A1–A2 linker region. The lysine-rich ¹⁴⁰⁵KKKK¹⁴⁰⁸ region in the VWF A1 domain was recently postulated to play a role in mediating interaction with plasmin.²² Moreover, this region is important in modulating heparin binding to VWF.³⁸ Heparin has been reported to significantly enhance VWF proteolysis by ADAMTS13.³⁴ In contrast, our novel data demonstrate that heparin binding to the A1 domain of VWF attenuates plasmin-mediated VWF proteolysis in a dose-dependent manner. In binding studies, we confirmed that plasminogen binding to A1A2-VWF was significantly reduced in

the presence of heparin. Importantly, however, we observed that, despite site-directed mutagenesis of the plasmin cleavage site, A1A2-K1491A VWF binding to plasminogen was not reduced indicating that the plasmin(ogen)-binding site and the plasmin proteolysis sites within VWF are distinct. Therefore, our data show that under shear stress conditions, conformational changes in A1A2 facilitate plasmin binding to lysine-rich regions in the VWF A1 domain, and subsequently enable specific plasmin cleavage at the K1491–R1492 peptide bond within the VWF A1–A2 linker region. This pattern of distinct binding and cleavage sites as well as shear-enhanced and conformation-dependent cleavage has clear similarities to the VWF–ADAMTS13 interaction.^{32,60}

To date, no VWF gene mutations involving the K1491–R1492 plasmin cleavage site have been described. However, both type 2A and type 2B von Willebrand disease are characterized by the loss of HMW multimers due, in part, to enhanced proteolysis.^{61,62} On the basis of our data on the critical role of A1A2 conformation in regulating susceptibility to plasmin proteolysis, it seems likely that some type 2A and 2B mutations may lead to enhanced proteolysis by plasmin and ADAMTS13. Furthermore, given the importance of shear in regulating plasmin-mediated VWF proteolysis, it is plausible that plasmin cleavage may contribute to the loss of HMW multimers observed in patients in whom pathological local shear stress levels are generated (eg, acquired von Willebrand disease secondary to aortic stenosis or left ventricular assist devices (LVADs)).^{63,64} Finally, plasmin-mediated VWF proteolysis after administration of thrombolytic treatment has been described in patients with myocardial infarction⁶⁵ and deep vein thrombosis,⁶¹ and thus may be a contributing factor in the clinical bleeding risk associated with systemic thrombolysis.

In previous studies, we and others have shown an important role for VWF glycans in modulating VWF cleavage by ADAMTS13^{39,40,42,66,67} and by plasmin.²⁹ Similarly, our novel findings demonstrate that VWF glycans also determine susceptibility to plasmin proteolysis, but in addition identify some important differences in the effects of VWF glycans in regulating proteolysis by plasmin compared with ADAMTS13. First, although the loss of terminal $\alpha 2$ to 6 linked sialic acid from human VWF significantly inhibits ADAMTS13 proteolysis,⁶⁶ desialylation actually enhances proteolysis by plasmin.⁶⁸ Second, N-linked glycans expressed at N1515 and N1574 within the A2 domain protect VWF against proteolysis by both plasmin and ADAMTS13.^{67,69} Finally, ABO(H) blood group determinants modulate VWF susceptibility to proteolysis by ADAMTS13 ($O \geq B > A \geq AB$)³⁵ but do not influence plasmin-mediated cleavage. We hypothesize that VWF glycan determinants modulate interactions with both plasmin and ADAMTS13 by modifying protein conformation. Consequently, removal of specific glycans in the A1A2A3 domains result in conformations that are more permissive for either plasmin or ADAMTS13 cleavage. Further studies are necessary to define the molecular mechanism(s) through which glycans within the A domains of VWF regulate plasmin-mediated proteolysis. Nevertheless, these findings are of clinical relevance in that significant quantitative and qualitative variations in VWF glycan expression have been previously

been reported in both the normal population^{70,71} and in patients with different disease states.^{72,73}

In conclusion, accumulating recent data have demonstrated that in addition to ADAMTS13, plasmin may also play an important role in regulating plasma VWF multimer distribution, particularly under specific pathological conditions that present with the accumulation of HMW VWF multimers.^{22,48,50,51,54} Consequently, elucidating the molecular mechanisms through which plasmin binds and cleaves multimeric VWF is not only of basic scientific interest but also of direct translational importance. Improved understanding of the plasmin–VWF interaction may offer exciting opportunities to develop novel adjunctive therapies for the treatment of refractory TTP, which continues to be associated with significant morbidity and mortality. In addition, using thrombolytic therapy to lyse VWF-dependent platelet aggregates in sites of high shear stress may also be useful, particularly in pathological conditions where UL-VWF is resistant to ADAMTS13 proteolysis.^{25,53}

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Disclosures

J.S. O'Donnell has served on the speaker's bureau for Baxter, Bayer, Novo Nordisk, Boehringer Ingelheim, Leo Pharma, and Octapharma. He has also served on the advisory boards of Baxter, Bayer, Octapharma CSL Behring, Daiichi Sankyo, Boehringer Ingelheim, and Pfizer. The other authors report no conflicts.

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Highlights

- An *in vivo* role for plasmin-mediated proteolysis of von Willebrand factor (VWF) has been demonstrated in a mouse model of thrombotic thrombocytopenic purpura.
- We examined plasmin-mediated proteolysis of VWF under shear and of recombinant VWF proteins.
- Plasmin cleaves VWF in a shear- and glycan-dependent manner at K1491-R1492.
- Heparin inhibits plasmin-mediated proteolysis of VWF.

THROMBOSIS AND HEMOSTASIS

A novel role for the macrophage galactose-type lectin receptor in mediating von Willebrand factor clearance

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KEY POINTS

- VWF sialylation modulates *in vivo* clearance through Ashwell-Morrell independent pathways.
- VWF binding to MGL plays a novel role in facilitating VWF clearance.

Previous studies have shown that loss of terminal sialic acid causes enhanced von Willebrand factor (VWF) clearance through the Ashwell-Morrell receptor (AMR). In this study, we investigated (1) the specific importance of *N*- vs *O*-linked sialic acid in protecting against VWF clearance and (2) whether additional receptors contribute to the reduced half-life of hyposialylated VWF. α 2-3-linked sialic acid accounts for <20% of total sialic acid and is predominantly expressed on VWF *O*-glycans. Nevertheless, specific digestion with α 2-3 neuraminidase (α 2-3Neu-VWF) was sufficient to cause markedly enhanced VWF clearance. Interestingly, *in vivo* clearance experiments in dual $VWF^{-/-}/Asgr1^{-/-}$ mice demonstrated enhanced clearance of α 2-3Neu-VWF even in the absence of the AMR. The macrophage galactose-type lectin (MGL) is a C-type lectin that binds to glycoproteins expressing terminal *N*-acetylgalactosamine or galactose residues. Importantly, the markedly enhanced clearance of hyposialylated VWF in $VWF^{-/-}/Asgr1^{-/-}$ mice was significantly attenuated in the presence of an anti-MGL inhibitory antibody. Furthermore, dose-dependent binding of human VWF to purified recombinant human MGL was confirmed using surface plasmon resonance. Additionally, plasma VWF:Ag levels were significantly elevated in $MGL1^{-/-}$ mice compared with controls. Collectively, these findings identify MGL as a novel macrophage receptor for VWF that significantly contributes to the clearance of both wild-type and hyposialylated VWF. (*Blood*. 2018;131(8):911-916)

Similarly, genetic inactivation of ST3GalIV sialyltransferase in a transgenic mouse model causes enhanced VWF clearance.⁹ Several studies have reported significantly reduced VWF sialylation levels in patients with type 1 VWD.^{7,9} Furthermore, van Schooten et al reported an inverse correlation between aberrant sialylation of T antigen and plasma VWF:Ag levels, suggesting that *O*-linked sialylation on VWF may be of particular importance.⁷

Introduction

Although substantial progress has been achieved in understanding von Willebrand factor (VWF) structure and function, the biological mechanisms underpinning VWF clearance from the plasma remain poorly understood.¹ Nevertheless, studies have demonstrated that enhanced VWF clearance plays an important role in the etiology of both type 1 and type 2 von Willebrand disease (VWD).^{1,2} During biosynthesis, VWF undergoes complex posttranslational modification, including significant *N*- and *O*-linked glycosylation. Mass spectrometry studies have shown that sialylated biantennary complex-type chains constitute the commonest *N*-linked glycans expressed on VWF, whereas a disialylated core 1 tetrasaccharide structure (known as the T antigen) accounts for 70% of the total *O*-glycan population.^{3,4} Thus, the majority of *N*- and *O*-linked glycans of human VWF are capped by negatively charged sialic acid residues.⁵ In keeping with other plasma glycoproteins, terminal sialic acid expression plays an important role in protecting VWF against clearance.^{6,7} Consequently, enzymatic removal of terminal sialylation from VWF has been associated with a markedly reduced plasma half-life *in vivo*.⁸

Current evidence suggests that the enhanced clearance of hyposialylated VWF occurs via the Ashwell-Morrell receptor (AMR).¹⁰ This C-type lectin is expressed on hepatocytes and is composed of 2 transmembrane protein subunits (Asgpr-1 and Asgpr-2). Grewal et al previously demonstrated that plasma VWF clearance is significantly attenuated in *Asgr-1* knockout mice.¹⁰ Nevertheless, important questions regarding the biological mechanisms through which VWF sialylation regulates its clearance *in vivo* remain unclear. In particular, the relative importance of *N*-linked vs *O*-linked sialylation in regulating physiological and/or pathological clearance of VWF has not been defined. In addition to the AMR, a number of other lectin receptors have been shown to bind with enhanced affinity to hyposialylated

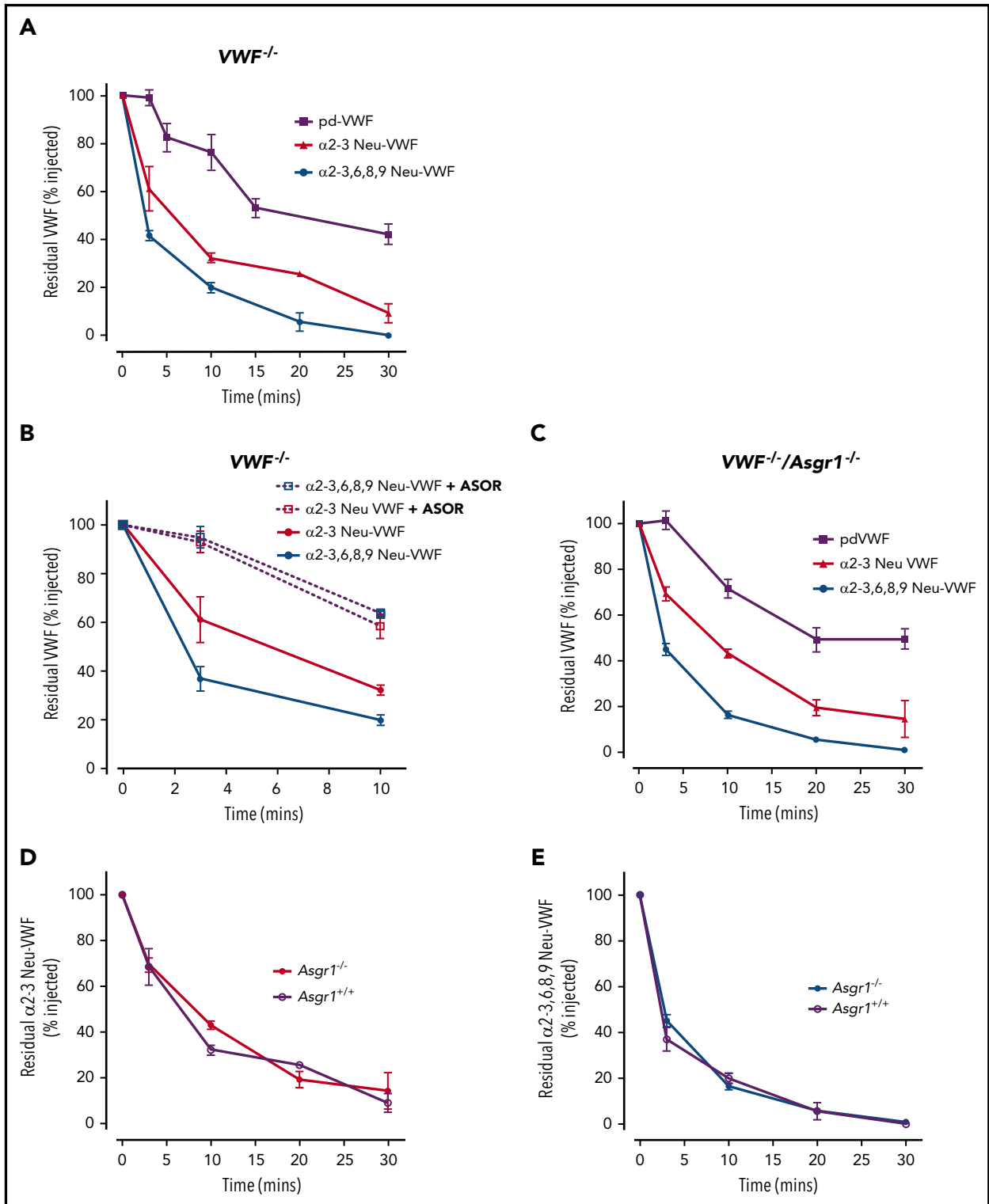


Figure 1. Clearance of hyposialylated VWF proceeds independently of AMR. (A) To study the effects of N- and O-linked sialylation on VWF clearance, purified human pd-VWF was treated with either α 2-3,6,8,9 or α 2-3 neuraminidase. In vivo clearance for each glycoform was then assessed in $VWF^{-/-}$ mice and compared with that of wild-type pd-VWF. At each time point, residual circulating VWF concentration was determined by WVF:Ag enzyme-linked immunosorbent assay. All results are plotted as percentage residual WVF:Ag levels relative to the amount injected. Three to five mice were used per time point. Data are represented as mean \pm SEM. In some cases, the SEM cannot be seen because of its small size. (B) In the presence of ASOR, the enhanced in vivo clearance of both α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF was significantly attenuated (α 2-3 Neu-VWF $t_{1/2}$ = 8.2 ± 1.4 minutes vs 12.4 ± 2.4 minutes, $P < .05$; and α 2-3,6,8,9 Neu-VWF $t_{1/2}$ = 3.7 ± 0.7 minutes vs 14.4 ± 2.7 minutes, $P < .005$, respectively). (C) To determine whether AMR-independent pathways contribute to the enhanced clearance of hyposialylated VWF, in vivo clearance studies were repeated in $VWF^{-/-}/Asgr1^{-/-}$ mice. Importantly, the markedly enhanced clearance of both α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF was still evident in the absence of the AMR ($t_{1/2}$ = 8.2 ± 0.6 and 3.2 ± 0.4 compared with 50.6 ± 2 minutes for pd-VWF; $P < .05$). Furthermore, the reduced half-life observed for α 2-3 Neu-VWF (D) and α 2-3,6,8,9 Neu-VWF (E) were not significantly different in the presence or absence of the AMR (α 2-3 Neu-VWF $t_{1/2}$ = 8.2 ± 1.4 minutes vs 8.2 ± 0.6 minutes, $P = .96$; and α 2-3,6,8,9 Neu-VWF $t_{1/2}$ = 3.7 ± 0.7 minutes vs 3.2 ± 0.4 minutes, $P = .42$, respectively).

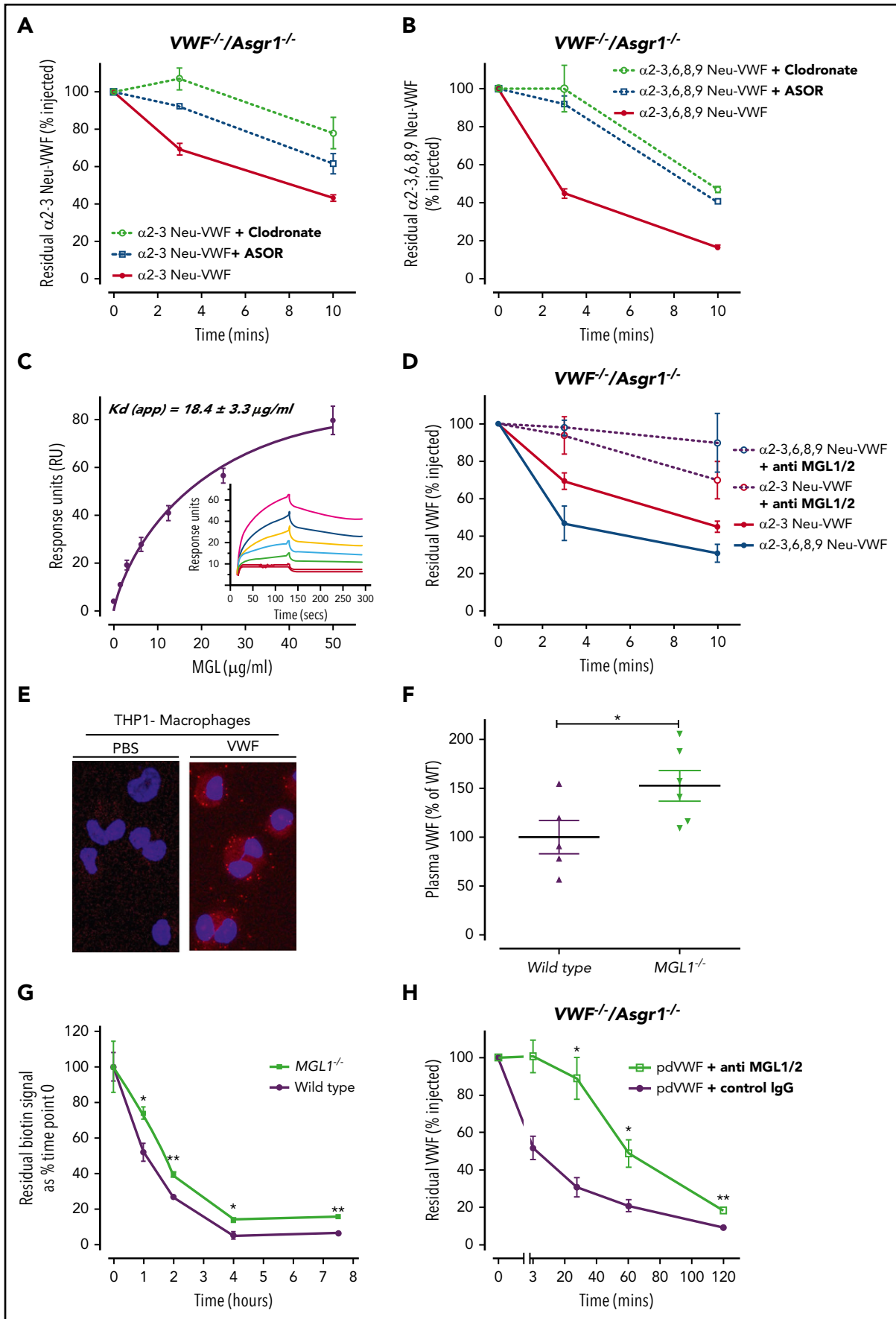


Figure 2.

glycoproteins.¹¹ In this study, we demonstrate a critical role for VWF O-linked sialylation in modulating in vivo clearance and further define a novel role for the macrophage galactose-type lectin (MGL) in regulating VWF clearance in a sialic acid-dependent manner.

Study design

Isolation and digestion of human plasma-derived VWF

As described in the supplemental Methods (available on the *Blood* Web site), plasma-derived VWF (pd-VWF) was purified from commercial concentrate Fandhi (Grifols, Barcelona, Spain) and subsequently treated with α 2-3 neuraminidase (*Streptococcus pneumoniae*; Sigma Aldrich, Ireland) or α 2-3,6,8,9 neuraminidase (*Arthrobacter ureafaciens*; New England Biolabs, United Kingdom) as previously described.⁵ VWF glycan expression was analyzed using lectin enzyme-linked immunosorbent assays (see supplemental Methods and supplemental Figure 1).⁸

VWF clearance studies

VWF^{-/-} and *Asgr1*^{-/-} mice on a C57BL/6J background were obtained from the Jackson Laboratory (Sacramento, CA) and crossbred to generate novel VWF^{-/-}/*Asgr1*^{-/-} double-knockout mice. *MGL1*^{-/-} mice were also obtained from the Jackson Laboratory. Where indicated, clearance studies were repeated in the presence of either clodronate or asialo-orosomucoid (ASOR) as previously described.^{8,12} Specific clearance studies were performed after inhibition of MGL using a polyclonal goat anti-mouse MGL1/2 antibody. All in vivo clearance experiments were performed as detailed in the supplemental Methods in accordance with the Health Product Regulatory Authority, Ireland.

In vitro VWF binding studies

As described in the supplemental Methods, surface plasmon resonance (SPR) was used to evaluate MGL binding to VWF.¹³ Briefly, purified pd-VWF was immobilized on a CM5 chip, and binding to recombinant MGL (R&D Systems, United Kingdom) was determined. Furthermore, proximity ligation assay (Duolink-PLA; Sigma Aldrich, Ireland) was performed to evaluate colocalization of VWF and MGL on THP1 macrophages.

Data presentation and statistical analysis

Experimental data were analyzed with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). Data were expressed as mean values \pm standard error of the mean (SEM). Data were

analyzed with Student unpaired 2-tailed t test, and *P* values of $<.05$ were deemed significant.

Results and discussion

VWF sialylation modulates clearance through Ashwell-Morrell independent pathways

In keeping with previous studies, we observed that combined removal of N- and O-linked sialic acid by digestion with α 2-3,6,8,9 neuraminidase resulted in markedly enhanced clearance of pd-VWF in VWF^{-/-} mice (Figure 1A). Specific removal of α 2-3-linked sialic acid was sufficient to markedly enhance VWF clearance ($t_{1/2} = 9.0 \pm 1$ minutes; $P < .05$) (Figure 1A). In fact, clearance of α 2-3 Neu-VWF was almost as rapid as that of α 2-3,6,8,9 Neu-VWF ($t_{1/2} = 4.0 \pm 0.3$ minutes). This finding is interesting because α 2-3-linked sialic acid is predominantly located on the O-linked glycans of human VWF and accounts for $<20\%$ of total sialic acid expression.⁵ Currently, the AMR is the only receptor described to regulate clearance of hyposialylated VWF.¹⁰ We found that the enhanced clearance of α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF were both significantly attenuated in the presence of a hyposialylated inhibitor glycoprotein (ASOR) (Figure 1B). However, ASOR has a short plasma half-life and is not a specific AMR inhibitor.¹⁴ Previous studies have shown that AMR demonstrates significantly greater affinity for exposed galactose residues on tri- and tetra-antennary galactoses (as present on VWF N-glycan) compared with terminal galactose moieties on mono- or biantennary galactoses (as present on VWF O-glycan).^{15,16} We therefore hypothesized that other lectin receptors may contribute to the enhanced clearance of hyposialylated VWF and be of particular importance in modulating the effects of O-linked sialylation on VWF clearance. To address this, in vivo clearance studies were repeated in dual VWF^{-/-}/*Asgr1*^{-/-} knockout mice. Critically, we observed that markedly enhanced clearance of both α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF persisted in VWF^{-/-}/*Asgr1*^{-/-} mice ($t_{1/2} = 8.2 \pm 0.6$ and 3.2 ± 0.4 vs 50.6 ± 2 minutes for pd-VWF; $P < .05$) (Figure 1C). Furthermore, the enhanced clearance rates observed for α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF were not significantly different in the presence or absence of the AMR (Figure 1D-E). Collectively, these data confirm that reductions in N- and/or O-linked sialylation have major effects on VWF half-life and demonstrate that α 2-3-linked sialic acid expressed on O-linked glycans may be of particular importance in regulating pd-VWF clearance. Furthermore, our findings suggest that previously unrecognized AMR-independent pathways contribute to the enhanced clearance of hyposialylated VWF in vivo.

Figure 2. MGL facilitates VWF clearance in vivo. To investigate other receptors and/or cell types that modulate the enhanced clearance of hyposialylated VWF, α 2-3 Neu-VWF (A) and α 2-3,6,8,9 Neu-VWF (B) clearance studies in VWF^{-/-}/*Asgr1*^{-/-} mice were repeated in the presence of ASOR, or following clodronate-induced macrophage depletion. The enhanced clearance of both α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF was still inhibited by ASOR (blue lines) even in the absence of the AMR (α 2-3 Neu-VWF $t_{1/2} = 8.2 \pm 1.4$ minutes vs 16.8 ± 1.6 minutes, $P < .005$; and α 2-3,6,8,9 Neu-VWF $t_{1/2} = 3.7 \pm 0.7$ minutes vs 7.7 ± 1.3 minutes, $P < .05$, respectively). In addition, clodronate-induced macrophage depletion (green lines) also significantly attenuated the enhanced clearance of hyposialylated VWF (α 2-3 Neu-VWF $t_{1/2} = 8.2 \pm 1.4$ minutes vs 24.0 ± 1.1 minutes, $P < .05$; and α 2-3,6,8,9 Neu-VWF $t_{1/2} = 3.7 \pm 0.7$ minutes vs 9.6 ± 4.1 minutes, $P < .05$, respectively). Three to 5 mice were used per time point, and data are represented as mean \pm SEM. (C) SPR was used to evaluate the binding of immobilized purified pd-VWF to recombinant human MGL. Dose-dependent binding was observed, with K_D (app) of $18.4 \pm 3 \mu\text{g/mL}$. (D) In mice, there are 2 homologs of human MGL, mMGL1 and mMGL2. Murine MGL1 shares significant sequence homology with human MGL and binds oligosaccharides with multiple terminal Gal residues including the T antigen. Interestingly, the markedly enhanced clearance of both α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF in VWF^{-/-}/*Asgr1*^{-/-} mice was significantly attenuated in the presence of an mMGL blocking antibody vs isotype immunoglobulin G (IgG) control antibody, respectively (α 2-3 Neu-VWF $t_{1/2} = 21.9 \pm 11.8$ minutes vs 9.1 ± 1.5 minutes, $P < .05$; and α 2-3,6,8,9 Neu-VWF $t_{1/2} = 24.4 \pm 8.1$ minutes vs 5.7 ± 2.1 minutes, $P < .05$, respectively). (E) THP1 macrophages incubated with VWF demonstrated VWF-MGL colocalization detected by Duolink-PLA, visualized as red spots via immunofluorescence microscopy. No signal was observed from cells incubated with phosphate-buffered saline (PBS) alone. (F) Plasma VWF:Ag levels were significantly elevated in *MGL1*^{-/-} mice compared with wild-type (WT) littermate controls ($P < .05$). (G) The clearance of endogenous murine VWF in *MGL1*^{-/-} mice was significantly attenuated compared with wild-type controls at all time points measured ($P < .05$). (H) In vivo clearance of wild-type pd-VWF in VWF^{-/-}/*Asgr1*^{-/-} mice was significantly attenuated in the presence of an mMGL blocking antibody compared with isotype control IgG ($t_{1/2} = 64.6 \pm 18.4$ minutes vs 42.8 ± 10.7 minutes; $P < .005$). A minimum of 3 mice were used per time point; data are plotted as mean \pm SEM.

The macrophage galactose receptor regulates in vivo clearance of VWF

To investigate other receptors and/or cell types that modulate the enhanced clearance of hyposialylated VWF, α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF clearance studies in $VWF^{-/-}/Asgr1^{-/-}$ mice were repeated in the presence of ASOR, or following clodronate-induced macrophage depletion (Figure 2A-B). The enhanced clearance of both α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF was still inhibited by ASOR even in the absence of the AMR. Interestingly, in vivo macrophage depletion also significantly attenuated the enhanced clearance of hyposialylated VWF. Finally, in vitro binding studies demonstrated enhanced binding of asialo-VWF to differentiated THP1 macrophages (supplemental Figure 2). Collectively, these data demonstrate that additional asialo-receptors, at least in part expressed on macrophages, regulate the enhanced clearance of hyposialylated VWF in vivo.

MGL is a C-type lectin receptor expressed as a homo-oligomer on antigen-presenting cells such as macrophages and dendritic cells (supplemental Figure 3).¹⁷ The carbohydrate recognition domain of MGL binds with high affinity to glycoproteins expressing terminal *N*-acetylgalactosamine or galactose (Gal) residues, and thus MGL can regulate glycoprotein endocytosis.¹⁸⁻²⁰ MGL binding to oligosaccharide chains is attenuated by terminal sialylation.²¹ Importantly, given the putative role of VWF O-linked glycans in modulating clearance, MGL also recognizes the T antigen.²² In mice, there are 2 homologs of human MGL, mMGL1 and mMGL2.²³ Murine MGL1 shares significant sequence homology with human MGL and has been shown to bind oligosaccharides with terminal Gal residues including the so called T antigen.²³ Of note, previous studies have demonstrated that ~70% of the O-glycans of VWF are composed of this sialylated tumor-associated T antigen structure.^{4,7} Interestingly, the enhanced clearance of both α 2-3 Neu-VWF and α 2-3,6,8,9 Neu-VWF in $VWF^{-/-}/Asgr1^{-/-}$ mice was significantly attenuated in the presence of anti-mMGL1/2 inhibitory antibody, suggesting a novel role for MGL in regulating macrophage-mediated clearance of hyposialylated VWF (Figure 2D). Importantly, we observed dose-dependent binding of human pd-VWF to purified recombinant human MGL using SPR (Figure 2C). Moreover, Duolink-PLA analysis demonstrated that VWF colocalizes with MGL on the surface of THP1 macrophages, as indicated by the distinct red fluorescent dots (Figure 2E). Plasma VWF:Ag levels were significantly elevated in $MGL1^{-/-}$ mice compared with wild-type controls ($152.6 \pm 15.7\%$ vs $100 \pm 16.9\%$; $P < .05$) (Figure 2F). Furthermore, in vivo clearance of endogenous murine VWF was attenuated in $MGL1^{-/-}$ mice (Figure 2G), suggesting that MGL-mediated VWF clearance is important even in the presence of AMR. Finally, clearance of pd-VWF in $VWF^{-/-}/Asgr1^{-/-}$ mice was attenuated in the presence of mMGL1/2 inhibitory antibody (Figure 2H). Collectively, these findings reveal MGL as a novel macrophage lectin receptor for VWF that contributes to the clearance of both wild-type

and hyposialylated VWF. Further studies will be required to determine the importance of MGL compared with other recently described receptors involved in regulating VWF clearance.²⁴ Nevertheless, the role of MGL in modulating VWF clearance has direct translational relevance in that quantitative variations in *N*- and *O*-linked sialylation have been described in patients with type 1 VWD.^{7,9} In addition, desialylation of VWF has been described with glycoprotein ageing in plasma²⁵ and can also occur during infections with specific pathogens that are associated with significantly enhanced neuraminidase activity (eg, *Streptococcus pneumoniae*).¹⁰

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Authorship

Contribution: S.E.W., J.M.O., S.A., C.D., C.N.J., J.S., P.G.F., T.M.B., P.S., O.S., and A.C. performed experiments; S.E.W., J.M.O., S.A., C.D., C.N.J., J.S., P.G.F., T.M.B., R.J.S.P., O.S., A.C., and J.S.O. designed the research and analyzed the data; and all authors were involved in writing and reviewing the manuscript.

Conflict-of-interest disclosure: J.S.O. has served on the speaker's bureau for Baxter, Bayer, Novo Nordisk, Boehringer Ingelheim, Leo Pharma, and Octapharma; has served on the advisory boards of Baxter, Bayer, Octapharma CSL Behring, Daiichi Sankyo, Boehringer Ingelheim, and Pfizer; and has received research grant funding awards from Baxter, Bayer, Pfizer, and Novo Nordisk. The remaining authors declare no competing financial interests.

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Footnotes

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There is a *Blood* Commentary on this article in this issue.

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A novel role for the macrophage galactose-type lectin receptor in mediating von Willebrand factor clearance

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von Willebrand factor clearance – biological mechanisms and clinical significance

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Summary

The mechanisms involved in regulating von Willebrand factor (VWF) clearance remain poorly understood. However recent studies have shown that macrophages play a critical role in regulating the half-life of VWF, and have identified specific lectin (including asialoglycoprotein, macrophage galactose-type lectin, Sigec-5 and C-type lectin domain family 4 member M) and scavenger receptors (including low-density lipoprotein receptor-related protein-1, scavenger receptor A1 and stabilin-2) that are involved in VWF clearance. Further studies will be required to determine the relative importance of these individual receptors with respect to physiological and pathological VWF clearance. Nevertheless, recent clinical data have highlighted the importance of enhanced VWF clearance in the pathogenesis of type 1 von Willebrand disease (VWD). Moreover, increased clearance also contributes to reduced VWF levels in many patients with type 2 and type 3 VWD. Improved understanding regarding VWF clearance is not only of direct biological relevance, but may also have important implications for the development of novel therapeutic agents with extended plasma half-lives for the treatment of both VWD and haemophilia A.

Keywords: von Willebrand factor, von Willebrand disease, clearance, macrophage.

von Willebrand factor (VWF) is a large multimeric plasma glycoprotein that plays a key role in maintaining normal haemostasis by regulating platelet adhesion to exposed collagen at sites of vascular injury (Lenting *et al*, 2015). VWF also acts as a carrier molecule for procoagulant factor VIII (FVIII) (Terraube *et al*, 2010). This binding to VWF serves to protect FVIII from premature proteolytic degradation and rapid clearance from the circulation. von Willebrand disease (VWD) is the commonest inherited human bleeding disorder

and results from either quantitative or qualitative reductions in plasma VWF levels (Nichols *et al*, 2009; Laffan *et al*, 2014). In contrast, elevated plasma levels of the VWF-FVIII complex constitute a dose-dependent risk factor for venous and arterial thrombosis (Jenkins *et al*, 2012). Substantial progress has been achieved in understanding VWF biosynthesis, structure and function (Lenting *et al*, 2015). However, until recently, the biological mechanisms responsible for regulating VWF circulatory clearance remained poorly understood. This review will focus upon recent insights into the molecular mechanisms involved in regulating VWF clearance *in vivo*. In addition, emerging evidence regarding the clinical importance of enhanced VWF clearance in the pathogenesis of VWD is also considered.

VWF biosynthesis, structure and function

Under normal physiological conditions, *in vivo* expression of VWF is restricted to endothelial cells (EC) and megakaryocytes only (Mayadas & Wagner, 1991). VWF is initially synthesized as a monomer composed of a series of repeating domains in the order D²-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK and undergoes complex post-translational modification within EC prior to its secretion (Kaufman, 1998; Lenting *et al*, 2015). This includes both N- and O- linked glycosylation, multimerization and, finally, propeptide cleavage. Consequently, in normal plasma VWF circulates as a series of heterogeneous oligomers containing a variable number of subunits, with the largest multimers having molecular weights in excess of 20 000 kDa. Following damage to blood vessel wall, the normal EC lining is disrupted and subendothelial matrix comes into contact with circulating plasma. VWF mediates platelet adhesion at sites of vascular injury. The multimeric composition of plasma VWF is a critical determinant of its functional activity. In normal plasma, VWF multimeric composition is regulated by the metalloprotease ADAMTS13 (A Disintegrin And Metalloproteinase with Thrombospondin type-1 repeats) which cleaves a specific scissile bond (Tyr 1605-Met 1606) within the VWF A2 domain (Sadler, 2008). In addition to ADAMTS13, recent studies have suggested that other plasma proteases, including plasmin, can also cleave VWF multimers (Brophy *et al*, 2017).

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Cellular basis of VWF clearance

Despite significant insights into the biology underlying VWF biosynthesis, structure and function, our understanding of the mechanisms involved in regulating VWF clearance remains limited (Pipe *et al*, 2016). Initial clearance studies in a variety of different animal models (including New Zealand White rabbits, Sprague Dawley rats and C57BL/6J VWF-deficient mice) consistently demonstrated biphasic clearance of VWF, with an initial rapid distribution phase followed by a second slower elimination phase (Sodetz *et al*, 1977; Stoddart *et al*, 1996; Lenting *et al*, 2004). The biological mechanism(s) underpinning this biphasic clearance pattern have not been defined. Nevertheless, following intravenous injection of radiolabelled VWF into VWF-deficient mice, Lenting *et al* (2004) showed that the liver and spleen were both efficient in modulating VWF uptake. However, given its relative size and blood flow, current data suggest that the majority of intravenously-injected VWF is primarily targeted to the liver (Lenting *et al*, 2004). In contrast, other organs, such as the spleen and kidneys, took up relatively small amounts of VWF (van Schooten *et al*, 2008). Subsequent immunohistochemical studies demonstrated that VWF specifically colocalised with CD68+ Kupffer macrophage cells within the murine liver (van Schooten *et al*, 2008). Moreover, macrophage depletion with either gadolinium or clodronate was associated with significantly prolonged survival of VWF in this VWF-deficient mouse model (van Schooten *et al*, 2008; Rawley *et al*, 2015). Interestingly, similar clearance rates were observed for high and low molecular weight multimers, suggesting that VWF clearance is independent of its multimer size (Lenting *et al*, 2004). This finding is in keeping with data from other studies that found no relationship between VWF proteolysis by ADAMTS13 and rate of VWF clearance (Badirou *et al*, 2010).

In keeping with these *in vivo* data, *in vitro* studies have shown that primary human macrophages bind VWF in a dose-dependent and saturable manner (van Schooten *et al*, 2008; Castro-Nunez *et al*, 2012; Chion *et al*, 2016). Furthermore, this macrophage binding was followed by VWF uptake and degradation (van Schooten *et al*, 2008). Several groups have reported that VWF binding to macrophages is significantly enhanced in the presence of shear stress or ristocetin,

suggesting that VWF conformation plays a critical role in regulating macrophage-mediated clearance (Castro-Nunez *et al*, 2012; Chion *et al*, 2016). Together, these data demonstrate that macrophages play a prominent role in regulating VWF clearance and thereby modulating plasma levels of the VWF-FVIII complex. Nevertheless, it remains possible that other cells may also contribute to physiological and/or pathological VWF clearance. For example, Sorvillo *et al* (2016) recently reported that immature monocyte-derived dendritic cells (DCs) bind to VWF. In their capacity as key antigen-presenting cells, DCs are known to have a wide variety of endocytic mechanisms. Interestingly however, despite binding to the cell surface, VWF was not efficiently internalised by DCs (Sorvillo *et al*, 2016). Neutrophil binding to VWF under both static and shear conditions has been demonstrated, but there is no evidence to date that neutrophils contribute to VWF clearance (Pendou *et al*, 2006).

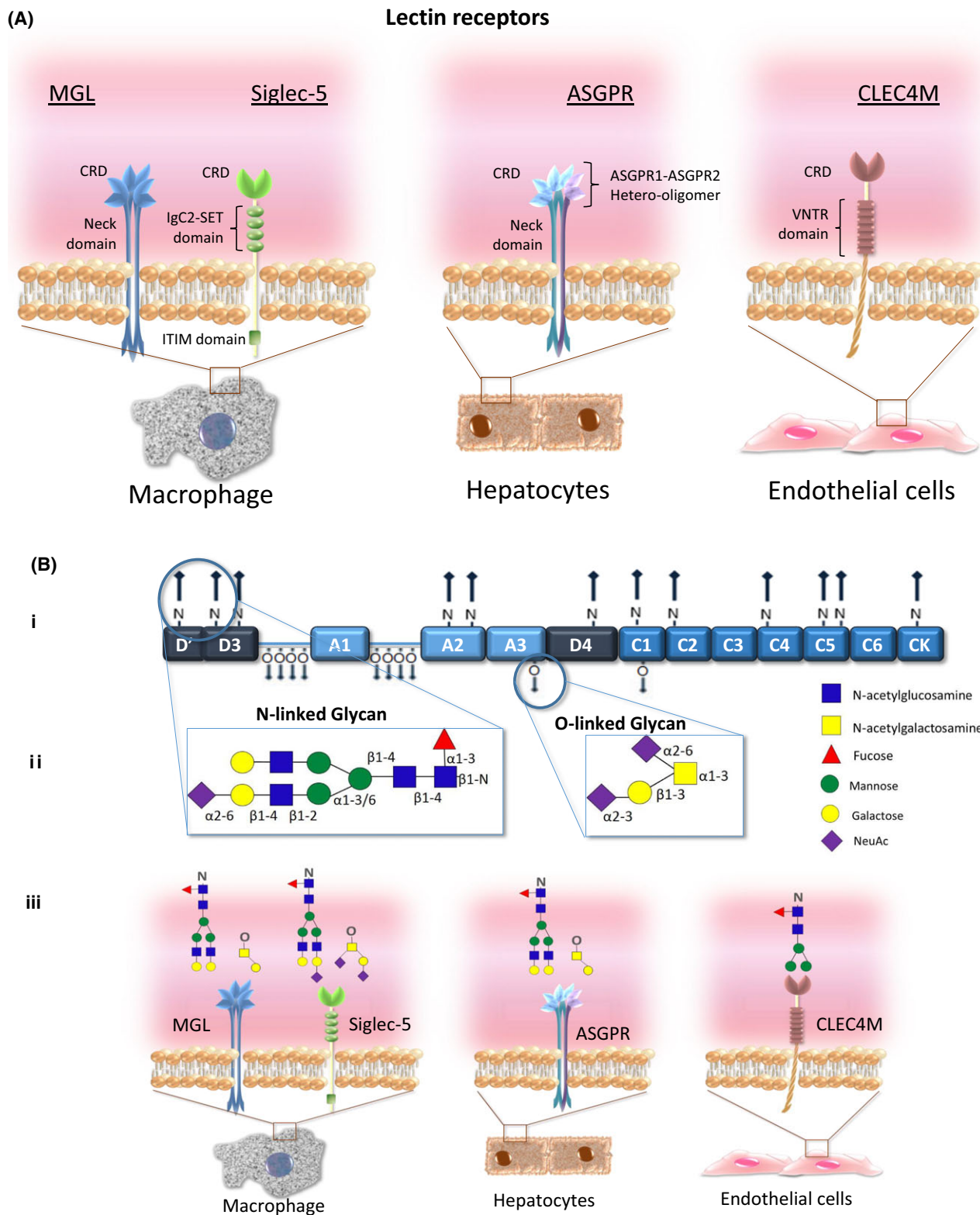
Lectin receptors and VWF clearance

The first VWF clearance receptor identified was the asialoglycoprotein receptor (ASGPR; also termed Ashwell-Morell receptor, AMR) (Grewal *et al*, 2008) (Fig 1A). This receptor is a member of the calcium-dependent (C-type) lectin family of receptors (Ashwell & Harford, 1982; Stockert, 1995). Importantly, although the ASGPR is expressed abundantly in the liver on hepatocytes, it is not expressed on macrophages. The ASGPR is composed of two trans-membrane polypeptides (Asgr-1 and Asgr-2) that assemble into hetero-oligomers on the cell surface (Stockert, 1995). The C-terminal extracellular domains of Asgr-1 and Asgr-2 form a carbohydrate recognition domain (CRD) that selectively binds glycoproteins expressing either β -D-galactose (β Gal) or N-acetyl-D-galactosamine (GalNAc) determinants (Spiess, 1990) (Fig 1B). These β Gal and GalNAc residues are more typically expressed on plasma glycoproteins as sub-terminal moieties on oligosaccharide chains capped by sialic acid. As glycoproteins age in plasma, terminal sialic acid residues are lost so that the ASGPR can then bind to the exposed β Gal or GalNAc and mediate endocytosis (Yang *et al*, 2015). In keeping with other plasma glycoproteins, enzymatic removal of terminal sialylation from VWF has been associated with a markedly reduced plasma half-life *in vivo* (Preston *et al*,

Fig 1. (A) Lectin receptors mediate von Willebrand factor (VWF) clearance. A number of lectin receptors have been proposed to play roles in mediating VWF clearance *in vivo*. These lectin receptors all possess carbohydrate recognition domains (CRDs) that typically interact with specific glycan structures expressed on circulating plasma glycoproteins. Macrophage galactose lectin (MGL) and Siglec-5 are both expressed on macrophages. In contrast, the asialoglycoprotein receptor (ASGPR) is predominantly expressed on hepatocytes and exists as a hetero-oligomer composed of major (ASGPR1) and minor subunits (ASGPR2). The C-type lectin domain family 4 member M (CLEC4M) receptor is found on endothelial cells in the liver sinusoids. This receptor is highly polymorphic, with 3–9 variable numbers of tandem repeats (VNTR) in the neck region. ITIM, immunoreceptor tyrosine-based inhibitory motif. (B) VWF glycan moieties mediate the interaction with lectin clearance receptors. (i) The VWF monomer is highly glycosylated and possesses 12 N-linked and 10 O-linked glycans, which together account for almost 20% of the monomeric mass. (ii) These glycan structures have been characterised by mass spectrometry. The commonest N- and O-linked structures are represented. These N- and O-glycans are usually capped by terminal sialic acid moieties which play an important role in regulating interaction with the CRDs of the various lectin clearance receptors. (iii) Both MGL and ASGPR bind with increased affinity to hyposialylated glycan structures. In contrast, Siglec-5 binds preferentially to sialylated chains, and CLEC4M to mannose-rich glycans.

2013). Grewal *et al* (2008) first reported that enhanced clearance of hyposialylated VWF occurs via the ASGPR. Thus, plasma VWF levels are significantly elevated due to attenuated clearance in *Asgr1* knockout mice (Grewal *et al*, 2008).

Recent studies have shown that ASGPR-independent pathways also contribute to the enhanced clearance of hyposialylated VWF and have identified a role for the macrophage galactose-type lectin (MGL) in regulating this clearance



(Ward *et al*, 2018) (Fig 1A, B). MGL is another C-type lectin receptor with a CRD that also binds to terminal GalNAc or Gal residues (Yamamoto *et al*, 1994; Higashi *et al*, 2002; van Vliet *et al*, 2005). In contrast to ASGPR, predominant expression of MGL is on macrophages and DCs rather than hepatocytes. Dose-dependent binding of VWF to purified human MGL has been confirmed using surface plasmon resonance (Ward *et al*, 2018). Interestingly, the enhanced clearance of hyposialylated VWF in VWF-deficient mice was significantly attenuated in the presence of anti-MGL inhibitory antibody. Furthermore, plasma VWF antigen (VWF:Ag) levels were significantly elevated in MGL1-deficient mice compared to wild type controls. Collectively, these findings suggest that MGL is involved in regulating macrophage-mediated clearance of both wild-type and hyposialylated VWF.

Unlike ASGPR and MGL, which both bind enhanced affinity to hyposialylated VWF, Siglec-5 is a member of the sialic-acid binding immunoglobulin-like lectin (Siglec) family (Lock *et al*, 2004) (Fig 1A, B). Siglec-5 is expressed on monocytes/macrophages, neutrophils and B lymphocytes (Jandus *et al*, 2011). Pegon *et al* (2012) demonstrated dose-dependent binding of Siglec-5 to human VWF in a sialic-acid dependent manner. Also, Siglec-5 expressed on HEK293 cells was able to bind VWF and regulate endocytosis into early endosomes. Although there is no Siglec-5 homolog in mice, hydrodynamic expression of human Siglec-5 in murine hepatocytes resulted in a marked reduction in murine plasma VWF levels.

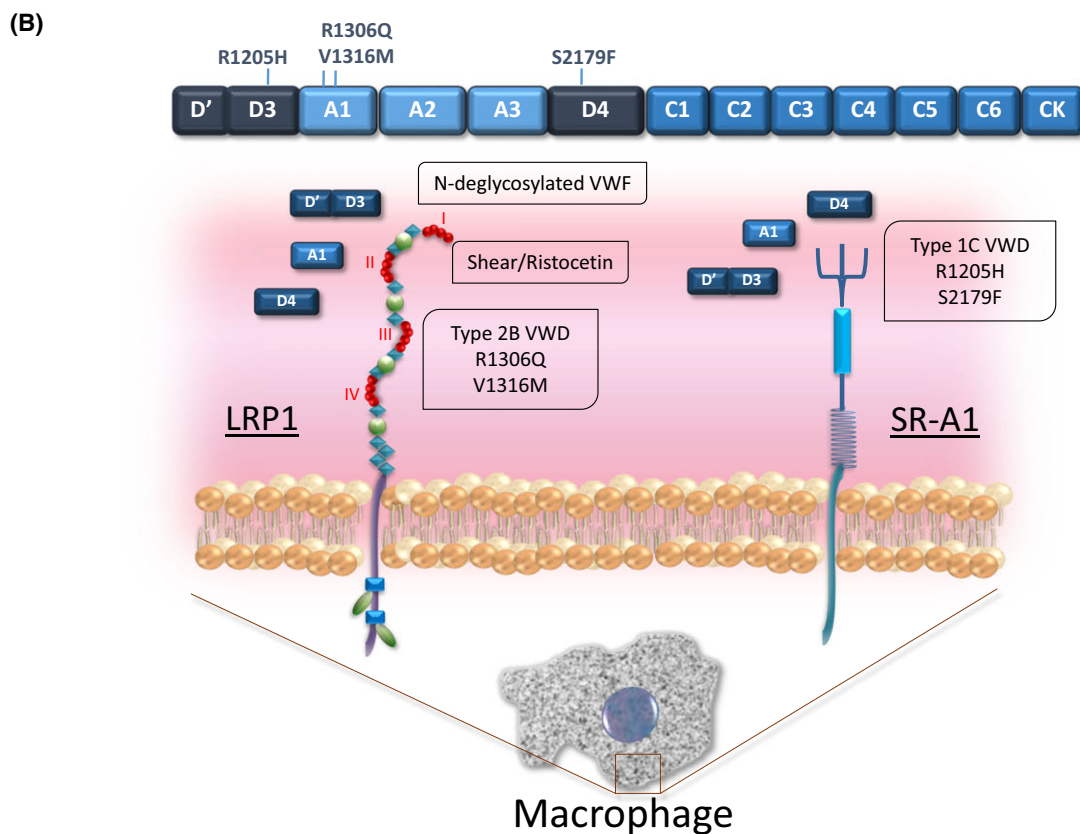
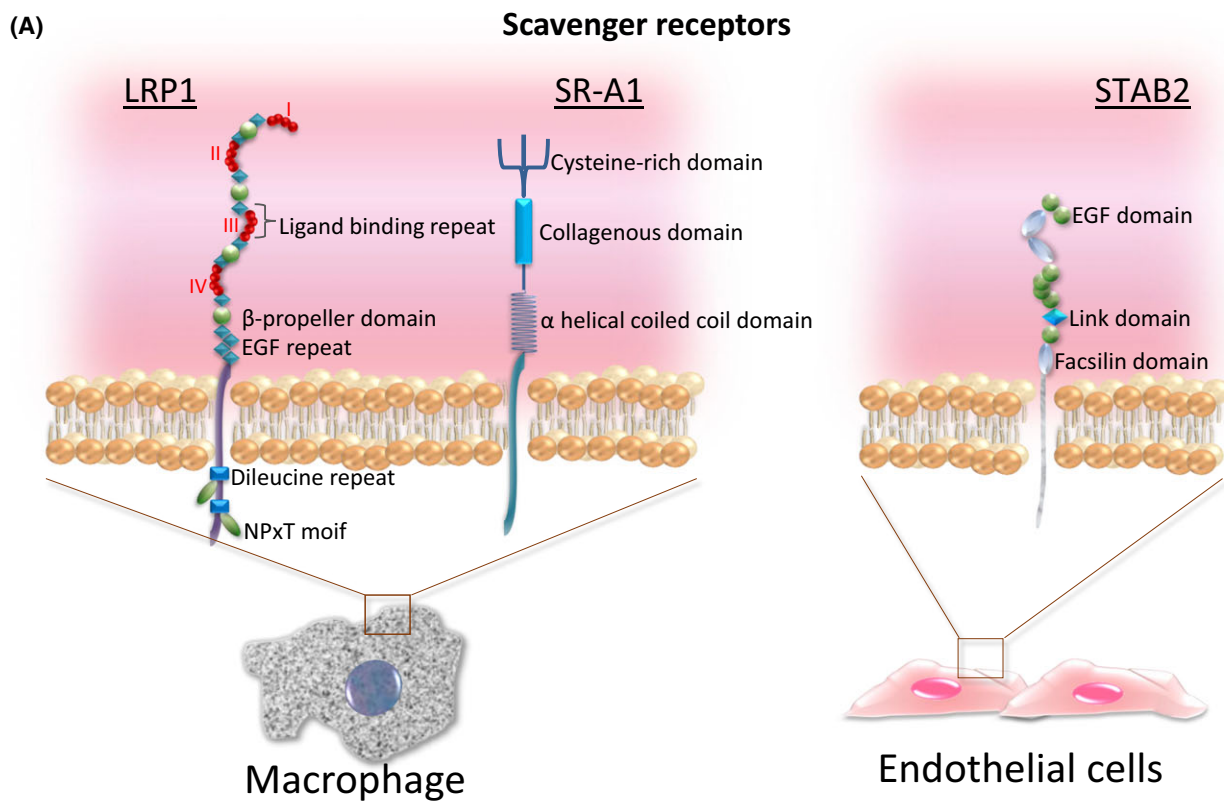
C-type lectin domain family 4 member M (CLEC4M) has specific affinity for mannose and is specifically expressed on EC in liver sinusoids and lymph nodes (Khoo *et al*, 2008) (Fig 1A, B). Rydz *et al* (2013) reported that CLEC4M binds to human VWF, and that recombinant CLEC4M expressed on HEK293 cells can bind VWF on the cell surface, facilitate internalization, and subsequently target VWF to early endosomes. Similar to Siglec-5, mice do not express CLEC4M. However hydrodynamic expression of human CLEC4M in murine hepatocytes resulted in a 46% decrease in murine plasma VWF levels. In addition, significant correlations between CLEC4M polymorphisms and human plasma VWF:Ag levels have been reported (Smith *et al*, 2011; Sanders *et al*, 2015a; Manderstedt *et al*, 2018).

Finally, galectin-1 (Gal-1) and galectin-3 (Gal-3) are lectins with specific affinity for β -galactoside structures (Vasta, 2009). These galectins are expressed in many different cell types (including EC and macrophages) and are also present in normal plasma (Thijssen *et al*, 2008). Saint-Lu *et al* (2012) observed that Gal-1 and Gal-3 could bind to VWF within EC and remained bound to circulating VWF in the plasma. Plasma VWF:Ag levels were normal in Gal-1/Gal-3 double-deficient mice, suggesting that although galectins can bind to VWF and may influence its functional properties, they may not play a direct role in modulating its clearance. In this context, it is interesting that Gal-1 and Gal-3 have also both recently been shown to bind to FVIII in a glycan-dependent manner (O'Sullivan *et al*, 2016a).

Scavenger receptors and VWF clearance

Accumulating data support the hypothesis that the low-density lipoprotein (LDL) receptor-related protein-1 (LRP1) plays an important role in regulating VWF clearance. LRP1 is a large endocytic scavenger receptor that is highly expressed on a number of tissues including macrophages (Strickland *et al*, 2014) (Fig 2A). The extracellular domain of LRP1 is composed of a modular structure consisting including four clusters (I, II, III and IV) of LDL receptor type A repeats that regulate ligand binding. *In vitro* studies have demonstrated that cluster IV of LRP1 can bind to VWF and that LRP1 modulates VWF endocytosis into early endosomes (Rastegarlarlari *et al*, 2012; Wohner *et al*, 2015). Interestingly however, the binding of wild type VWF to LRP1 occurs only in the presence of shear stress or ristocetin, suggesting that VWF needs to be at least partially unfolded in order to interact with LRP1 (Rastegarlarlari *et al*, 2012; Wohner *et al*, 2015) (Fig 2B). LRP1 binding can also be accelerated by truncation of the N-linked glycans of VWF (O'Sullivan *et al*, 2016b). In keeping with the importance of shear, the A1 domain of VWF has been shown to be important in modulating interaction with LRP1 (Wohner *et al*, 2015; Chion *et al*, 2016). *In vitro* studies further suggest that additional domains of VWF (including D'D3 and D4) may also contribute to LRP1 binding (Wohner *et al*, 2018) (Fig 2B). Although the molecular mechanisms through which multimeric VWF interacts with the large LRP1 receptor remain poorly understood, plasma VWF levels were significantly increased (1.6-fold) in mice

Fig 2. (A) Scavenger receptors are involved in von Willebrand factor (VWF) clearance. Various scavenger receptors have been implicated in the clearance of VWF. Low-density lipoprotein receptor-related protein-1 (LRP1) is a large endocytic receptor consisting of four ligand binding repeats termed cluster I-IV. Scavenger receptor A1 (SR-A1) exists as a homotrimer with a cysteine-rich N-terminal domain and collagen-like repeats that are critical for ligand recognition. Finally, stabilin-2 (STAB2) which is expressed on liver sinusoidal endothelial cells has also been proposed to contribute to VWF clearance. STAB2 is composed of multiple epidermal growth factor (EGF)-like repeats and a fasciclin domain that is important for ligand binding. (B) Specific determinants of VWF interaction with scavenger receptors. Emerging data suggest that specific scavenger receptors may play particular roles in contributing to the physiological and pathological clearance of VWF. The D'D3, A1 and D4 domains of VWF have all been shown to bind to LRP1. In addition, N-deglycosylation and a number of type 2B mutations have also been reported to enhance LRP1-mediated clearance of VWF. Interestingly, the D'D3, A1 and D4 domains of VWF have also all been implicated as regulating interaction with SR-A1. Furthermore, a number of type 1C VWF mutations including the VWF-Vicenza R1205H mutation also promote VWF binding to SR-A1.



with macrophage-specific deficiency of LRP1 compared to controls (Rastegarlarlari *et al*, 2012). Of note, several human studies have also reported that polymorphisms in the *LRP1* gene are associated with variations in plasma VWF:Ag levels (Cunningham *et al*, 2005; Morange *et al*, 2005).

The scavenger receptor class A member I (SR-A1; also known as SCARA1 or CD204) is another scavenger receptor expressed in macrophages and DCs (Zani *et al*, 2015) (Fig 2A). Recent *in vitro* studies have demonstrated that VWF binds to purified SR-A1 in a dose-dependent and saturable manner (Wohner *et al*, 2018). Similar to LRP1, multiple different domains of VWF have been implicated in regulating SR-A1 binding (including the D'D3 region, the A1 domain and the D4 domain) (Wohner *et al*, 2018) (Fig 2B). However, in marked contrast to LRP1, binding of VWF to SR-A1 can occur under static conditions, without the need for either shear stress or ristocetin. Besides LRP1 and SR-A1, genome-wide association studies have reported significant associations between VWF-FVIII plasma levels and genes encoding a number of other scavenger receptors, including Stabilin-2 (STAB 2) and SCARA5 (Souto *et al*, 2003; Smith *et al*, 2011). Furthermore, recent studies have demonstrated that VWF can also bind to STAB 2 on liver sinusoidal endothelial cells (LSECs) (Fig 2A), and that human VWF clearance may be reduced in STAB 2-deficient mice (Swystun *et al*, 2018).

In summary, although recent studies have provided important insights into the biology involved in regulating the clearance of VWF *in vivo*, important questions remain to be addressed. These include defining which clearance receptors are most important in terms of regulating both physiological and pathological VWF clearance. Additional studies will be also required to define whether these specific receptors may form synergistic complexes in regulating VWF clearance. Of note, Rastegarlarlari *et al* (2012) have already demonstrated that β 2-integrins and LRP1 may function together in modulating macrophage-mediated VWF clearance. In addition, although many of these receptors have also been shown to bind to free FVIII (reviewed in Pipe *et al*, 2016), their relative importance in determining FVIII clearance and immunogenicity remain to be elucidated.

Enhanced VWF clearance in the pathogenesis of type 1 VWD

Understanding the biological basis involved in regulating VWF clearance has direct clinical significance. In particular, a series of studies have reported that that enhanced VWF clearance constitutes an important pathogenic mechanism in patients with type 1 VWD (Casonato *et al*, 2002, 2010; Brown *et al*, 2003; Federici *et al*, 2004; Schooten *et al*, 2005; Haberichter *et al*, 2006, 2008; Castaman *et al*, 2008; Millar *et al*, 2008; Flood *et al*, 2011; Eikenboom *et al*, 2013; Sanders *et al*, 2015b). The observation that VWF circulatory half-life was significantly reduced in selected patients with VWD originated from fall-off studies performed following desmopressin (DDAVP) administration (Fig 3). Subsequently, it

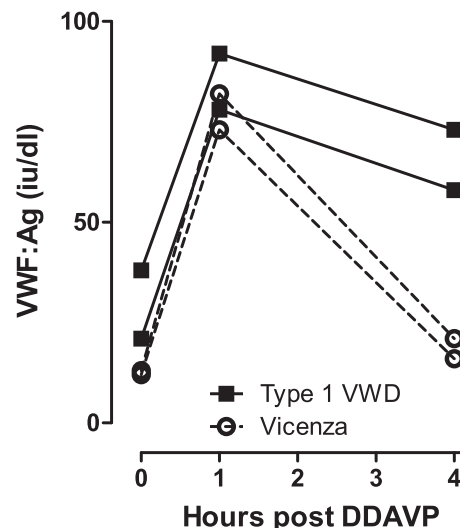


Fig 3. Enhanced von Willebrand factor (VWF) clearance in patients with von Willebrand disease (VWD) Vicenza (R1205H). Typical desmopressin (DDAVP) response in patients with the archetypal Type 1C VWD R1205H substitution, demonstrating an initial significant rise in plasma VWF antigen (VWF:Ag) levels followed by a rapid fall to baseline levels due to accelerated *in vivo* VWF clearance.

was demonstrated that enhanced clearance in VWD patients could also be identified by measuring steady state plasma VWF propeptide (VWFpp) and VWF:Ag levels (Haberichter *et al*, 2006, 2008; Haberichter, 2015). On the basis of DDAVP and/or VWFpp/VWF:Ag ratio studies, several large cohort studies have demonstrated that enhanced VWF clearance is prevalent in patients with type 1 VWD. For example, the US Zimmerman Program included 140 patients with type 1 VWD (plasma VWF levels < 30 iu/dl) (Flood *et al*, 2011). Importantly, 57 (41%) of these patients had significantly reduced plasma VWF levels (typically <15 iu/dl) together with increased plasma VWFpp/VWF:Ag ratios (>3.0) consistent with enhanced VWF clearance. Similarly, the Willebrand in The Netherlands (WiN) study studied 380 type 1 VWD, all with plasma VWF levels \leq 30 iu/dl (Sanders *et al*, 2015b). In keeping with the US results, an increased VWFpp/VWF:Ag ratio (>2.2) consistent with enhanced clearance was identified in 46% of individuals. Other studies, including the MCMMDM-1VWD European study, have also reported that reduced VWF half-life is a common finding among individuals with type 1 VWD (Castaman *et al*, 2008; Eikenboom *et al*, 2013). Furthermore, the Low VWF Ireland Cohort (LoVIC) study recently reported evidence that subtle enhanced VWF clearance is also common in patients with Low VWF levels (range 30–50 iu/dl) (Lavin *et al*, 2017). Taken together, these findings define pathologically-enhanced clearance as an important mechanism in the pathogenesis of type 1 VWD and have led to the suggestion that affected patients should be considered as a distinct type 1C (1-Clearance) subgroup (Haberichter *et al*, 2006). Given the fact that many patients with type 1 VWD and Low VWF do not have

VWF gene coding mutations, nor indeed demonstrate linkage to the VWF gene, further studies into the biological mechanisms underpinning enhanced clearance may provide exciting insights into VWD pathogenesis. Importantly, accumulating data have reported associations between polymorphisms and rare mutations of several clearance receptors (including LRP1, CLEC4M and STAB 2) in patients with VWD.

Enhanced VWF clearance in types 2 and 3 VWD

Besides the importance of increased VWF clearance in type 1 VWD pathogenesis, emerging data suggest that significant reductions in VWF half-life are also present in many patients with type 2 VWD. For example, the WiN study also studied VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios in 240 patients with type 2 VWD (including 158 with type 2A, 42 with type 2B and 27 with type 2M) (Sanders *et al*, 2015b). Interestingly 59% of type 2A patients, 95% of 2B patients and 48% of 2M patients demonstrated increased VWFpp/VWF:Ag ratios but normal FVIII:C/VWF:Ag ratios. Overall, Sanders *et al* (2015b) observed that the VWFpp/VWF:Ag ratio was actually higher in patients with type 2 VWD compared to those with type 1 VWD, suggesting that the pathogenic importance of enhanced clearance is even more important in type 2 VWD. This hypothesis is supported by data derived from other studies confirming enhanced VWF clearance in patients with type 2 VWD (Haberichter *et al*, 2006; Casari *et al*, 2013a).

According to the International Society on Thrombosis and Haemostasis and United Kingdom Haemophilia Centre Doctors' Organisation diagnostic guidelines, type 3 VWD is characterised by plasma VWF levels <5% (Nichols *et al*, 2009; Laffan *et al*, 2014). The WiN study included 37 patients with type 3 VWD (Sanders *et al*, 2015b). In 59% of these patients, plasma VWFpp levels were undetectable, consistent with a complete absence of VWF synthesis. Surprisingly however, high levels of VWFpp were identified in 41% of type 3 VWD patients, suggesting that markedly enhanced clearance may contribute to the very low plasma VWF levels seen in some type 3 VWD patients (Sanders *et al*, 2015b).

VWF mutations and enhanced clearance

Despite recent advances in our understanding of the biology underpinning VWF clearance, and the evidence that enhanced clearance is important in VWD pathogenesis, the molecular mechanisms through which individual VWF mutations serve to trigger enhanced VWF clearance remain poorly understood. Nevertheless, more than 30 different VWF point mutations have already been reported in patients with increased VWF clearance (Castaman *et al*, 2008; Casari *et al*, 2013b). The first mutation characterized was the VWD Vicenza variant which involves a single amino acid substitution R1205H in the D3 domain of VWF (Casonato *et al*, 2002). Patients with VWD-Vicenza typically have reduced plasma VWF:Ag levels (<10 iu/dl) coupled with significantly elevated VWFpp/VWF:Ag ratios (Casonato

et al, 2002; Haberichter *et al*, 2008). Following DDAVP, the half-life of the secreted VWF-R1205H is also markedly reduced compared to wild-type VWF (Castaman *et al*, 2008). Two other substitutions of arginine 1205 (with cysteine and serine respectively) have also been reported in individuals with significantly reduced plasma VWF levels and reduced half-lives following DDAVP (Millar *et al*, 2008). *In vivo* clearance studies demonstrated that substitutions of R1205 with histidine, cysteine or serine all resulted in markedly reduced survival of recombinant VWF in VWF-deficient mice (Rawley *et al*, 2015). The enhanced clearance of these R1205 variants *in vivo* was significantly attenuated following clodronate-induced macrophage depletion. Subsequent *in vitro* studies have confirmed enhanced binding of VWF-R1205H to differentiated THP-1 macrophages (Wohner *et al*, 2018). Although the precise mechanisms remain unclear, recent data suggest that the SR-A1 scavenger receptor may be important in regulating macrophage-mediated clearance of VWF-R1205H (Wohner *et al*, 2018) (Fig 2B). Further studies will be required to investigate whether additional macrophage receptors are also involved.

Besides the R1205 substitutions, a number of other point mutations have also been described in patients with type 1C VWD (Casari *et al*, 2013b). Amongst these other mutations in the D3 domain (including C1130F, W1144G and C1149R) have been reported. An increasing number of different point mutations in other VWF domains have also been implicated (e.g. I1416N in A1, S2179F in the D4 domain and C2671Y in the CK domain) (Schooten *et al*, 2005; Haberichter *et al*, 2006, 2008; Castaman *et al*, 2008; Casari *et al*, 2013b). In addition, Wohner *et al* (2015) recently demonstrated that specific type 2B VWD variants also result in enhanced macrophage-mediated clearance. In particular, the R1306Q and V1316M substitutions within the A1 domain were shown to significantly enhance VWF clearance via macrophages through an LRP1-dependent mechanism (Fig 2B). Further studies will be required to define the molecular mechanisms through which all of these different mutations all lead to increased VWF clearance.

VWF glycans and enhanced clearance

In keeping with the putative role for lectin receptors (including ASGPR, MGL, CLEC4M and Siglec-5) in regulating VWF clearance, several lines of evidence have shown that VWF glycosylation is a critical determinant of its half-life (Lenting *et al*, 2010; Preston *et al*, 2013). For example, plasma VWF levels are 20–30% lower in blood group O compared to non-O individuals (Jenkins & O'Donnell, 2006). Gallinaro *et al* (2008) reported that this effect of ABO blood group on plasma VWF levels was modulated through altered clearance, with significantly increased clearance in group O individuals. More recent studies have proposed alternative mechanisms may also contribute to this ABO effect (Groeneveld *et al*, 2015). Interestingly, plasma VWF levels are further reduced in persons with the rare Bombay phenotype who lack expression of ABO(H) antigens (O'Donnell *et al*, 2005).

Furthermore, platelet VWF:Ag levels are not influenced by ABO group (McGrath *et al*, 2010a, 2013).

The majority of the N- and O-glycan chains of VWF are capped by terminal sialic acid residues (Canis *et al*, 2010, 2012) (Fig 1B). This sialylation modulates the susceptibility of VWF to proteolysis (McGrath *et al*, 2010b; Brophy *et al*, 2017), and also plays a major role in regulating VWF clearance (Fig 1B). Enzymatic removal of sialic acid residues from VWF markedly reduces plasma half-life (Sodetz *et al*, 1977; Stoddart *et al*, 1996; O'Sullivan *et al*, 2016b; Ward *et al*, 2018). Moreover, genetic inactivation of a specific sialyltransferase (ST3Gal-IV) in mice causes reduced plasma VWF levels due to significantly enhanced clearance (Ellies *et al*, 2002). Further studies will be needed to investigate whether ABO blood group may impact upon quantitative VWF sialylation in human subjects. Despite these data, the importance of carbohydrates in the pathogenesis of VWD remains largely unexplored. Two previous studies reported increased binding of the lectin *Ricinus communis* agglutinin I (RCA-I) to VWF (suggestive of increased Gal or GalNAc expression) in patients with VWD compared to controls (Ellies *et al*, 2002; Millar *et al*, 2008). In addition, van Schooten *et al* (2007) reported enhanced binding of the lectin peanut agglutinin (PNA) (suggesting increased O-linked T antigen expression) in a cohort of patients with type 1 VWD compared to healthy controls. Critically however, given the complexity and heterogeneity of the N- and O-linked glycans expressed on VWF, the molecular mechanisms through which VWF glycans modulate interaction with specific lectins and/or other cellular receptors to regulate *in vivo* clearance have not been defined.

Management of VWD patients with enhanced clearance

The evidence that enhanced VWF clearance may play an important role in the pathogenesis of VWD has direct therapeutic relevance. DDAVP therapy is widely used in the treatment of type 1 VWD. Previous studies have demonstrated that there is significant inter-individual variation in VWF response post-DDAVP administration (Federici *et al*, 2004; Haberichter *et al*, 2006, 2008; Castaman *et al*, 2008). This variability is in keeping with the concept that type 1 VWD may result from either reduced EC synthesis and/or an increased rate of plasma clearance. VWF mutations that interfere with VWF synthesis within EC will be associated with reduced Weibel Palade body stores and thereby attenuated DDAVP responses. In contrast, patients with type 1C VWD will have reduced plasma VWF levels due to enhanced clearance, but Weibel Palade body stores and hence DDAVP response will not be affected. Nonetheless, the plasma half-life of the VWF secreted in response to DDAVP in these subjects may be significantly reduced. For example, in patients with type 1C VWD treated with DDAVP, there is often a marked initial increase in plasma VWF:Ag levels (up to 13.5-fold at 1 h post-DDAVP) but the plasma half-life of the secreted mutant VWF can be less than

1 h (Castaman *et al*, 2008). Collectively, these observations have led to concerns that the clinical efficacy of DDAVP administered at 12–24 hourly intervals may be attenuated in VWD patients with enhanced VWF clearance. Consequently, VWF-containing concentrates may represent a longer-lasting and more efficacious therapeutic option for these patients, particularly in the context of surgery or major bleeding complications (Lavin & O'Donnell, 2016). Critically, studies to date have shown that the half-life of infused plasma-derived VWF concentrates in type 1C patients is normal, suggesting that the enhanced clearance phenotype observed in most cases is primarily attributable to changes in the endogenous VWF molecule (e.g. amino acid substitutions or glycan abnormalities) rather than gain of functions in the VWF clearance pathway (Brown *et al*, 2003; Haberichter *et al*, 2006).

The clinical efficacy of DDAVP in patients with type 1C VWD has been assessed in a limited number of studies. Castaman *et al* (2011) studied 20 patients with type 1 VWD due to enhanced clearance (16 with R1205H and 4 with C1130F) who underwent dental extractions. These patients all received a single dose of DDAVP together with oral tranexamic acid for 5 days. Despite the reduced duration of their DDAVP-induced VWF responses, no bleeding complications were observed in any of this cohort. Seventeen patients also underwent additional surgical or invasive procedures ($n = 20$) during the study period and were treated with DDAVP. Only three of these procedures were complicated by bleeding, and in only one case was additional treatment with VWF-containing concentrate required. In addition, DDAVP has also been used successfully to manage delivery in pregnant women with type 1 VWD (Castaman *et al*, 2006). Thus, despite the markedly reduced VWF half-life observed in patients with type 1 VWD, these limited data from a single centre suggest that DDAVP therapy may still be efficacious. Further prospective randomised studies will be required to fully define the role of DDAVP in patients with specific VWF mutations.

Conclusions

Recent studies have defined the importance of enhanced clearance in relation to the pathogenesis of type 1 VWD. Furthermore, emerging data have also emphasised the prevalence of increased clearance in type 2 VWD. Finally, and perhaps surprisingly, enhanced VWF clearance has also been shown to be important in a significant proportion of patients with type 3 VWD. All together, these clinical findings underscore the critical need for better understanding of the physiological and pathological mechanisms involved in regulating VWF clearance *in vivo*. Although recent studies have provided important novel insights into some of the specific receptors that may be important, further studies will be required to dissect the molecular mechanisms involved and define the relative biological importance of individual VWF receptors. Clearly, understanding VWF clearance is not only of direct translational relevance, but may also have important

implications for the development of novel therapeutic agents with extended plasma half-lives for the treatment of both VWD and haemophilia A. In addition, further studies will also be required to elucidate the importance of reduced VWF clearance (for example as a result of ageing) in the aetiology of elevated plasma levels of the VWF-FVIII complex which constitute a common and dose-dependent risk factor for thrombosis.

Authorship

All authors drafted the first version of different sections of the manuscript and all critically reviewed the final manuscript.

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Conflict-of-interest disclosure

J.S.O'D has served on the speaker's bureau for Baxter, Bayer, Novo Nordisk, Boehringer Ingelheim, Leo Pharma and Octapharma. He has also served on the advisory boards of Baxter, Bayer, Octapharma CSL Behring, Daiichi Sankyo, Boehringer Ingelheim, Shire and Pfizer. J.S.O'D has also received research grant funding awards from Baxter, Bayer, Pfizer, Shire and Novo Nordisk.

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REVIEW ARTICLE

von Willebrand factor sialylation—A critical regulator of biological function

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Abstract

von Willebrand factor (VWF) undergoes complex post-translational modification prior to its secretion into the plasma. Consequently, VWF monomers contain complex N-glycan and O-glycan structures that, together, account for approximately 20% of the final monomeric mass. An increasing body of evidence has confirmed that these carbohydrate determinants play critical roles in regulating multiple aspects of VWF biology. In particular, studies have demonstrated that terminal ABO blood group has an important effect on plasma VWF levels. This effect is interesting, given that only 15% of the N-glycans and 1% of the O-glycans of VWF actually express terminal ABO(H) determinants. In contrast, the vast majority of the N-glycans and O-glycans on human VWF are capped by terminal negatively charged sialic acid residues. Recent data suggest that sialylation significantly regulates VWF functional activity, susceptibility to proteolysis, and clearance, through a number of independent pathways. These findings are of direct clinical relevance, in that quantitative and qualitative variations in VWF sialylation have been described in patients with VWD, as well as in patients with a number of other physiologic and pathologic conditions. Moreover, platelet-derived VWF is significantly hyposialylated as compared with plasma-derived VWF, whereas the recently licensed recombinant VWF therapeutic is hypersialylated. In this review, we examine the evidence supporting the hypothesis that VWF sialylation plays multiple biological roles. In addition, we consider data suggesting that quantitative and qualitative variations in VWF sialylation may play specific roles in the pathogenesis of VWD, and that sialic acid expression on VWF may also differ across a number of other physiologic and pathologic conditions.

KEYWORDS

ADAMTS-13, glycosylation, von Willebrand disease, von Willebrand factor

1 | INTRODUCTION

von Willebrand factor (VWF) is a large multimeric plasma glycoprotein that plays essential roles in maintaining normal hemostasis.¹ First, VWF binds to exposed subendothelial tissues at sites

of vascular injury and subsequently facilitates platelet tethering to form a primary platelet plug. Second, VWF also binds to procoagulant factor VIII (FVIII), thereby protecting it from proteolysis and significantly extending its plasma half-life.² von Willebrand disease (VWD) is the commonest inherited human bleeding disorder, and is caused by quantitative or qualitative reductions in plasma VWF levels.^{3,4} In contrast, elevated plasma levels of the VWF-FVIII complex represent a dose-dependent risk factor for both arterial and venous

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thrombosis.⁵ Consequently, understanding the factors that modulate plasma VWF activity is of direct clinical relevance. Human VWF is heavily glycosylated, and a series of studies have highlighted that the sugar determinants expressed on VWF play key roles in regulating multiple aspects of its biology.⁶ In this review, we examine recent evidence supporting the hypothesis that VWF sialylation may be of particular importance in this context. In addition, we also consider data suggesting that quantitative and qualitative variations in VWF sialylation may play specific roles in the pathogenesis of VWD, and that sialic acid expression on VWF may also differ across a number of other physiologic and pathologic conditions.

2 | VWF BIOSYNTHESIS AND POST-TRANSLATIONAL MODIFICATION

Under normal physiologic conditions, *in vivo* biosynthesis of VWF is restricted to vascular endothelial cells (ECs) and megakaryocytes only.⁷ VWF synthesized within ECs can be secreted into the plasma or stored within intracellular Weibel-Palade (WP) bodies.¹ Subsequently, acute EC activation by different secretagogues (including thrombin, fibrin, and histamine) can trigger secretion of this stored VWF into the plasma. In contrast, VWF synthesized within megakaryocytes is stored within α -granules.⁸ Consequently, under steady-state conditions, plasma VWF is predominantly derived from ECs. Within both EC and megakaryocytes, VWF is initially synthesized as a 2813 amino acid monomer that consists of a series of homologous repeating domains (in the order D¹-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK).¹ This initial VWF monomer is subjected to extensive post-translational modification, which includes multimerization and significant glycosylation.^{6,9} As a result, VWF circulates in normal plasma as a series of heterogeneous oligomers, with the largest multimers having molecular weights in excess of 20 000 kDa.

3 | SIALYLATION OF HUMAN PLASMA-DERIVED VWF

Initial analysis of the VWF amino acid monomer sequence identified 13 potential N-glycosylation consensus sequons (N857, N1147, N1231, N1515, N1574, N2223, N2290, N2357, N2400, N2546, N2585, N2635, and N2790) (Figure 1A).¹⁰ Recent mass spectrometry (MS) studies on human plasma-derived VWF have confirmed occupancy of all of these sites except N1147.¹¹ Importantly, these

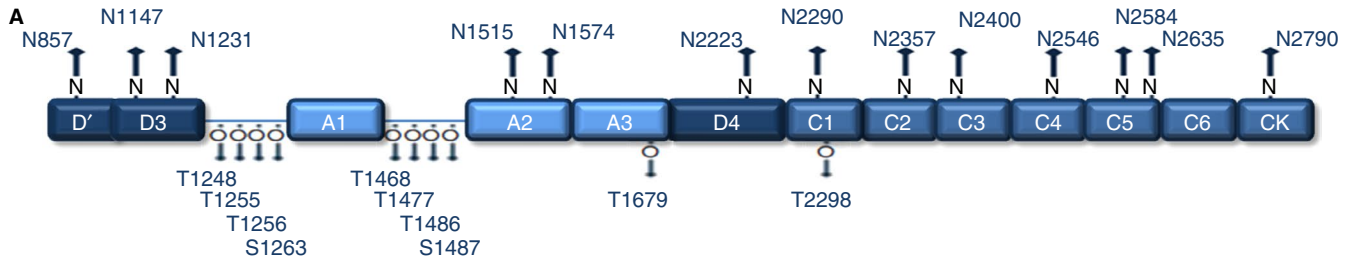
Essentials

- von Willebrand Factor (VWF) is extensively glycosylated with serial studies demonstrating that these carbohydrate determinants play critical roles in regulating multiple aspects of VWF biology.
- Terminal sialic acid residues, expressed on both the N- and O-linked glycans of VWF, regulate VWF functional activity, susceptibility to proteolysis and plasma clearance *in vivo*.
- Quantitative and qualitative variations in VWF sialylation have been reported in patients with von Willebrand Disease, as well as in a number of other physiological and pathological states.
- Further studies are warranted to define the molecular mechanisms through which N- and O-linked sialylation impacts upon the multiple biological activities of VWF.

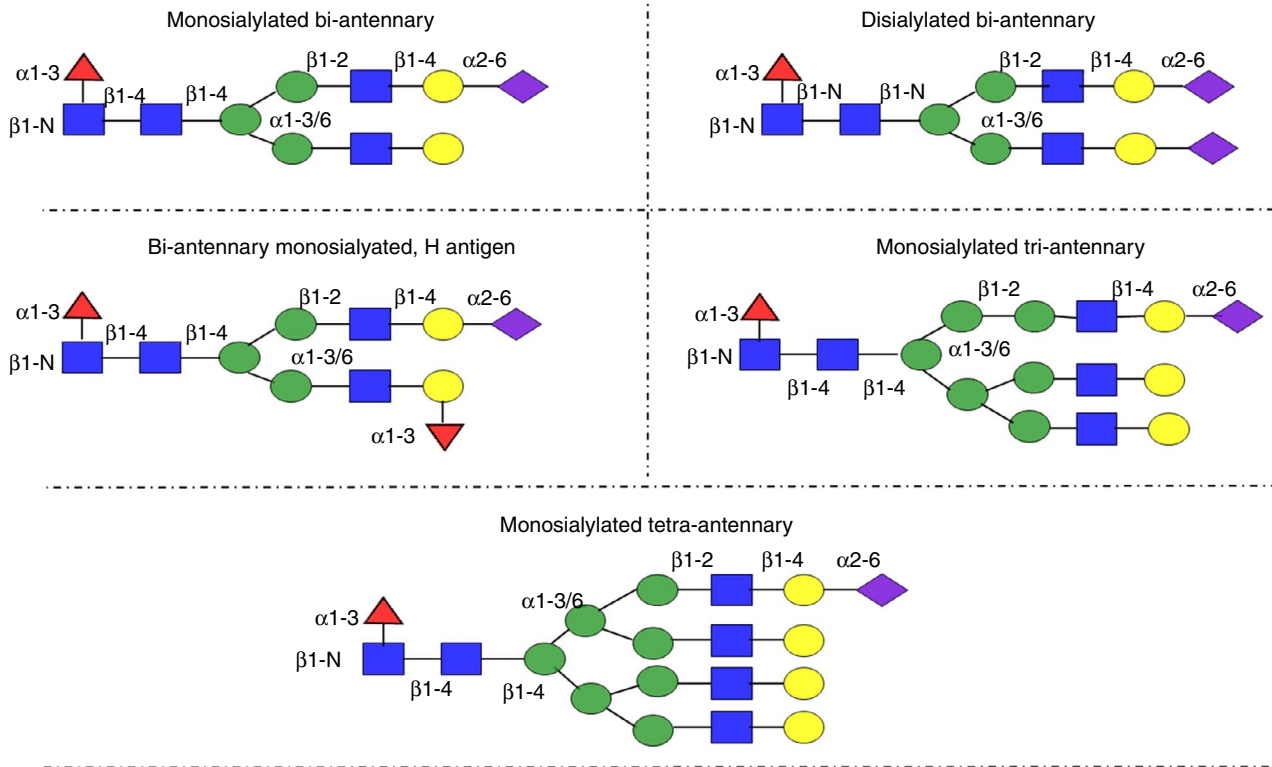
studies also highlighted that the glycans expressed at individual positions across the VWF monomer differed significantly. For example, smaller N-glycan structures were observed at sequons N857 and N1147, whereas larger and more complicated sugar structures were present at N1515 and N1574.¹¹ Overall, complex-type carbohydrates predominated, such that less than 1% of the chains were high-mannose in type. The most common structures observed were monosialylated and disialylated biantennary chains. Recent studies have demonstrated that approximately 80% of the total sialic acid on VWF is expressed on its N-linked glycans, and that the majority of this N-linked capping sialic acid is present in α 2-6 linkage (Figure 1B).^{11,12} Finally, terminal ABO(H) blood group determinants have also been identified on approximately 15% of the N-linked carbohydrates of human plasma-derived VWF (Figure 1B).¹¹ These ABO(H) structures were not localized to specific N-glycan sites on plasma-derived VWF, but, rather, were disseminated across all of the occupied sequons. Importantly, Canis et al observed evidence of concentrated ABO(H) expression at some specific N-glycan sites (e.g., N1515 and N1574),¹¹ and also that ABH determinants were present only on non-sialylated antennae.

Besides its extensive N-glycosylation, each VWF monomer also contains 10 O-glycosylation sites (Figure 1A). Unlike the N-glycans, which are distributed across multiple different domains of VWF, eight of the 10 O-linked glycans (T1248, T1255, T1256, S1263,

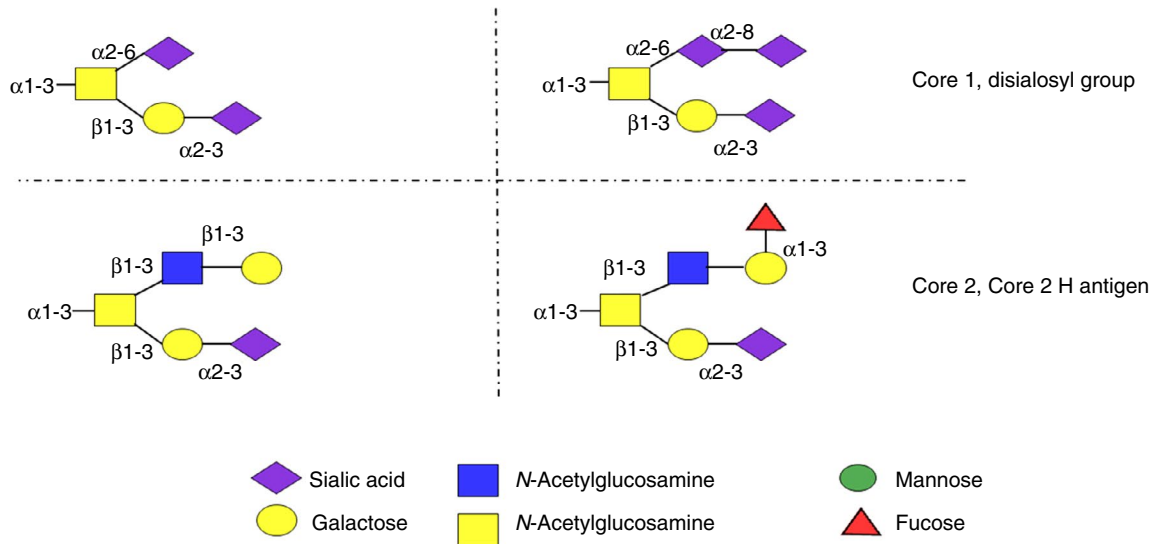
FIGURE 1 (A) The von Willebrand factor (VWF) amino acid sequence includes 13 potential N-glycosylation consensus sequons that span the entirety of the VWF domains. Mass spectrometry has revealed that all of these glycosites are occupied, with the exception of N1147 and N2635, which show only partial occupancy. VWF also contains 10 O-linked glycosylation sites, eight of which flank the A1 domain in two clusters. (B) The commonest N-linked glycan structures are illustrated. Monosialylated bi-antennary complex structures account for approximately 80% of VWF N-linked glycans, with tri-antennary and tetra-antennary sugars also being present in the minority, and high-mannose structures representing less than 1% of VWF N-linked glycan structures. The N-linked glycans are highly sialylated, with 50% of the overall complex antennae being capped with α 2-6-linked sialic acid. (C) In contrast, VWF O-linked glycans are of the short mucin type, with the disialylated core 1 T-antigen structure accounting for 70% of the total VWF O-linked glycans. Core 2 structures are also present. Both VWF N-glycans and O-glycans express ABO blood group determinants (13% and 1%, respectively)



B N-linked glycans



C O-linked glycans



T1468, T1477, S1486, and T1487) are clustered in two groups at either side of the A1 domain.¹³ The other O-glycan chains are located within the A3 and C1 domains (T1679 and T2298, respectively). In contrast to the complexity of its N-glycans, the O-glycans of plasma-derived VWF predominantly exist as short mucin-type carbohydrates (Figure 1C).¹³ Nevertheless, recent MS analyses have again significantly improved our understanding of the structures of these O-glycan structures.^{14,15} The predominant structure seen was a disialylated core 1 structure, also referred to as the Thomas Friedenreich (T) antigen, and accounted for approximately 70% of the total O-glycan population (Figure 1C). Altogether, O-linked sialic acid expression has been estimated to account for less than 20% of the total sialylation on human plasma-derived VWF, and may be present in either α 2-3 or α 2-6 linkage.^{11,14,15} It is of note that disialosyl

groups, which are uncommon among human glycans, were identified on core 1 glycan structures (Figure 1C).^{14,15} Finally, terminal ABO(H) determinants were also identified on O-linked glycans, but were restricted to approximately 1% of core 2 O-glycan structures.

4 | SIALYLATION AND VWF AGING IN PLASMA

Emerging evidence suggests that, like that of other plasma glycoproteins, VWF glycosylation varies with protein aging. Importantly, Yang et al recently showed that aging of secreted plasma glycoproteins is associated not only with a loss of sialic acid, but also with a progressive stepwise loss of terminal sugar residues from N-glycan chains.¹⁶

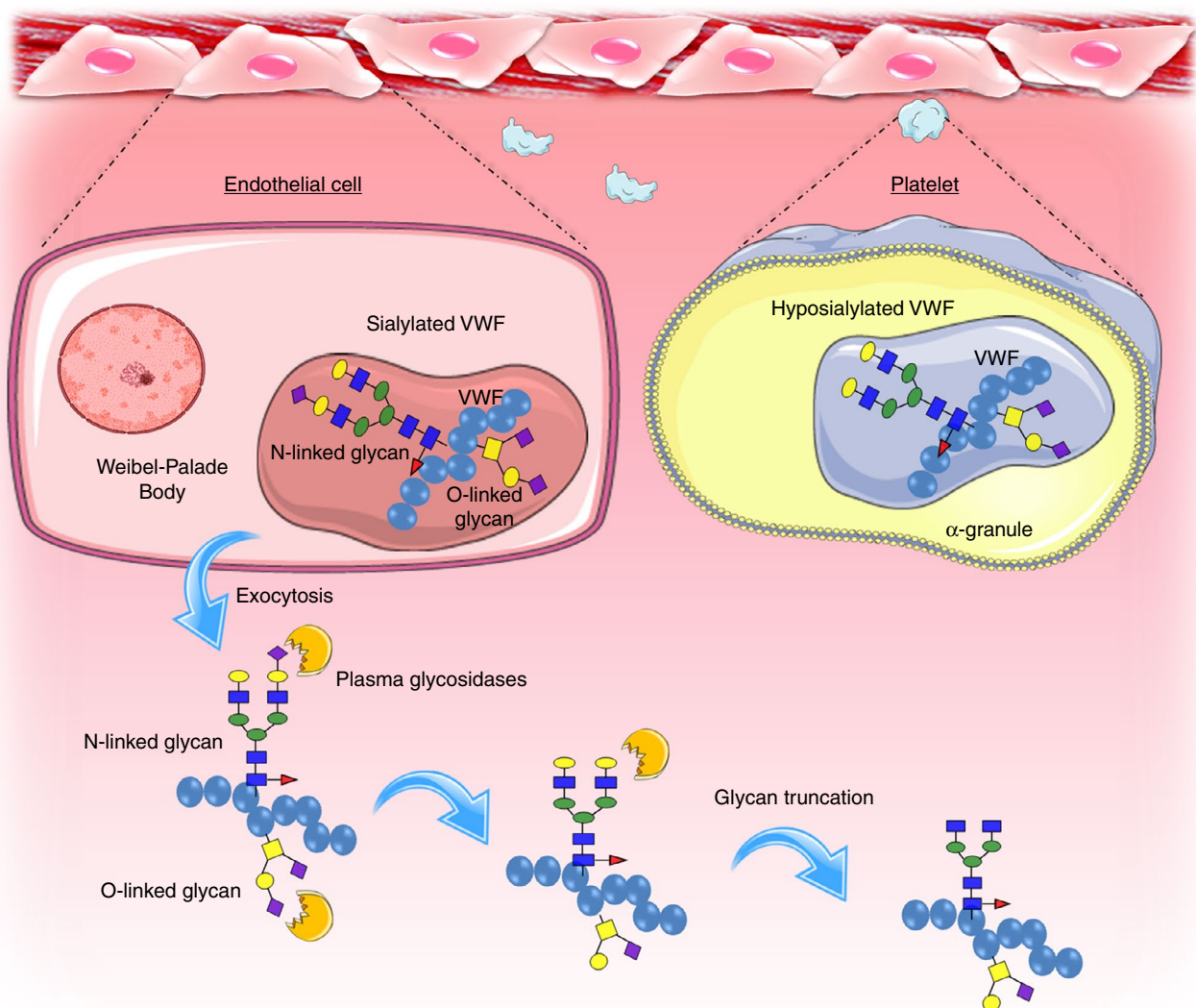


FIGURE 2 The sialylation status of von Willebrand factor (VWF) is heterogeneous and dynamic. VWF of endothelial cell (EC) origin is fully sialylated, whereas platelet-derived VWF has been shown to have significantly reduced VWF N-linked sialylation, probably because of differential sialyltransferase activity in ECs and megakaryocytes. Once secreted, VWF is exposed to circulating plasma glycosidases, which have been reported to perform stepwise removal or trimming of sialic acid/galactose from various plasma glycoproteins

Loss of terminal α 2-6-linked sialic acid (catalyzed by plasma neuraminidases) constitutes the first step in this process. Subsequently, through the actions of other plasma glycosidase enzymes, further progressive N-glycan trimming ensues (Figure 2).¹⁶ In keeping with these data, lectin studies performed on plasma samples collected before and after DDAVP administration have demonstrated significant differences in VWF glycan expression. For example, Aguila et al showed that binding of *Sambucus nigra* agglutinin (SNA), a plant lectin that specifically binds α 2-6-linked sialic acid, to VWF secreted following DDAVP administration was significantly elevated as compared with binding to circulating steady-state plasma VWF, suggesting that VWF high molecular weight multimers (HMWMs) stored within WP bodies are more highly sialylated than VWF in the circulation.¹⁷ In addition, decreased peanut agglutinin (PNA), a plant lectin with affinity for the short mucin T antigen structure, binding and increased blood group antigen expression on VWF have also been described in post-DDAVP samples.^{18,19} Thus, although the majority of the N-linked and O-linked glycans of VWF initially secreted from ECs are capped with terminal sialic residues, the level of plasma VWF sialylation is likely to progressively diminish with glycoprotein aging in the plasma (Figure 2).

5 | SIALYLATION OF HUMAN PLATELET VWF

Platelet α -granules contain an estimated 10%-20% of the total VWF present in platelet-rich plasma.^{20,21} This pool of platelet-derived VWF is discrete from plasma-derived VWF, there being no interchange between the two compartments. Previous studies have demonstrated that sialic acid expression on platelet-derived VWF is markedly reduced as compared with that on plasma-derived VWF (Figure 2).^{22,23} Using HPLC analysis, McGrath et al further showed that this reduced sialic acid expression was mainly attributable to a specific decrease (>50%) in N-linked sialylation on platelet-derived VWF.²⁴ In contrast, O-linked sialylation on platelet-derived VWF and plasma-derived VWF were similar. Again in contrast to plasma-derived VWF, studies have also consistently shown that A and B blood group determinants are not present on platelet-derived VWF.^{8,24} These glycosylation differences presumably reflect differences in the post-translational modification of platelet-derived VWF (synthesized within megakaryocytes) and that of plasma-derived VWF (synthesized within ECs).⁸

6 | VWF SIALYLATION INFLUENCES FUNCTIONAL ACTIVITY

Previous studies conducted under both static and shear-based conditions have demonstrated that VWF glycan structures significantly influence functional properties.²⁵⁻²⁷ A specific role for sialylation in regulating aspects of VWF activity has also been defined. Federici et al showed that neuraminidase treatment of VWF removed more

than 95% of total sialic acid from VWF, neuraminidase-treated VWF (Neu-VWF).²⁵ In the presence of protease inhibitors, desialylation had no direct effect on the multimer pattern.²⁸ However, Neu-VWF was shown to induce spontaneous platelet aggregation in platelet-rich plasma, and also to be more effective in modulating platelet adhesion to a collagen surface under shear.²⁵ Desialylation of VWF is associated with exposure of penultimate galactose (Gal) residues. Importantly, removal of these Gal moieties significantly attenuated the increased adhesion and aggregation properties of Neu-VWF.²⁵

As previously discussed, N-linked sialylation on platelet-derived VWF is markedly reduced as compared with that on plasma-derived VWF. Given this reduction in sialic acid expression, it is interesting that previous studies have reported functional differences between platelet-derived VWF and plasma-derived VWF.^{8,29} For example, although platelet-derived VWF is enriched in HMWMs, it binds glycoprotein (GP) 1b α with significantly reduced affinity as compared with plasma-derived VWF.²² In contrast, platelet-derived VWF shows significantly enhanced binding to both GPIIb/IIIa and heparin.²² Further studies will be necessary to elucidate the molecular mechanisms underlying these differences in specific activity, and, in particular, to determine the contribution of differences in VWF glycosylation.

7 | VWF SIALYLATION REGULATES SUSCEPTIBILITY TO PROTEOLYSIS

VWF multimer distribution is a key determinant of its functional activity.^{1,3} HMWMs bind collagen and GPIb α with increased affinity as compared with low molecular weight multimers, and are thus more effective in facilitating platelet plug formation. In normal plasma, VWF multimer distribution is regulated by ADAMTS-13, which cleaves VWF at a specific Tyr1605-Met1606 bond within the A2 domain.^{30,31} Recent studies have suggested that other proteases, including plasmin, may also play roles in regulating VWF multimer distribution under specific circumstances.^{32,33} The clinical importance of regulating VWF multimer distribution is illustrated by the fact that ADAMTS-13 deficiency in patients with thrombotic thrombocytopenic purpura (TTP) results in accumulation of ultralarge VWF (UL-VWF) multimers and life-threatening thrombotic microvascular occlusion.³¹ Conversely, loss of HMWMs because of enhanced proteolysis in patients with type 2A VWD is associated with significant bleeding.³⁴ It is well recognized that terminal sialic acid expression on glycoproteins plays a key role in protecting against proteolytic destruction. In keeping with this concept, previous studies demonstrated that VWF desialylation resulted in significantly enhanced proteolysis by a number of proteases, including cathepsin B, trypsin, and chymotrypsin.^{12,28} Paradoxically, however, more recent studies have shown that removal of sialic acid from VWF causes it to be significantly more resistant to cleavage by ADAMTS-13.¹² In particular, enzymatic desialylation of plasma-derived VWF with α 2-3,6,8,9 neuraminidase markedly impaired ADAMTS-13-mediated VWF proteolysis in a dose-dependent manner. Interestingly, treatment with

α 2-3 neuraminidase to specifically digest α 2-3-linked sialic acid from the O-glycans of plasma-derived VWF had no significant effect on ADAMTS-13 proteolysis.¹² Together, these data suggest that α 2-6-linked sialylation on plasma-derived VWF may play a critical role in enhancing the susceptibility of VWF to proteolysis by ADAMTS-13. Although the molecular mechanism underpinning this sialylation effect remains unclear, site-directed mutagenesis studies have suggested an important role for the N-linked glycans at Asn1574 in the VWF A2 domain in regulating ADAMTS-13 proteolysis.³⁵

As previously discussed, levels of N-linked sialylation are significantly reduced on platelet-derived VWF as compared with plasma-derived VWF.⁸ Interestingly, as a result of this quantitative change in terminal α 2-6-linked sialic acid expression, platelet-derived VWF shows specific resistance to ADAMTS-13-mediated proteolysis as compared with plasma-derived VWF.²⁴ Hence, not only are high local concentrations of platelet-derived VWF released at sites of vascular injury, but, because it has undergone different post-translational modification within megakaryocytes, this released platelet-derived VWF exists as a discrete natural glycoform that is at least partially resistant to ADAMTS-13 proteolysis.

Recent studies have suggested that plasmin-induced cleavage of VWF may also be of both physiologic and potential pharmacologic significance.^{32,36} For example, significant activation of plasminogen to plasmin has been reported in patients during acute TTP.³² Furthermore, a role for plasmin in preventing the accumulation of pathologic UL-VWF multimers in the absence of ADAMTS-13 regulation has been proposed.³⁶ Interestingly, VWF glycans also play a role in regulating susceptibility to plasmin-mediated proteolysis.^{28,33} However, in contrast to ADAMTS-13 proteolysis, ABO blood group does not influence VWF cleavage by plasmin,³³ and Berkowitz et al previously showed that desialylation of VWF is associated with enhanced plasmin-mediated proteolysis.²⁸

8 | VWF SIALYLATION DETERMINES CLEARANCE THROUGH MULTIPLE PATHWAYS

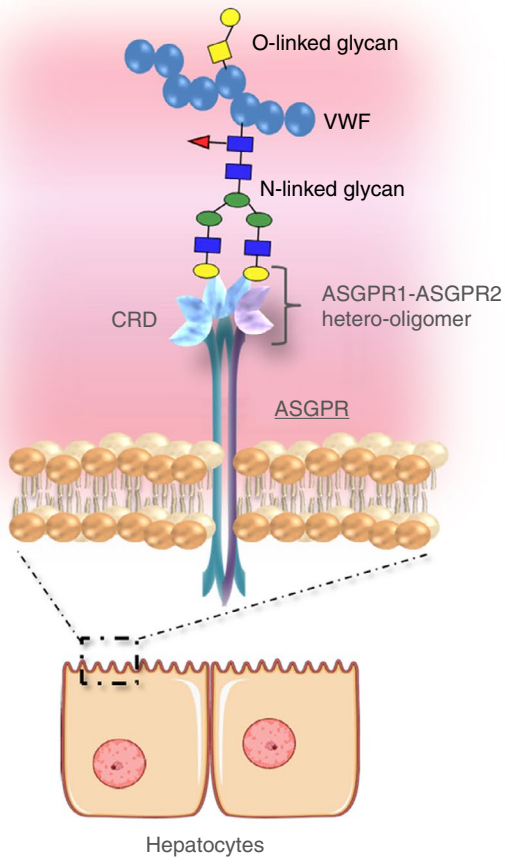
In addition to influencing the susceptibility of VWF to proteolysis, sialic acid expression has been shown to play a critical role in modulating VWF clearance.³⁷ Sodetz et al first demonstrated that enzymatic removal of terminal sialic acid residues was associated with a significant reduction in VWF half-life in rabbits.³⁸ This observation was subsequently confirmed in a number of other independent studies in other animals.³⁹⁻⁴¹ Furthermore, genetic inactivation of the

sialyltransferase ST3Gal-IV in a transgenic mouse model was shown to result in enhanced VWF clearance and a significant reduction in plasma VWF levels.⁴² In contrast to the specific effect of α 2-6-linked sialylation in enhancing VWF proteolysis by ADAMTS-13, current evidence suggests that both α 2-6-linked and α 2-3-linked sialylation play roles in protecting VWF against clearance.^{37,38,40,43,44} Similarly to their specific role in modulating susceptibility to ADAMTS-13 proteolysis, the N-linked glycans at positions 1515 and 1574 within the A2 domain also play a key role in protecting VWF against *in vivo* clearance.⁴⁵ The major effect of α 2-3-linked sialylation in regulating clearance is interesting,⁴³ as this sialic acid accounts for less than 20% of the total sialylation on human plasma-derived VWF, and is expressed predominantly on O-glycans.¹⁴

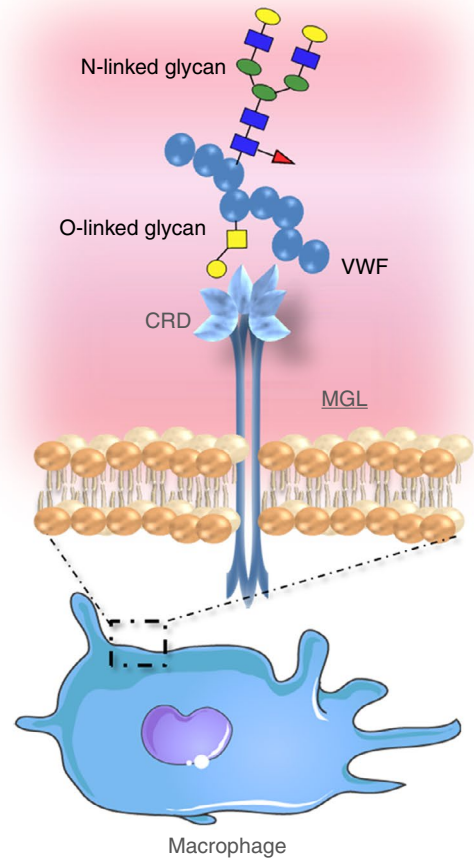
A number of lectin receptors have been implicated in the enhanced clearance of hyposialylated VWF (Figure 3).³⁷ The first receptor proposed to be important in this context was the Ashwell-Morrell receptor or asialoglycoprotein receptor (ASGPR).⁴⁴ ASGPR is a C-type lectin receptor expressed predominantly on hepatocytes, and consists of two transmembrane protein subunits (Asgpr-1 and Asgpr-2).^{46,47} The ASGPR carbohydrate recognition domain preferentially binds to GPs expressing β -D-galactose (β -Gal) or N-acetyl-D-galactosamine (GalNAc) residues.⁴⁷ These residues are typically present on VWF as subterminal moieties on glycan chains capped by sialic acid residues.^{11,14} Consequently, loss of terminal sialylation results in enhanced exposure of these β -Gal and GalNAc residues, which can then trigger ASGPR-mediated clearance (Figure 3A). In keeping with the hypothesis that ASGPR plays a role in VWF clearance, Grewal et al reported significantly elevated plasma VWF levels in *Asgpr-1* knockout mice, and further demonstrated that VWF clearance was reduced in these mice.⁴⁴ More recent studies have implicated a role for the macrophage-galactose-type lectin (MGL) in also modulating the enhanced clearance of hyposialylated VWF (Figure 3B).⁴³ MGL is another C-type lectin receptor that binds to β -Gal and GalNAc residues exposed following desialylation.^{48,49} Interestingly, given that loss of 2-3-linked sialylation may be of particular importance in triggering enhanced VWF clearance, emerging data suggest that MGL may have specific affinity for the O-glycan of plasma-derived VWF.⁴⁴ This observation is in keeping with previous studies showing that MGL preferentially binds to the T antigen structure.¹⁸ Finally, specific members of the sialic acid-binding immunoglobulin-like lectin (Siglec) family have also been shown to bind to human VWF (Figure 3C). In particular, Pegon et al observed that Siglec-5 bound to human plasma-derived VWF in a dose-dependent manner, and

FIGURE 3 A number of lectin receptors have been proposed to play roles in mediating von Willebrand factor (VWF) clearance *in vivo*. Glycoprotein binding to these receptors is mediated by carbohydrate recognition domains (CRDs) that specifically interact with terminal glycan structures expressed on circulating plasma glycoproteins. (A) Asialoglycoprotein receptor (ASGPR) is expressed on hepatocytes as a heterotrimer composed of the major (ASGPR-1) and minor (ASGPR-2) subunits, with the CRD having specificity for glycoproteins terminating in galactose (Gal) and N-acetyl-D-galactosamine (GalNAc) moieties following N-linked sialic acid removal. (B) Macrophage-galactose-type lectin (MGL) is expressed as a homotrimer on macrophages and dendritic cells. The MGL CRD is also specific for exposed Gal/GalNAc terminal moieties; however, MGL binds preferentially to desialylated O-linked glycan structures, with particular affinity for the T antigen. (C) Sialic acid-binding immunoglobulin-like lectin-5 (Siglec-5), which is also expressed on macrophages, preferentially binds sialylated glycan structures

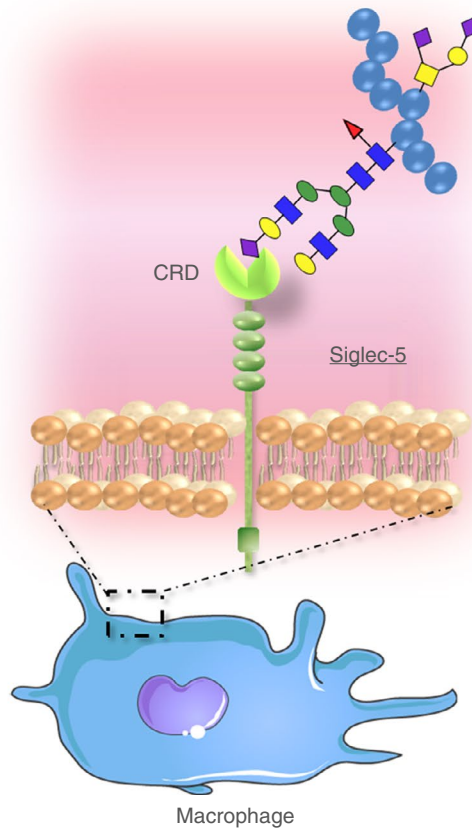
A



B



C



could regulate its endocytosis into early endosomes, suggesting that this receptor may also contribute to VWF clearance.⁵⁰ Importantly, in contrast to ASGPR and MGL, which both bind with increased affinity to hyposialylated VWF, Siglec-5 binding to VWF is sialic acid-dependent.³⁷

9 | VWF SIALYLATION IN HEALTHY NORMAL INDIVIDUALS

In light of the important effects of sialic acid expression in regulating VWF biology, it is interesting that variation in VWF sialylation has been reported between normal individuals. In a study of 68 healthy blood donors, Aguila et al observed significant interindividual variation in VWF binding for all three lectins that recognize terminal sialic acid residues, namely SNA, *Maackia amurensis* lectin II (MAL-II) and wheat germ agglutinin (WGA).¹⁷ In keeping with these findings, exposure of subterminal β -Gal (*Ricinus communis* agglutinin I [RCA-I] and *Erythrina cristagalli* lectin [ECA] binding) also varied significantly between different normal individuals.¹⁷ Collectively, these data suggest that, even among normal individuals, there may be marked interindividual heterogeneity in quantitative N-linked and O-linked sialic acid expression on plasma-derived VWF. Further studies will be required to determine the biological mechanisms underpinning this interindividual heterogeneity in VWF sialylation, and to define whether the changes in sialic acid expression may contribute to quantitative and qualitative variations in plasma VWF levels in the normal population.⁵¹

10 | ABNORMAL VWF SIALYLATION IN PATIENTS WITH VWD

Perhaps unsurprisingly, given the key roles for sialylation in regulating VWF biology, several groups have investigated VWF glycosylation in patients with VWD. Two independent studies reported significantly increased binding of the lectin RCA-I to plasma-derived VWF (suggestive of increased Gal or GalNAc exposure) in patients with VWD as compared with controls.^{42,52} Importantly, these studies both included patients with different types of VWD, although the total numbers enrolled were limited ($n = 19$ and $n = 26$, respectively). Furthermore, significantly enhanced binding of the lectin PNA (suggesting increased O-linked T antigen expression) has also been observed in patients with type 1 VWD as compared with healthy controls.¹⁸ Collectively, these data raise the intriguing question of whether alteration in VWF sialylation could be important in the pathogenesis of VWD, particularly in patients with low VWF levels in whom the disease is not linked to the VWF gene locus (Figure 4A).⁵³

To further address this question, Aguila et al developed a novel panel of lectin assays to assess VWF sialylation in 110 patients with low VWF levels (plasma VWF levels in the range of 30-50 IU/dL) as compared with ABO-matched healthy controls.¹⁷ For each patient and control, plasma-derived VWF sialylation was assessed with the lectins SNA, MAL-II, and WGA. Significant interindividual

variation in VWF sialylation was observed among the cohort of patients with low VWF levels. Importantly, however, binding SNA to VWF was significantly reduced in the cohort with low VWF levels as compared with controls, suggesting a specific reduction in terminal α 2-6-linked sialic acid expression in these patients.¹⁷ In keeping with this reduction in terminal α 2-6-linked sialylation, a significant increase in RCA-I binding (consistent with increased Gal exposure) was also seen in the cohort with low VWF levels as compared with healthy controls. Interestingly, the highest levels of RCA-I binding were seen in patients with low VWF levels who did not have any VWF mutations to explain their reduced plasma VWF levels. Finally, an inverse correlation was observed between enhanced RCA-I binding and estimated VWF half-life in patients with low VWF levels. Together, these findings support the idea that loss of terminal sialylation may contribute to the underlying pathophysiology in at least a subgroup of patients with low VWF levels by promoting enhanced clearance.¹⁷

11 | ALTERED VWF SIALYLATION IN MISCELLANEOUS PHYSIOLOGIC AND PATHOLOGIC CONDITIONS

In addition to the evidence that VWF sialylation may be abnormal in some patients with VWD, several studies have suggested that a number of other conditions may also be associated with alterations in sialic acid expression on plasma-derived VWF (Figure 4). Several pathogens, including *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa*, have been shown to express neuraminidase enzymes that can target host sialoglycoproteins.^{54,55} Furthermore, platelet desialylation by bacterial NanA sialidase has been implicated in causing enhanced platelet clearance and thrombocytopenia in patients with *S. pneumoniae* infection.⁴⁴ Desialylation of VWF resulting in enhanced clearance and reduced plasma VWF levels has also been observed in mice infected with *S. pneumoniae* (Figure 4B).⁴⁴

Several studies have shown that plasma VWF levels are markedly elevated in patients with liver cirrhosis.^{18,56} Furthermore, an inverse correlation between PNA binding to VWF and plasma VWF levels has also been described in patients with cirrhosis (Figure 4C).¹⁸ In addition, a correlation between the severity of cirrhosis and relative PNA binding was also observed.¹⁸ As PNA is known to preferentially bind to the desialylated O-linked T antigen carbohydrate structures on VWF, these data suggest that abnormal VWF glycosylation, and in particular reduced presence of the sialylated T antigen, may be common in cirrhotic patients. Reduced VWF sialylation has been reported in patients with pulmonary hypertension.⁵⁷ In a study of 16 patients with moderate to severe pulmonary hypertension, Lopes et al observed reduced binding of WGA to patient VWF as compared with VWF from healthy controls.⁵⁷ Subsequent studies demonstrated that this decrease in WGA binding was attributable to a significant reduction in VWF sialic acid expression. Overall, the authors estimated that VWF sialylation in patients with pulmonary hypertension was reduced by 20%-25% (Figure 4D).⁵⁷ However, in some of the

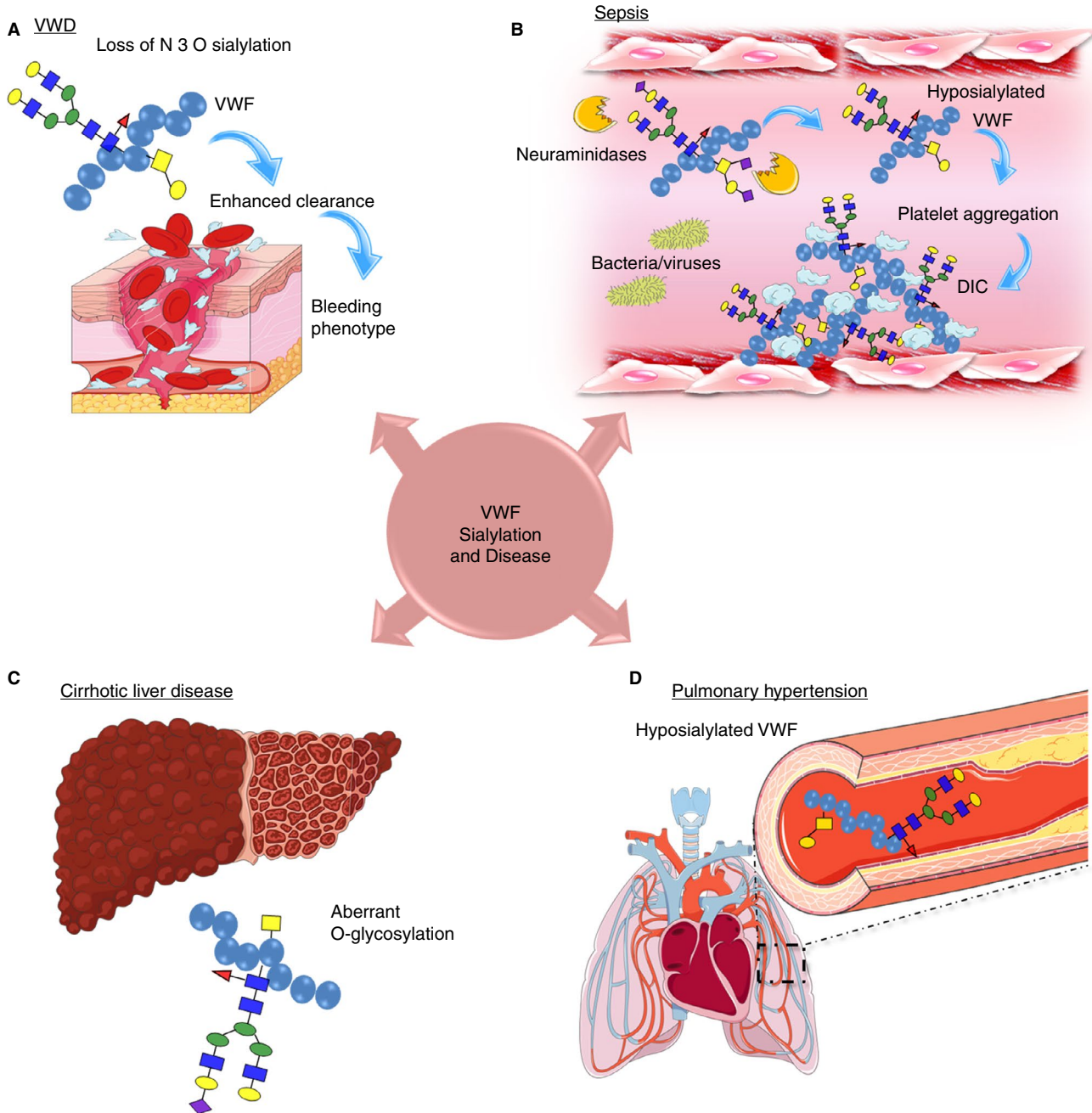


FIGURE 4 (A) A number of studies have demonstrated that subgroups of patients with von Willebrand disease (VWD) show reduced terminal sialic acid expression and subsequent increased galactose exposure on von Willebrand factor (VWF) glycan structures. Galactose exposure is correlated with an increase in VWF clearance in vivo and decreased VWF antigen (VWF:Ag) levels, contributing to the pathophysiology of VWD. (B) Viral and bacterial neuraminidases can mediate the desialylation of circulating VWF and platelets during sepsis. Desialylated VWF has been shown to trigger platelet aggregation and thus contribute to the coagulopathy of sepsis. (C) VWF:Ag levels are markedly elevated in patients with cirrhotic liver disease. Peanut agglutinin binding has been shown to inversely correlate with VWF:Ag in these patients, suggesting reduced presence of the T antigen on VWF disseminated intravascular coagulation (DIC). (D) Hyposialylated plasma VWF and loss of HMW multimers have been reported in patients with pulmonary hypertension

patients studied, VWF sialic acid expression was actually reduced by up to 70%. These findings are of clinical relevance, as pulmonary hypertension has also been associated with a significant reduction in HMW VWF. It seems likely that other diseases (e.g., cancer) may also be associated with quantitative and/or qualitative alterations in VWF sialylation. Further studies will be required to define the nature

of these glycan changes and determine how they may impact upon: (a) VWF structure and function; and (b) disease pathogenesis.

In addition to these pathologic conditions, current evidence suggests that VWF glycosylation and sialylation may also undergo physiologic variations, for example, during pregnancy or with increasing age. It is well established that plasma VWF levels increase

progressively during the course of normal pregnancy. For example, Druary-Smith et al recently showed that mean plasma VWF antigen levels were 120.3 IU/dL in the first trimester, 139.9 IU/dL in the second trimester, and 191.3 IU/dL in the third trimester.⁵⁸ This increase in levels appears to relate in part to a reduction in VWF clearance. Alterations in plasma VWF multimer distribution (including a reduction in HMWMs and an altered triplet pattern) have also been associated with pregnancy.⁵⁸ Although the biology underpinning these changes in VWF clearance and multimer distribution remains unclear, changes shown in isoelectric focusing studies raise the possibility that VWF glycan expression may also differ during pregnancy.⁵⁸

12 | VWF SIALYLATION AND THERAPEUTIC DEVELOPMENT

Recent studies have investigated the glycosylation and sialylation of VWF in a number of different therapeutic concentrates.⁵⁹ As all of these concentrates were plasma-derived, it is perhaps unsurprising that Riddell et al observed no significant differences in SNA (which prefers α 2-6-linked over α 2-3-linked sialic acid) or RCA-1 (terminal Gal residues) binding between 10 commercially available VWF-containing concentrates. In addition to the plasma-derived VWF concentrates available for clinical use, a first recombinant VWF (rVWF) concentrate has recently been licensed (VONVENDI; Shire).⁶⁰ This rVWF is expressed in Chinese hamster ovary cells, and consequently shows differences from plasma-derived VWF in glycosylation and sialylation. In particular, ABO(H) blood group antigens are not expressed on the rVWF product, and sialylation levels are increased.⁶¹ Given these differences in glycosylation, it is interesting that data from the phase 1 and phase 3 studies suggest that the half-life of infused rVWF (19.6 hours) is considerably longer than that of plasma-derived VWF (range of 12.8-15.8 hours).^{62,63} Moreover, the data further suggest that rVWF may be more effective in stabilizing endogenous FVIII than plasma-derived VWF. Additional studies will be required to confirm these initial observations and to define the biological mechanisms underlying these findings.

13 | CONCLUSIONS

In conclusion, despite the fact that sialic acid is only present as a single terminal capping residue on the end of the complex branching glycans of VWF, it nevertheless plays significant roles in regulating VWF structure and function. Further studies will be required to define the molecular mechanisms through which N-linked and/or O-linked sialylation impacts on the multiple biological activities of multimeric VWF. Nonetheless, the elucidation of these mechanisms may offer new insights into how VWF contributes to the pathogenesis of a range of different pathologies. In addition, these studies may provide exciting opportunities to develop novel recombinant VWF

therapeutics with targeted glyco-engineering. In this context, it is interesting that initial studies suggest that polysialylation of VWF may provide a strategy through which to develop a long-acting recombinant VWF product.

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CONFLICT OF INTERESTS

J. S. O'Donnell has served on speaker's bureau for Baxter, Bayer, Novo Nordisk, Boehringer Ingelheim, Leo Pharma, and Octapharma. He has also served on the advisory boards of Baxter, Bayer, Octapharma CSL Behring, Daiichi Sankyo, Boehringer Ingelheim, Shire, and Pfizer. J. S. O'Donnell has also received research grant funding awards from Baxter, Bayer, Pfizer, Shire, and Novo Nordisk. The other authors state that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

All authors drafted the first version of different sections of the manuscript, and all critically reviewed the final manuscript.

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