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## **VON WILLEBRAND FACTOR SIALYLATION – A CRITICAL REGULATOR OF BIOLOGICAL FUNCTION**

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**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.

**ABSTRACT**

Von Willebrand factor (VWF) undergoes complex post-translational modification prior to its secretion into the plasma. Consequently, VWF monomers contain complex N- 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- and O-glycans on human VWF are capped by terminal negatively-charged sialic acid residues. Recent data suggest 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 a number of other physiological and pathological states. Moreover, platelet-VWF is significantly hyposialylated compared to 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 physiological and pathological conditions.

**KEYWORDS** – von Willebrand factor; von Willebrand disease; Glycosylation; ADAMTS13

## INTRODUCTION

von Willebrand factor (VWF) is a large multimeric plasma glycoprotein that plays essential roles in maintaining normal haemostasis.[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 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 physiological and pathological conditions.

### *VWF biosynthesis and post-translational modification*

Under normal physiological conditions, *in vivo* biosynthesis of VWF is restricted to vascular endothelial cells (EC) and megakaryocytes only.[7] VWF synthesised within EC can be secreted into the plasma or else 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 synthesised within megakaryocytes is stored within  $\alpha$ -granules.[8] Consequently, under steady state conditions, plasma VWF is predominantly derived from EC. 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 D'-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK).[1] This initial VWF monomer is subject to extensive post-translational modification that 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 20,000kDa.

### ***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, N2790) (Figure 1A).[10] Recent mass spectrometry (MS) studies on human plasma-derived (pd-) VWF have confirmed occupancy of all these sites except N1147.[11] 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 and more complicated sugar structures were present at N1515 and N1574 respectively.[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  $\alpha$ 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 pd-VWF (Figure 1B).[11] These ABO(H) structures were not localized to specific N-glycan sites on pd-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 only present on non-sialylated antennae.

Besides its extensive N-glycosylation, each VWF monomer also contains ten O-glycosylation sites (Figure 1A). Unlike the N-glycans that are distributed across multiple different domains of VWF, eight of the ten O-linked glycans (T1248, T1255, T1256, S1263, T1468, T1477, S1486, 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 its N-glycans, the O-glycans of pd-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, accounted for approximately 70% of the total O-glycans population (Figure 1C). Altogether, O-linked sialic acid expression has been estimated to account for < 20% of the total sialylation on human pd-VWF, and may be present in either  $\alpha$ 2-3 or  $\alpha$ 2-6 linkage.[11,14,15] Of note, disialosyl groups, which are uncommon amongst 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% core 2 O-glycan structures.

### ***Sialylation and VWF ageing in plasma***

Like other plasma glycoproteins, emerging evidence suggests that VWF glycosylation vary with protein ageing. Importantly, Yang *et al* recently identified that ageing of secreted plasma glycoproteins is not only associated with a loss of sialic acid, but also a progressive stepwise loss of terminal sugar residues from N-glycan chains.[16] Loss of terminal  $\alpha$ 2-6 linked sialic acid (catalysed 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 pre- and post-DDAVP administration 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  $\alpha$ 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 multimers stored within Weibel Palade bodies are more highly sialylated compared with 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- and O-linked glycans of VWF initially secreted from EC are capped with terminal sialic residues, the level of plasma VWF sialylation is likely to progressively diminish with glycoprotein ageing in the plasma. (Figure 2).

### ***Sialylation of human platelet-VWF***

Platelet  $\alpha$ -granules contain an estimated 10-20% of the total VWF present in platelet-rich plasma.[20,21] This pool of platelet-VWF is discrete from plasma 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 (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-VWF.[24] 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.[8,24] These glycosylation differences presumably reflect differences in the post-translational modification of platelet-VWF (synthesized within megakaryocytes) compared to pd-VWF (synthesized within EC).[8]

***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.[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 >95% of total sialic acid expression from VWF (Neu-VWF).[25] In the presence of protease inhibitors, desialylation had no direct effect on 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-VWF is markedly reduced compared to pd-VWF. Given this reduction in sialic acid expression, it is interesting that previous studies have reported functional differences between platelet VWF and pd-VWF.[8] For example, although platelet-VWF is enriched in high molecular weight multimers (HMWM), it binds GP1b $\alpha$  with significantly reduced affinity compared to pd-VWF.[22] In contrast, platelet-VWF displays 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 determine the contribution of differences in VWF glycosylation.

***VWF sialylation regulates susceptibility to proteolysis***

VWF multimer distribution is a key determinant of its functional activity.[1,3] HMWM bind collagen and GpIb $\alpha$  with increased affinity compared to low molecular weight (LMWM) multimers, and are thus more effective in facilitating platelet plug formation. In normal plasma, VWF multimer distribution is regulated by ADAMTS13 (A Disintegrin and Metalloproteinase with Thrombospondin type-1 repeats), which cleaves VWF at a specific Tyr1605-Met1606 bond within

the A2 domain.[31,32] Recent studies have suggested that other proteases including plasmin may also play roles in regulating VWF multimer conditions under specific circumstances.[33,34] 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.[32] Conversely, loss of HMWM due to enhanced proteolysis in patients with type 2A VWD is associated with significant bleeding.[35] It is well recognised 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 ADAMTS13. [12] In particular, enzymatic desialylation of pd-VWF with  $\alpha$ 2-3,6,8,9 neuraminidase markedly impaired ADAMTS13-mediated VWF proteolysis in a dose-dependent manner. Interestingly, treatment with  $\alpha$ 2-3 neuraminidase to specifically digest  $\alpha$ 2-3 linked sialylation from the O-glycans of pdVWF had no significant effect on ADAMTS13 proteolysis. [12] Together, these data suggest that  $\alpha$ 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 Asn-1574 in the VWF A2 domain in regulating ADAMTS13 proteolysis.[29]

As previously discussed, levels of N-linked sialylation are significantly reduced on platelet-VWF compared to pd-VWF.[8] Interestingly, as a result of this quantitative change in terminal  $\alpha$ 2-6 linked sialic acid expression, platelet-VWF has been shown to exhibit specific resistance to ADAMTS13-mediated proteolysis compared to pd-VWF.[24] Hence, not only are high local concentrations of platelet-VWF released at sites of vascular injury, but due to the fact 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.[33,36] For example, significant activation of plasminogen to plasmin has been reported in patients during acute TTP.[33] Furthermore, a role for plasmin in preventing accumulation of pathological UL-VWF multimers in the absence of ADAMTS13 regulation has also been proposed.[36] Interestingly, VWF glycans also play a role in regulating susceptibility to plasmin-mediated proteolysis.[28,34] However, in contrast to ADAMTS13 proteolysis, ABO blood group does not influence VWF cleavage by plasmin[34] and Berkowitz *et al* previously showed that desialylation of VWF is associated with enhanced plasmin-mediated proteolysis.[28]

#### ***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  $\alpha$ 2-6 linked sialylation in enhancing VWF proteolysis by ADAMTS13, current evidence suggests that both  $\alpha$ 2-6 and  $\alpha$ 2-3 linked sialylation play roles in protecting VWF against clearance.[37,38,40,43,44] Similar to their specific role in modulating susceptibility to ADAMTS13 proteolysis, the N-linked glycans at 1515 and 1574 within the A2 domain also play a key role in protecting VWF against *in vivo* clearance.[45] The major effect of  $\alpha$ 2-3-linked

sialylation in regulating clearance is interesting,[43] since this sialic acid accounts for < 20% of the total sialylation on human pd-VWF and is expressed predominantly on O-glycans.[14]

A number of lectin receptors have been implicated in contributing to the enhanced clearance of hyposialylated VWF (Figure 3).[37] The first receptor proposed to be important in this context was the Ashwell-Morrell receptor (AMR) or asialoglycoprotein receptor (ASGPR). [44] ASGPR is a C-type lectin receptor expressed predominantly on hepatocytes that consists of 2 trans-membrane protein subunits (Asgpr-1 and Asgpr-2).[46,47] The ASGPR carbohydrate recognition domain (CRD) preferentially binds to glycoproteins expressing  $\beta$ -D-galactose ( $\beta$ -Gal) or *N*-acetyl-D-galactosamine (GalNAc) residues.[47] These residues are typically present on VWF as sub-terminal moieties on glycan chains capped by sialic acid residues.[11,14] Consequently, loss of terminal sialylation results in enhanced exposure of these  $\beta$ -Gal and GalNAc residues which can then trigger ASGPR-mediated clearance (Figure 3A). In keeping with the hypothesis that the 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  $\beta$ -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 pd-VWF. [44] This observation is in keeping with previous studies that have shown 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 pd-VWF in a dose-dependent manner and 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]

### ***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 all three lectins that recognize terminal sialic acid residues SNA, *Maackia amurensis lectin II* (MAL-II) and Wheat germ agglutinin (WGA). [17] In keeping with these findings, exposure of sub-terminal  $\beta$ -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 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.

### ***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 pd-VWF (suggestive of increased Gal or GalNAc exposure) in patients with VWD compared to 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 compared to healthy controls.[18] 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 (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 (plasma VWF levels in the range 30-50IU/dL) compared to ABO-matched healthy controls.[17] 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  $\alpha$ 2-6 linked sialic acid expression in these patients. [17] In keeping with this reduction in terminal  $\alpha$ 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. [17]

#### ***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, several studies have suggested that a number of other conditions may also be associated with alterations in sialic acid expression on plasma 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. pneumonia* infection.[44] Desialylation of VWF resulting in enhanced clearance and reduced plasma VWF levels has also been observed in mice infected with *S. pneumonia* (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 lectin binding to VWF and plasma VWF levels has also been described in patients with cirrhosis (Figure 4C).[18] In addition, a correlation between severity of cirrhosis and relative PNA binding was also observed.[18] Since PNA lectin 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 lectin to patient-VWF compared to VWF from healthy controls.[57] Subsequent studies demonstrated that this decrease in WGA lectin 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% (Figure 4D). [57] However, in some of the patients studied, VWF sialic acid expression was actually reduced up to 70%. These findings are of clinical relevance as pulmonary hypertension has also been associated with a significant reduction in HMWVWF. 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-Smith *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.[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 HMWM and 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 in isoelectric focussing studies raise the possibility that VWF glycan expression may also differ during pregnancy. [58]

### ***VWF sialylation and therapeutic development***

Recent studies have investigated the glycosylation and sialylation of VWF in a number of different therapeutic concentrates.[59] Since all of these concentrates were plasma-derived, it is perhaps unsurprising that Riddell *et al* observed no significant differences in SNA lectin (which prefers  $\alpha$ 2-6 linked over  $\alpha$ 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; Shire).[60] 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.[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 pd-VWF (range 12.8-15.8 hours).[62,63] 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 conform these initial observations and to define the biological mechanisms underlying these findings.

### ***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, nevertheless it plays significant roles in regulating VWF structure and function. Further studies will be required to define the molecular mechanisms through which N- and/or O-linked sialylation impacts upon 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.

## **AUTHORSHIP**

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

### **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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## Figure Legends

**Figure 1**

(A) The VWF amino acid sequence identified 13 potential N-glycosylation consensus sequons with span the entirety of VWF domains. Mass spectrometry has revealed all of these glycosites are occupied with the exception of N1147 and N2635 which exhibit only partial occupancy. VWF also contains 10 O-linked glycosylation sites, eight of which flank the A1 domain in two clusters. (B) Commonest N-linked glycan structures are illustrated. Monosialylated bi-antennary complex structures account for approximately 80% of VWF N-linked glycans, with tri- 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 capped with  $\alpha$ 2-6 linked sialic acid. (C) In contrast VWF O-linked glycans are 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- and O- glycans express ABO blood group determinants (13% and 1% respectively)

**Figure 2**

The sialylation status of VWF is heterogeneous and dynamic. VWF of endothelial cell (EC) origin is fully sialylated while in contrast platelet-derived VWF has been shown to have significantly reduced VWF N-linked sialylation, likely due to differential sialyltransferase activity in ECs and megakaryocytes. Once secreted, VWF is exposed to circulating plasma glycosidases which have been reported to result in step-wise removal or trimming of sialic acid/galactose from various plasma glycoproteins.

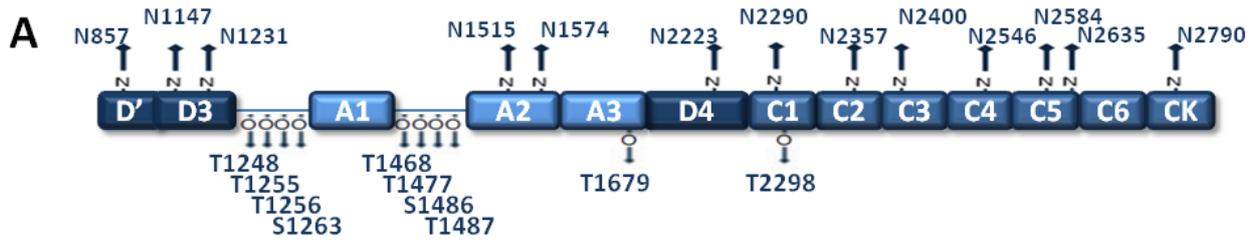
**Figure 3**

A number of lectin receptors have been proposed to play roles in mediating VWF clearance in vivo. Glycoprotein binding to these receptors is mediated by carbohydrate recognition domains (CRD) that specifically interact with terminal glycan structures expressed on circulating plasma glycoproteins. (A) The ASGPR is expressed on hepatocytes as a hetero-trimer composed of the major (ASGPR1) and minor (ASGPR2) subunits, with the CRD bearing specificity for glycoproteins terminating in Gal and GalNAc moieties following N-linked sialic acid removal. (B) MGL is expressed as a homo-trimer on macrophages and dendritic cells. The MGL CRD is also specific for exposed Gal/GalNAc terminal moieties; however MGL binds preferentially to de-sialylated O-linked glycan structures, with particular affinity for the T antigen. (C) Siglec-5, also expressed on macrophages, preferentially binds sialylated glycan structures.

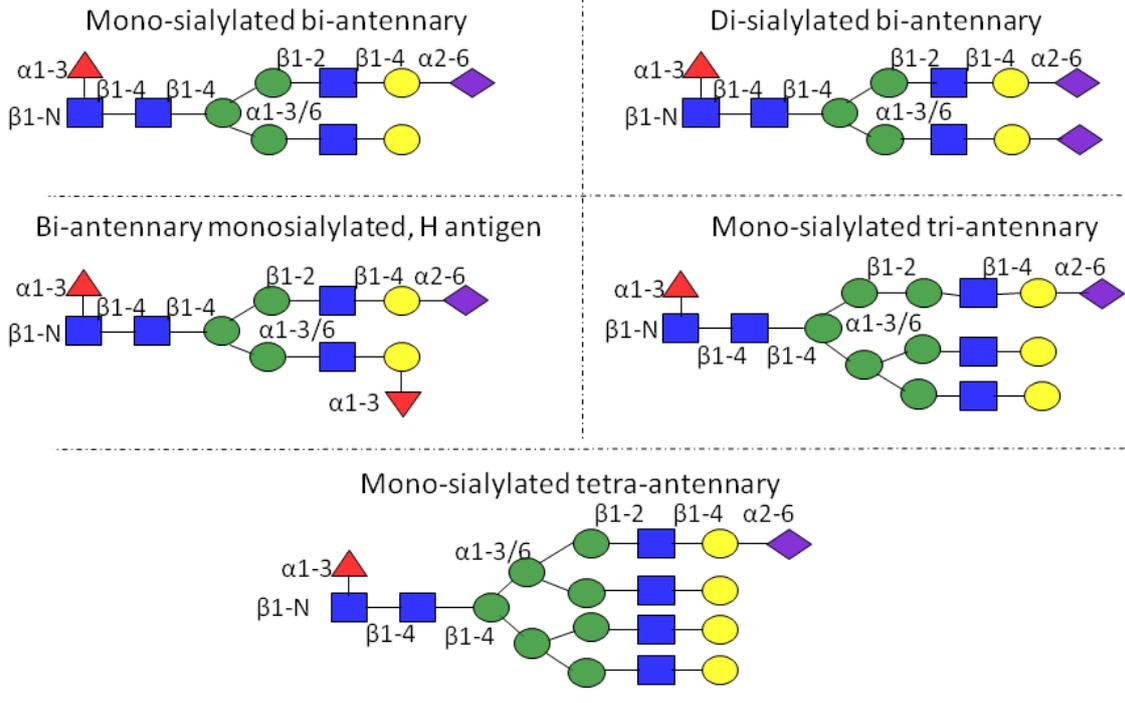
**Figure 4**

(A) A number of studies have identified that sub groups of patients with VWD display reduced terminal sialic acid expression and subsequently increased galactose exposure on VWF glycan structures. 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) VWF:Ag levels are markedly elevated in patients with cirrhotic liver disease. PNA lectin binding has been shown to inversely correlate with VWF:Ag in these patients suggesting a reduced presence of the T antigen on VWF

**Figure 1**



**B N-linked Glycans**



**C O-linked Glycans**

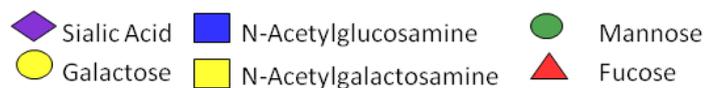
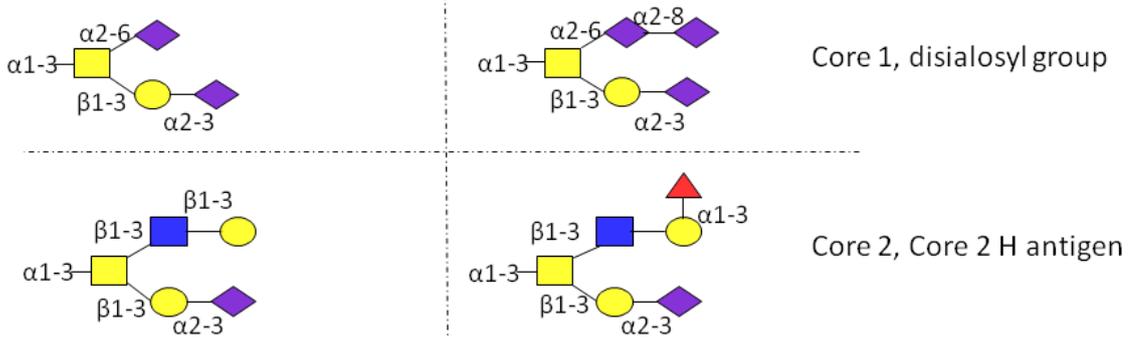
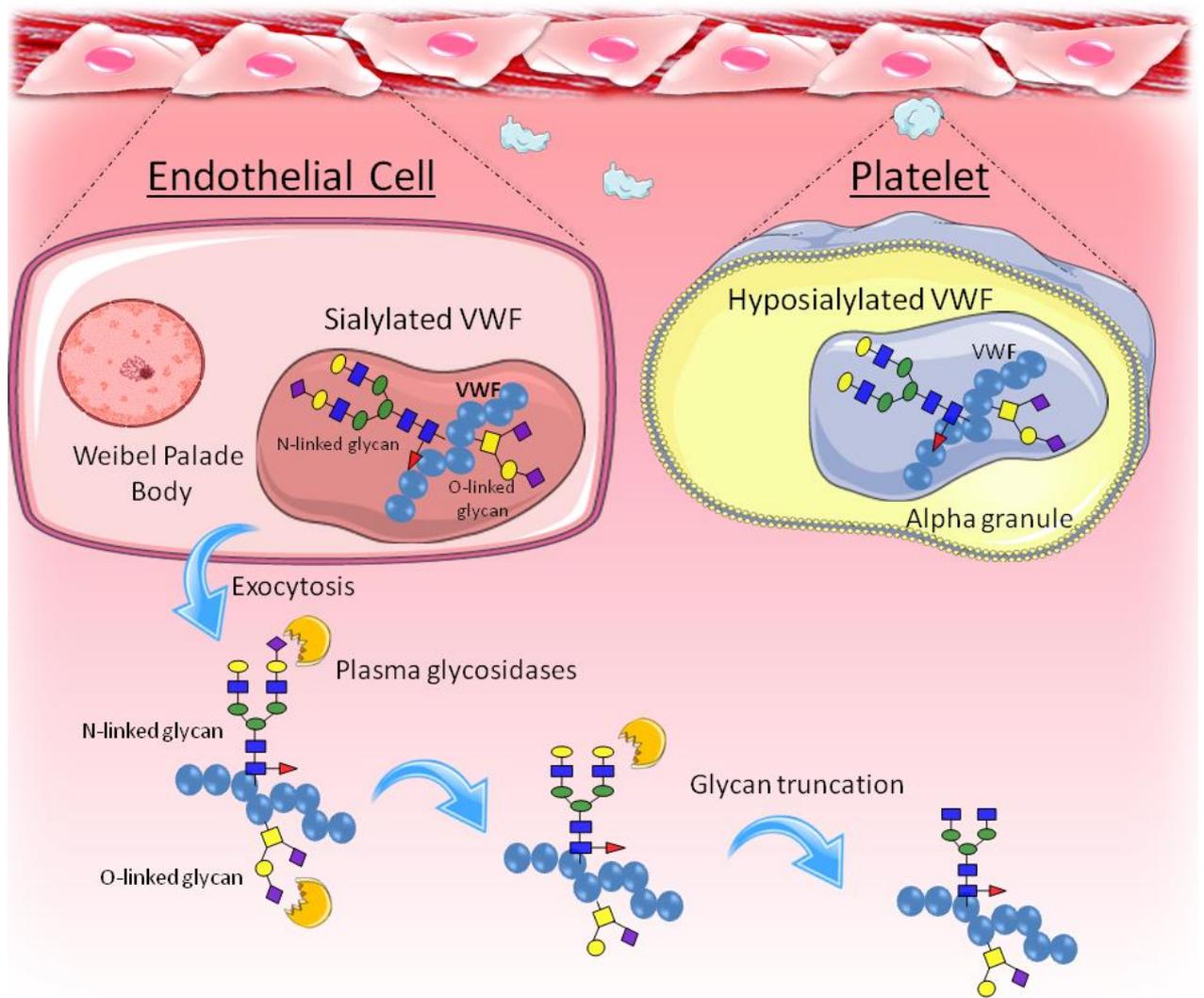


Figure 2



**Figure 3**

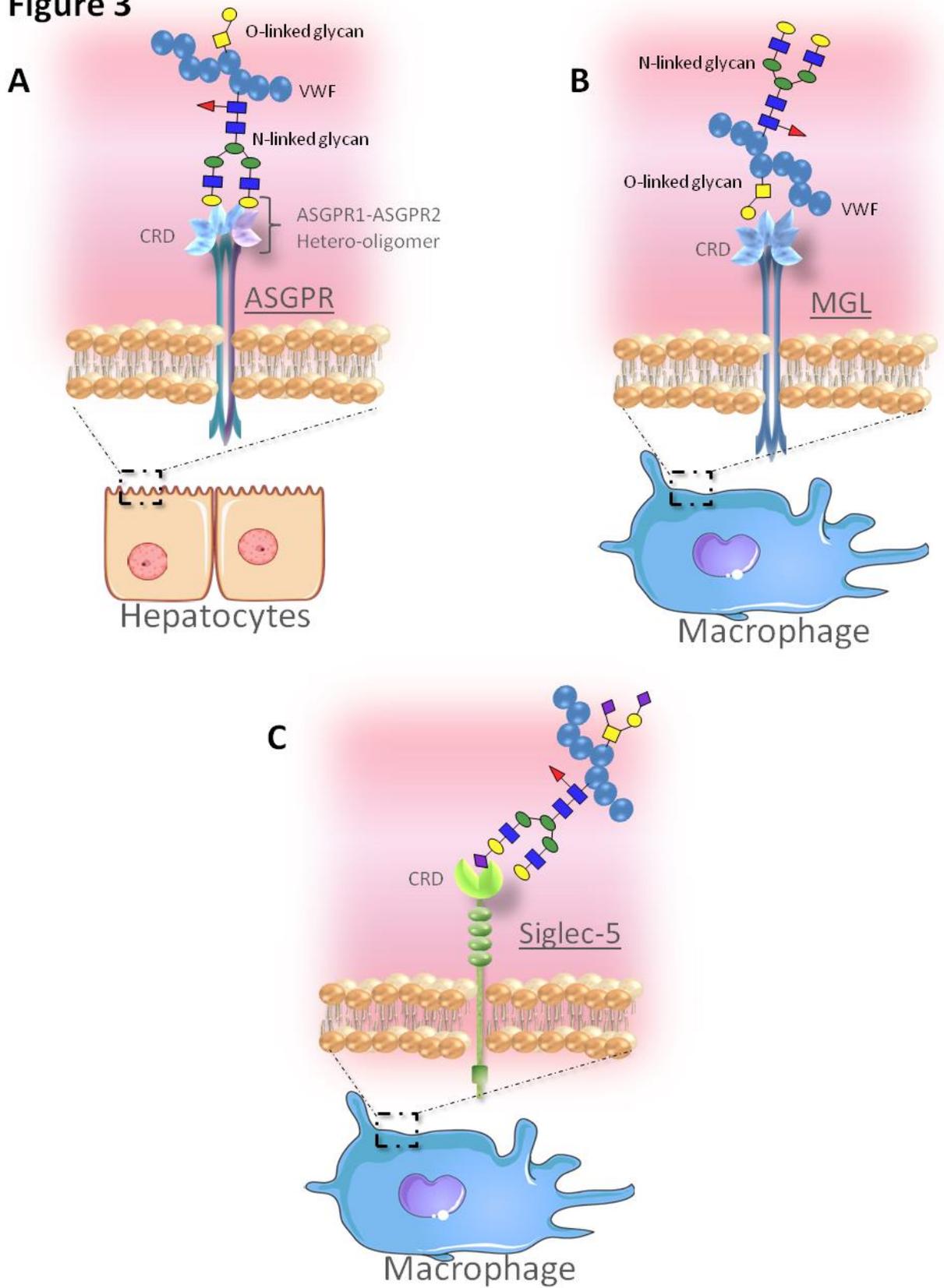


Figure 4

