Sialylation on O-linked glycans protects von Willebrand factor from macrophage galactose lectin-mediated clearance

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Supplementary Materials and Methods

Glycosidase digestion and quantitative analysis of glycan expression.

To generate VWF glycoforms, pdVWF was treated with α2-3 neuraminidase (0.4U/1µgVWF; Streptococcus pneumonia; New England Biolabs, UK), α2-3,6,8,9 neuraminidase (2U/1µgVWF; Arthrobacter ureafaciens; New England Biolabs, UK), β1-3 galactosidase (1U/1µgVWF; Xanthomonas manihotis; New England Biolabs, UK), PNGase F (5U/1µgVWF; Flacobacterium meningosepticum; New England Biolabs, UK) and or O-glycosidase (40mU/1µgVWF; Enterococcus faecalic; New England Biolabs,UK) under non-denaturing conditions overnight at 37°C. Following glycosidase digestion, changes in VWF glycans were assessed using specific lectin ELISAs as previously described.[1] In brief, purified VWF diluted in phosphate-buffered saline containing tween (PBS-T 0.5%) was captured using deglycosylated polyclonal anti-VWF 1:250 (Dako, Agilent Technologies) onto microtiter wells. Non-specific binding was blocked with Protein-Free Blocking Buffer™ (Thermo Fisher Scientific, UK). Glycan digested VWF variants (starting concentration 1µg/ml) were incubated for 2 hours at 37ºC. Biotinylated lectins including Sambucus nigra (0.1µg/ml), Maackia amurensis (2.5µg/ml), Wheat germ agglutinin (1µg/ml), Peanut agglutinin (1µg/ml) and Ricinus communis (0.5µg/ml) (Vector Laboratories, UK) were diluted in PBS-T and incubated for 1 hour at 37ºC. Lectin binding was detected with high sensitivity streptavidin–horseradish peroxidase (Pierce, Thermo Fisher Scientific, UK) and subsequent incubation with substrate 3,3',5,5'-Tetramethylbenzidine (TMB; R&D Systems, UK). The reaction was subsequently stopped with 50µL 1M H_2SO_4. Absorbance was read at 450nm and lectin binding was expressed as a percentage of control unmodified pdVWF. All ELISAs were repeated three times and dilutions were measured per duplicate.

Expression and purification of recombinant von Willebrand factor variants

The expression vectors pcDNA-VWF encoding full length recombinant (rVWF), VWF-A1A2A3, VWF-D'A3 or VWF-A3-CK fragments have previously been described.[2] Similarly, recombinant expression vectors for single domain constructs including VWF-A1 (residues 1239-1472), VWF-A2 (residues 1473-1668), and VWF-A3 (residues 1671-1878) have been previously described. Additional VWF-A1 constructs contain either of the two O-linked glycan (OLG) clusters at either side of the A1 domain, A1-OLG cluster 1 (T1248A, T1255A, T1256A, T1263A), and A1-OLG cluster 2 (T1468A, T1477A, S1486A, T1487A). All recombinant VWF variants were transiently expressed in HEK293T cells. Conditioned serum free medium was

harvested 72 hours post-transfection and concentrated via anion exchange chromatography as before. Full length VWF variants were further concentrated using 100-kDa cut-off spin filters (Amicon, United Kingdom). Truncated VWF constructs were further purified via nickel affinity chromatography. Subsequently all VWF variants were dialyzed into 20mM Tris pH 7.4.

Supplementary Table S1: Expression and detection of VWF variants used for MGL in vitro binding assays

VWF Variant	Expression	Tag	Detection
Fandhi; Plasma derived VWF	Human plasma	n/a	Polyclonal rabbit anti human VWF (Dako, Agilent Technologies); 1:1000
Full length VWF	HEK293T cells	n/a	Polyclonal rabbit anti human VWF (Dako, Agilent Technologies); 1:1000
D'-A3 VWF	HEK293T cells	His	anti-His-HRP antibody (Qiagen, UK), diluted 1:2500
A3-CK VWF	HEK293T cells	His	anti-His-HRP antibody (Qiagen, UK), diluted 1:2500
A1A2A3-VWF	HEK293T cells	His	anti-His-HRP antibody (Qiagen, UK), diluted 1:2500
A1-VWF	HEK293T cells	His	anti-His-HRP antibody (Qiagen, UK), diluted 1:2500
A2-VWF	HEK293T cells	His	anti-His-HRP antibody (Qiagen, UK), diluted 1:2500
A3-VWF	HEK293T cells	His	anti-His-HRP antibody (Qiagen, UK), diluted 1:2500
A1-OLG Cluster 1	HEK293T cells	Strep	High-sensitivity streptavidin-HRP, 1:10000
A1-OLG Cluster 2	HEK293T cells	Strep	High-sensitivity streptavidin-HRP, 1:10000

In vitro VWF binding studies

Recombinant human MGL (Stratech, UK) was immobilized at 5µg/mL on a PolySorp® 96 well plate (Nunc, Thermo Scientific™ 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. VWF or glycoforms thereof diluted in PBS-T supplemented with 2.5mM $CaCl_2$, 1mg/mL ristocetin (MP Biomedicals, UK) were added to wells and incubated at 37°C for 1 hour. Single concentration MGL-VWF binding assays were carried out at 10µg/mL for pdVWF glycoforms and 150nM for truncated recombinant VWF variants. For detection of full length VWF, HRP conjugated polyclonal anti-VWF (Dako, Agilent Technologies) 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 (R&D Systems, UK). The reaction was subsequently stopped with 50µL 1M H_2SO_4. Optical density was measured at 450nM using a VERSAmax microplate reader (Molecular Devices, UK).

Supplementary Results

Supplementary Figure 1

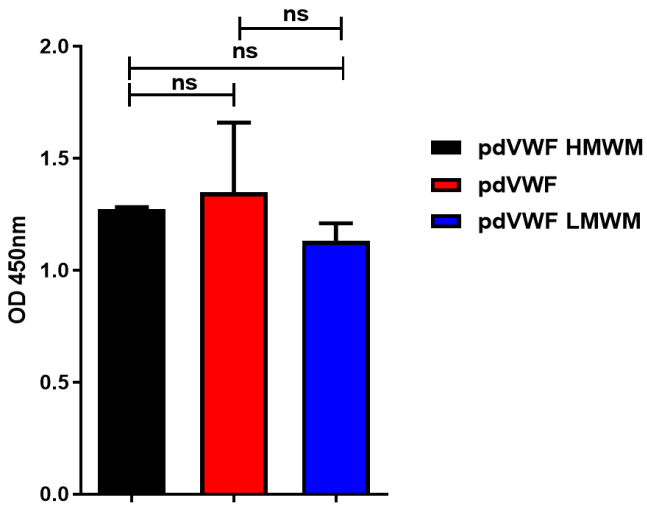


Figure S1: VWF multimer distribution does not influence MGL-binding.

To investigate whether VWF multimer distribution influences interaction with MGL, high molecular weight multimer (HMWM) and low molecular weight multimer (LMWM) fractions were purified by gel filtration and binding to human MGL assessed (ns = not significant).

Supplementary Figure 2

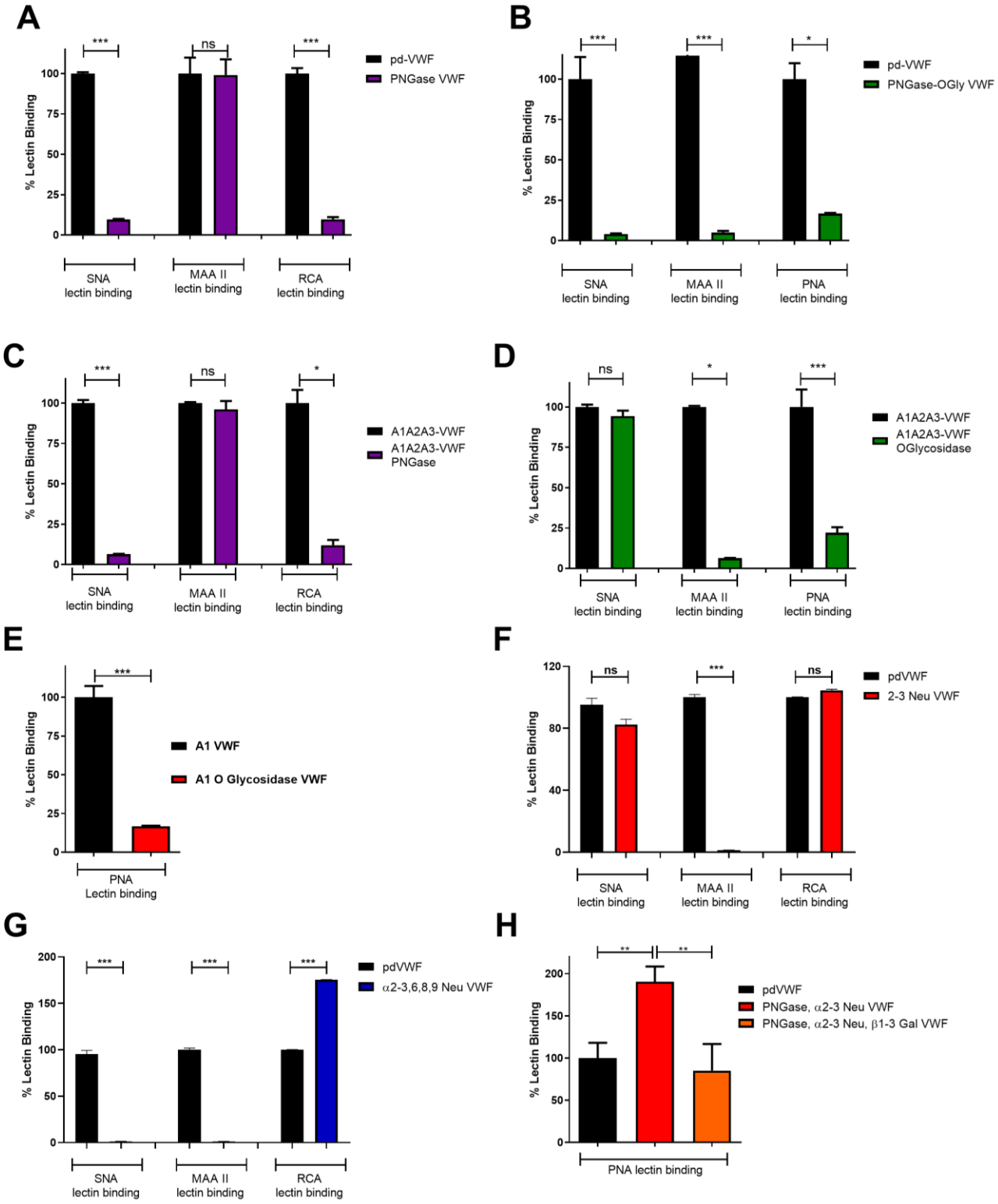
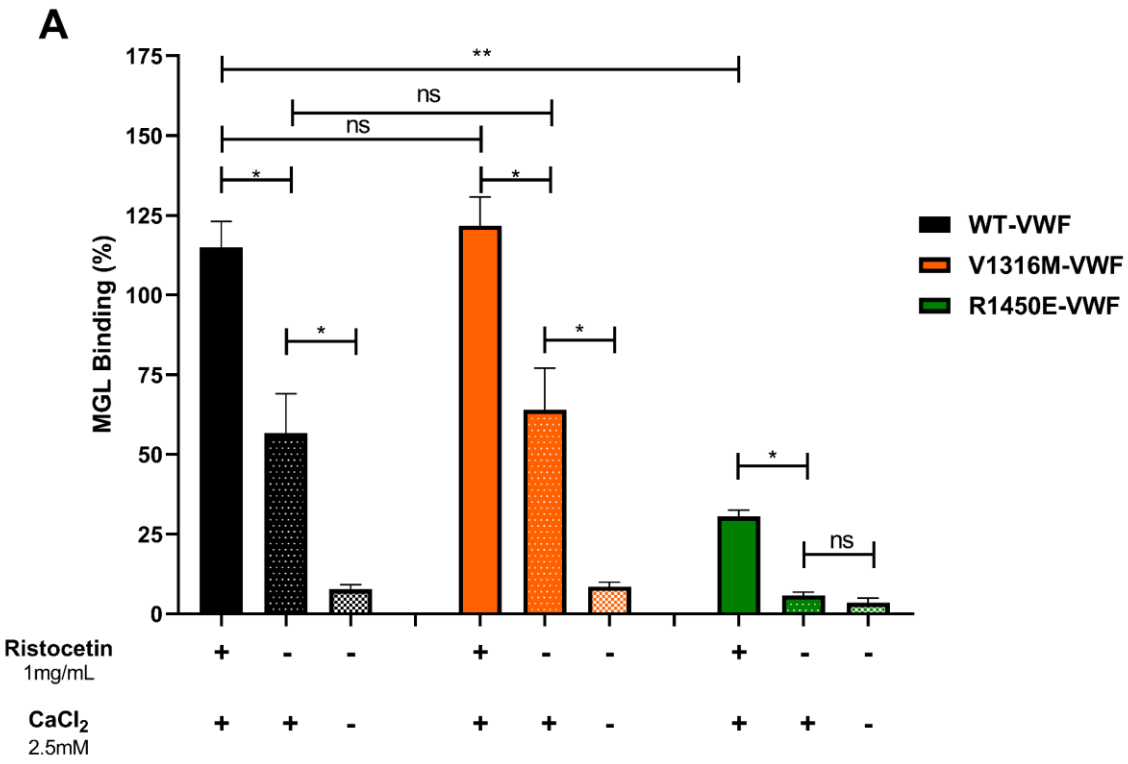


Figure S2: Lectin-analysis of VWF pre- and post-treatment with specific glycosidases. Full-length VWF, or a range of VWF truncations were digested with a variety of specific glycosidases including PNGase F, O-glycosidase, α2–3 neuraminidase α2–3,6,8,9-neuraminidase, α2–3,6,8,9-neuraminidase and β(1–4) galactosidase as described in the text. Following each *ex vivo* VWF digestion, lectin plate-binding assays were performed to confirm different VWF glycoforms generated. Lectins used included *Sambucus nigra agglutinin* (SNA), *Maackia amurensis lectin* II (MAA-II), *Ricinus communis agglutinin* I (RCA), and Peanut agglutinin (PNA) respectively. All ELISAs were performed in triplicate and results expressed as a percentage of binding to untreated pdVWF. Results presented represent the mean values ± SEM (*p<0.05, **p<0.01, ***p<0.0001 (Mann Whitney U-test); ns = not significant).

Supplementary Figure 3



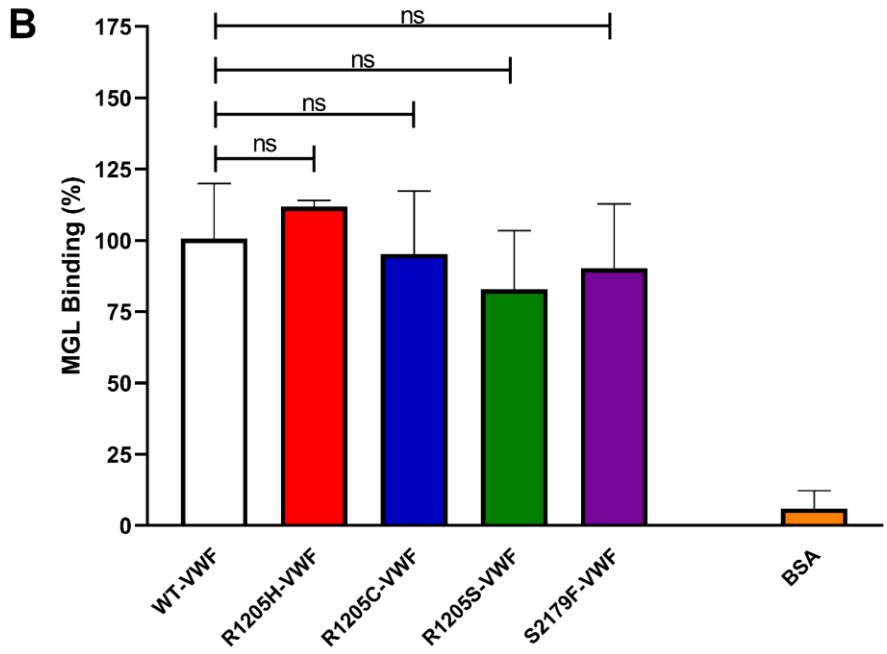
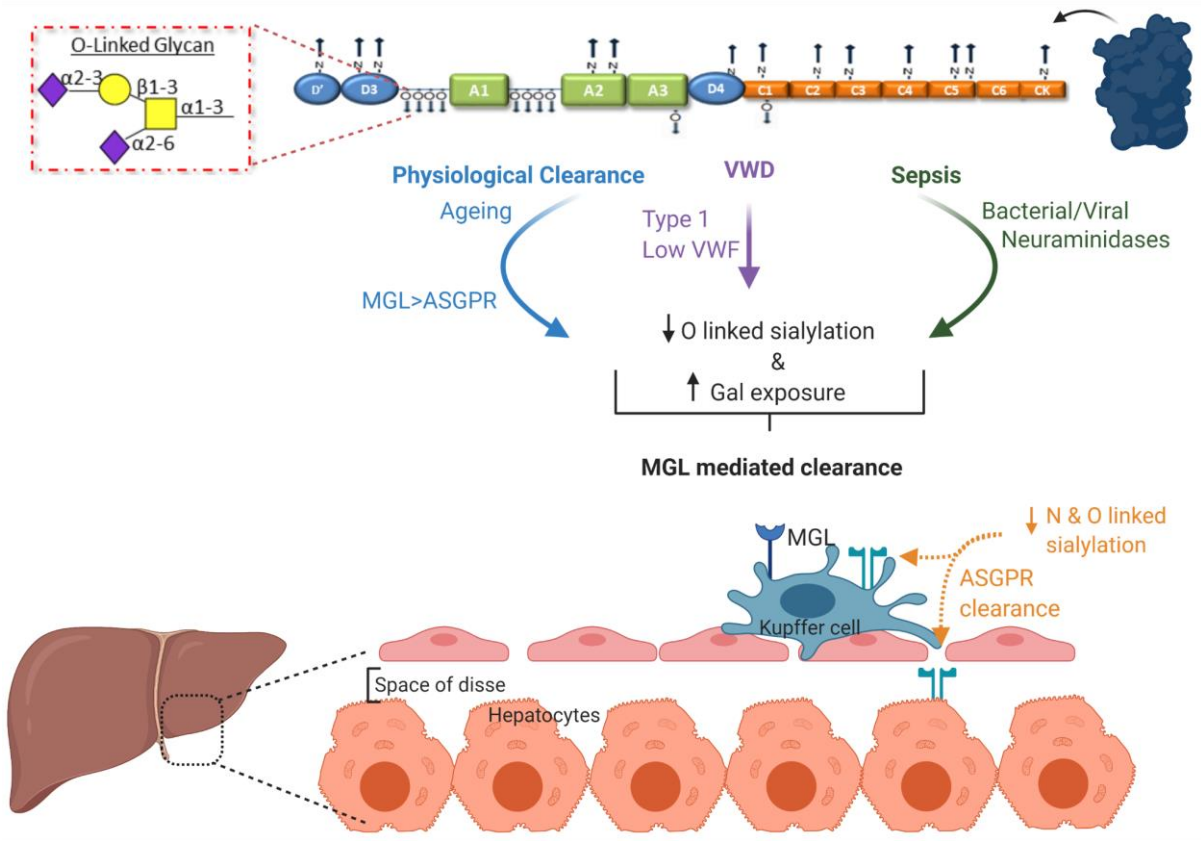


Figure S3: MGL does not exhibit enhanced binding to Type 1C and Type 2B VWF mutants

(A) Full length wild-type VWF binding to MGL in the presence and absence of 1mg/mL ristocetin and 2.5mM CaCl2 was assessed in comparison to Type 2B VWD mutations; V1316M and R1450E. Spontaneous binding of VWD 2B constructs to MGL in the absence of ristocetin was not observed. (B) Full length wild-type VWF biding to MGL in the presence of 1mg/mL ristocetin was assessed in comparison to Type 1C VWD mutations; R1205H, R1205C, R1205S and S2179F (ns = not significant). Results presented represent the mean values ± SEM (*p<0.05, **p<0.01 (Student T-test).

Supplementary Figure 4 – visual abstract



SUPPLEMENTARY REFERENCES

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