

ORIGINAL ARTICLE

Evaluation of von Willebrand factor concentrates by platelet adhesion to collagen using an in vitro flow assay

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Abstract:

Background: Von Willebrand disease (VWD) results from quantitative or qualitative deficiency of von Willebrand factor (VWF) and is treated using VWF-containing concentrates. Several studies have compared the function of various VWF containing concentrates however this has not been performed using shear based assays.

Objectives: To compare the platelet-capture potential of 10 commercially available, plasma-derived VWF concentrates under shear conditions.

Methods: VWF containing concentrates were assessed for VWF:Ag, VWF:CB, VWF:RC₀, factor VIII:C ADAMTS13 content, VWF multimeric profile and glycan content using lectin binding assays. Free-thiol content of each concentrate was investigated using MPB binding assays. An in vitro flow assay was used to determine the ability of each concentrate to mediate platelet capture to collagen.

Results: VWF multimeric analysis revealed reduction of high molecular weight (HMW) forms in four of the concentrates (Alphanate, Octanate and Haemoctin, and 8Y). The high MW multimer distribution of the remaining six concentrates (Optivate, Wilate, Fandhi, Wilfactin, Haemate P, and Voncento) was similar to the plasma control. Lectin analysis demonstrated that 8Y had increased amount of T-antigen. Although platelet capture after 5 minutes perfusion was similar for all concentrates; Alphanate, Octanate, and Haemoctin, demonstrated the lowest levels of platelet capture after 60 seconds of perfusion. Free-thiol content and ADAMTS13 levels varied widely between the concentrates but was not correlated with function.

Conclusion: Alphanate, Octanate, and Haemoctin, lacked HMW multimers and had the lowest initial platelet capture levels suggesting that the presence of VWF HMW multimers are required for initial platelet deposition.

KEYWORDS

ADAMTS13, von Willebrand factor, von Willebrand disease

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Essentials

- VWF-containing concentrates are commonly used to treat VWD.
- The activity of 10 VWF concentrates were investigated under static and flow conditions.
- Differences were observed in free-thiol and ADAMTS13 content and multimeric profile and the initial extent of platelet capture.
- Overall platelet capture did not differ between concentrates suggesting equal effectiveness.

1 | INTRODUCTION

Von Willebrand factor (VWF) is a complex multimeric protein of 500 kDa to 20 MDa molecular weight (MW) that circulates in plasma and is stored in platelet α -granules and endothelial cell Weibel-Palade bodies.¹ Not only does VWF serve as the carrier protein of circulating factor VIII (FVIII), preventing its proteolytic degradation,² its chief function is to mediate platelet adhesion to extracellular matrix components exposed by wounding.³ Binding to the platelet GP Ib/IX/V receptor complex occurs via the VWF-A1 domain and to type III and VI collagens exposed at the wound site through the VWF-A3 domain.^{4,5} Shear conditions and immobilization of VWF are required to induce conformational changes that increase its affinity for the GPIb complex.⁶ The ultra large and high MW forms of VWF multimers, derived from plasma and endothelial cells, are most efficient at mediating platelet adhesion to collagen surfaces.⁷

Patients with von Willebrand disease (VWD) for whom 1-deamino-8-D-arginine vasopressin (desmopressin) is ineffective, mainly those with severe quantitative defects (types 1 and 3) and qualitative conditions (type 2), may require treatment with commercially available plasma-derived concentrates containing VWF to control bleeding or when undergoing elective surgical procedures.^{8,9} VWF containing concentrates that are currently available include those originally manufactured for use with patients with hemophilia A (HA) but which also contain significant amounts of VWF: Haemoctin, and Optivate are licensed only for treatment of hemophilia A, BPL 8Y, Haemate P, Octanate, Fandhi, Alphante, and Wilate are licensed for the treatment of both VWD and HA while others with high specific VWF content such as Wilfactin and Voncento are licensed for treating patients with VWD only (<https://www.medicines.org.uk/emc/>).

In order to evaluate their clinical efficacy, many previous studies have compared the VWF content of commercially available concentrates in terms of the standard laboratory measure of activity such as ristocetin cofactor activity (VWF:RCo) and collagen binding activity (VWF:CB) performed under static conditions, as well as VWF multimer distribution.¹⁰⁻¹⁵

However, relatively few studies have addressed the functionality of these concentrates in terms of their ability to mediate platelet adhesion to collagen surfaces under physiological shear conditions.¹⁶

In this study we have assessed the effect of ten commercially available VWF containing concentrates on platelet adhesion to collagen under shear stress using an in vitro assay. These results were compared

with conventional assessment of VWF function assessed by VWF antigen (VWF:Ag), VWF:RCo, VWF:CB, and VWF multimeric analysis.

2 | MATERIALS AND METHODS

2.1 | VWF concentrates

Ten VWF-containing commercially available concentrates were assessed in this study. One vial each of Haemate P and Voncento (CSL Behring GmbH, Marburg, Austria), Wilate and Octanate (Octapharma, GmbH, Vienna, Austria), Optivate and 8Y (Bio Products Laboratory, Elstree, UK), Haemoctin (Biotest, Dreieich AG, Germany), Wilfactin (LFB Biomedicaments, Les Ulis, France), Fandhi and Alphanate (Instituto Grifols SA, Barcelona, Spain) and were all reconstituted according to the manufacturers' instructions.

2.2 | Clotting factor VIII:C, VWF antigen and activity assays

VWF:Ag, VWF:RCo and VWF:CB were all assayed against the 2nd WHO International VWF concentrate standard (09/182) (NIBSC, South Mimms, UK) and in addition one stage APTT based clotting factor VIII (FVIII:C) levels were measured against 8th International FVIII concentrate standard (07/350) (NIBSC). For the VWF:Ag assay, ELISA plates (Nunc MaxiSorp flat-bottom, Thermo-Fisher Ltd., Hemel Hempstead, UK) were coated with polyclonal rabbit anti-human VWF (DAKO Ltd., Ely, UK) as the capture antibody. For measurement of VWF:CB, ELISA plates (Nunc Immulon 4HBX) were coated overnight at room temperature with 3 μ g/mL human placenta type III collagen (Southern Biotech, Birmingham, AL); both assays utilized horse radish peroxidase labelled polyclonal rabbit anti-human VWF (DAKO Ltd., Ely, UK) as the tag antibody. VWF:RCo assays were measured by platelet-based VWF:RCo assay using von Willebrand reagent (Siemens Healthcare Diagnostics, Marburg, Germany) with added Ristocetin sulfate (abp, London, UK) (final concentration 6.25 mg/mL) on an ACL 9000 (Instrumentation Laboratory, Bedford, MA). For use in the FVIII:C, VWF:Ag, VWF:CB and VWF:RCo assays the concentrates were pre-diluted in the respective assay buffers (1:100 for Fandhi, Optivate, Wilfactin, Alphanate, and Wilate; 1:200 for Haemate P and Voncento; 1:50 for Octanate and 8Y; 1:25 for Haemoctin) and then further serially diluted for the respective assay. Each sample dilution was assayed in duplicate in five separate assays. One stage APTT based FVIII:C levels were measured on an ACL TOP 700 coagulometer (Instrumentation Laboratory, Bedford, MA). All assays have been previously published.¹⁷

2.3 | VWF multimeric profiles

VWF multimers were analyzed with SDS agarose (0.9%) gel electrophoresis overnight at 9.0 mA and then transfer to nitrocellulose overnight was by western blotting. Each concentrate was adjusted to a concentration of 1 IU/mL VWF:Ag. Following blocking with 10% Marvel milk powder (Premier International Foods [UK] Ltd., Spalding, UK) and labelling with polyclonal rabbit anti-human VWF (DAKO Ltd., Ely, UK), VWF multimers were incubated with rabbit anti human VWF biotinylated antibody, followed by secondary incubation with Reagent A (Avidin DH, an avidin) plus Reagent B (biotinylated alkaline phosphatase H) (Vectastain, Vector Labs, Peterborough, UK). Visualization was with ABC alkaline phosphatase kit (Vectastain). VWF multimer gels were visually compared to a reference “normal pooled plasma” sample. Densitometric analysis was performed essentially as previously described using ImageJ software.¹⁸ In brief, individual peaks on the densitometry plot were counted from the dimer band up to the tenth peak (representing the 10-mer), everything beyond the 10-mer was designated as being one high molecule weight peak. The pixel area under the 10-mer peak was quantified and taken as a percentage of the total area under the full densitometry scan.

2.4 | Total protein measurement

Total protein content of each VWF concentrate was assayed using a commercially available Bradford reagent as per the manufacturer's instructions (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK).

2.5 | In vitro flow assay

Platelet adhesion to collagen-coated flow chamber slides and preparation of washed platelets and red blood cells (RBCs) were performed essentially as previous described.¹⁹ In brief, Ibidi V1^{0.1} μ -slides (Ibidi GmbH, Munich, Germany) were coated overnight with 100 μ g/mL human placental type III collagen (Southern Biotech, Birmingham, AL) and subsequently blocked with 2% BSA. Blood was drawn from healthy donors into 10% v/v acid citrate dextrose and washed red blood cells (RBCs) and platelets prepared as previously described.¹⁹ Platelets were rendered fluorescent by the addition of 4 mmol/L Calcein-AM (Sigma, Gillingham, UK). RBCs and platelets were mixed and resuspended to the original blood starting volume with HEPES/Tyrodes buffer (10 mmol/L HEPES, 137 mmol/L NaCl, 2.68 mmol/L KCl, 0.42 mmol/L NaH₂PO₄, 1.7 mmol/L MgCl₂, 11.9 mmol/L NaHCO₃, 5 mmol/L glucose, pH7.4 supplemented with 3.5 mg/mL BSA. VWF concentrates were added to the resuspended washed platelets and RBCs to a final concentration of 1 IU/mL (VWF:Ag) and perfused over the collagen surface at 1800/seconds for 5 minutes. Flow slides were mounted on an epifluorescent microscope (CKX41; Olympus, GT-Vision, Suffolk, UK), and real-time recordings were captured via a

Rollera XR camera Qimaging (GT Vision) and StreamPix6 4 software. Video images were analyzed offline using freely available Virtual Dub (<http://www.virtualdub.org/>) and MacBiophotonics ImageJ software. Data are expressed as percentage surface coverage. Three time course experiments were performed on separate days for each concentrate using platelets from two normal donors.

2.6 | Assessment of VWF glycosylation

To assess the glycan content of the VWF concentrates a panel of lectins were used: Concanavalin A (Con A) binds to α -linked mannose and is used to determine the presence of N-linked glycans chain. Galanthus Nivalis lectin (GNL) binds to α 1-3 linked mannose residues and can be used to detect high mannose structures. Ricinus Communis agglutinin-1 (RCA-1) recognizes terminal galactose residues. Elderberry bark lectin (EBL) recognizes sialic acid residues; preferring α 2-6 sialic acid over α 2-3 linked residues. Maccia Amurensis lectin-II (MAL-II) has preference for α 2-3 linked sialic acid and peanut agglutinin (PNA) bind to the desilayated T-antigen and can be used to detect O-linked glycans structures. Nunc Maxisorp plates were coated with polyclonal anti-VWF antibodies overnight and subsequently blocked with 20% polyethylene glycol-5000 (PEG-5000) solution for 60 minutes at room temperature. Well were then incubated with 1 IU/mL of each VWF concentrate and then probed with the panel of biotinylated lectins all diluted 1 in 1000 in Tris Buffered Saline supplemented with divalent ions and then with streptavidin-HRP. As a control to ensure equivalent capture wells were also incubated with anti-VWF-HRP antibodies. Sigma colourfast OPD substrate was added to the wells and following stopping with 2 mol/L H₂SO₄ wells were read at 492 nm.

2.7 | Assay for VWF-free thiols

The free-thiol content of each concentrate was determined essentially as previous described.²⁰ In brief 1 U/mL of each VWF concentrate was incubated with 100 μ mol/L maleimide-PEO2-biotin for 15 minutes at room temperature and the reaction quenched with 200 μ mol/L reduced glutathione. Labelled VWF was captured to Maxisorp plates pre-coated with polyclonal anti-VWF antibodies. Samples for incubated for 60 minutes at room temperature then following washing were incubated with Streptavidin-HRP or polyclonal anti-VWF-HRP antibodies.

2.8 | Measurement of ADAMTS-13

Plasma ADAMTS13 was measured using an “in-house” ADAMTS13 ELISA as previously described.²¹

2.9 | Statistical analysis

Statistical analysis was performed using Prism7 software, using a standard one-way ANOVA with multiple comparisons.

3 | RESULTS

3.1 | FVII & VWF laboratory parameters from the nine commercially available concentrates

The laboratory parameters of all 10 VWF concentrates measured after reconstitution according to the manufacturers' instructions are summarized in Table 1. For each VWF-containing concentrate the mean from five separate assays was calculated for factor VIII clotting activity (FVIII:C) levels and ranged from 4 to 183 IU/mL whilst mean VWF:Ag values ranged from 22 to 248 IU/mL with similar ranges for VWF:RCo of 20 to 223 IU/mL and for VWF:CB of 16 to 242 IU/mL. The reconstituted concentrate with the highest measured VWF level in all three parameters was Voncento and the lowest was Haemoctin. For each VWF containing concentrate the mean measured individual VWF:RCo to VWF:Ag ratio, VWF:CB to VWF:Ag ratio 0.9-1.1 and 0.7-1, respectively, and correlation coefficients (r^2 values) of 0.991 and 0.988, respectively, were obtained. Additionally, the mean measured VWF:RCo and VWF:CB concentration ratios were between 0.7 and 1.1 with a correlation coefficient of 0.980 (Table 1).

3.2 | Lectin analysis of VWF concentrates

All the concentrates tested had similar binding to the four lectins (Con A, EBL, RCA-1, and GNL) confirming the presence of N-linked glycan chains, a similar sialic acid content, terminal galactose and high mannose content respectively (Figure 1). MAL-II binds to sialic

acid but prefers α 2-3 linked residues that are more predominant on O-linked glycans. 8Y showed significantly reduced MAL-II binding and this was combined with a significant increase in binding to PNA, that recognizes the desialyated T-antigen O-linked glycan structure previously shown to be present on VWF.²² However, overall the binding to PNA was low, consistent with sialic acid capping of the VWF O-linked glycans.

3.3 | Multimeric distribution of VWF concentrates

The multimeric distribution of the VWF-containing concentrates was compared to a normal plasma pool sample (Figure 2A). As a guide to interpreting the positions of multimers, the top line indicates the highest MW multimer visible on the blot; that of the Wilate concentrate. The middle line is level with the highest MW multimer of the normal pooled plasma and the bottom line is level with the highest MW multimer detected in Haemoctin. Of the 10 studied, four concentrates; Alphanate, Octanate, 8Y and Haemoctin, lacked high-MW multimers compared to those contained in the plasma pool standard. Additionally, Octanate and Haemoctin also lacked or had reduced low-MW bands. High-MW VWF multimers were present in higher quantities in Haemate P, Voncento, Optivate, and Wilate and the remaining two concentrates, Fandhi and Wilfactin, had similar amounts of high-MW VWF multimers to those of the normal plasma pool control. Densitometry analysis supported the visual inspection of the multimer gels with Haemate P, Voncento, Optivate, and Wilate presenting a greater percentage of HMW multimers compared to the normal plasma pool (Figure 2B,C).

TABLE 1 Mean VWF parameters of 10 commercially available VWF-containing concentrates

VWF concentrate	Total protein (mg/vial)	FVIII:CIU/mL	VWF:Ag (IU/mL)	VWF:RCo (IU/mL)	VWF: RCo/Ag ratio (range)	VWF:CB (IU/mL)	VWF: CB/ Ag ratio (range)	VWF: CB/ RCo ratio (range)	Presence of HMW multimers	ADAMTS13 (IU/mL)
Fandhi	56	101	133	134	1.0 (0.8-1.3)	103	0.8 (0.6-1.1)	0.8 (0.7-0.9)	Yes	0.57
Wilfactin	81	4.0	143	141	1.0 (0.8-1.1)	106	0.8 (0.6-1.0)	0.8 (0.6-1.0)	Yes	0.06
Haemate P	165	183	222	223	1.0 (0.7-1.2)	182	0.9 (0.6-1.1)	0.8 (0.6-1.1)	Yes	0.85
Alphanate	64	147	111	124	1.1 (0.8-1.4)	96	0.8 (0.4-1.4)	0.8 (0.6-1.0)	Reduced	1.5
Octanate	10	103	49.5	62.5	1.2 (1.1-1.3)	42	0.9 (0.8-1.2)	0.7 (0.6-0.8)	Reduced	0.16
8Y	130	58	47.5	45.9	1.0 (0.7-1.3)	49	0.8 (0.7-1.2)	1.0 (0.8-1.2)	Reduced	1.5
Optivate	19	115	212	206	1.0 (0.8-1.2)	180	0.9 (0.8-0.9)	0.9 (0.8-1.0)	Yes	0.67
Haemoctin	4	49	21.7	20	0.9 (0.8-1.2)	16	0.7 (0.7-0.8)	0.8 (0.7-0.9)	Reduced	1.3
Wilate	20	101	138	145	1.1 (1.0-1.1)	113	0.8 (0.7-0.9)	0.8 (0.7-0.9)	Yes	0.04
Voncento	Not tested	108	248	242	0.9 (0.8-1.0)	242	1.0 (0.9-1.1)	1.0 (1.0-1.2)	Yes	0.9

VWF:Ag, VWF:RCo, VWF:CB levels as well as total protein content were assessed as stated in the Materials and Methods section.

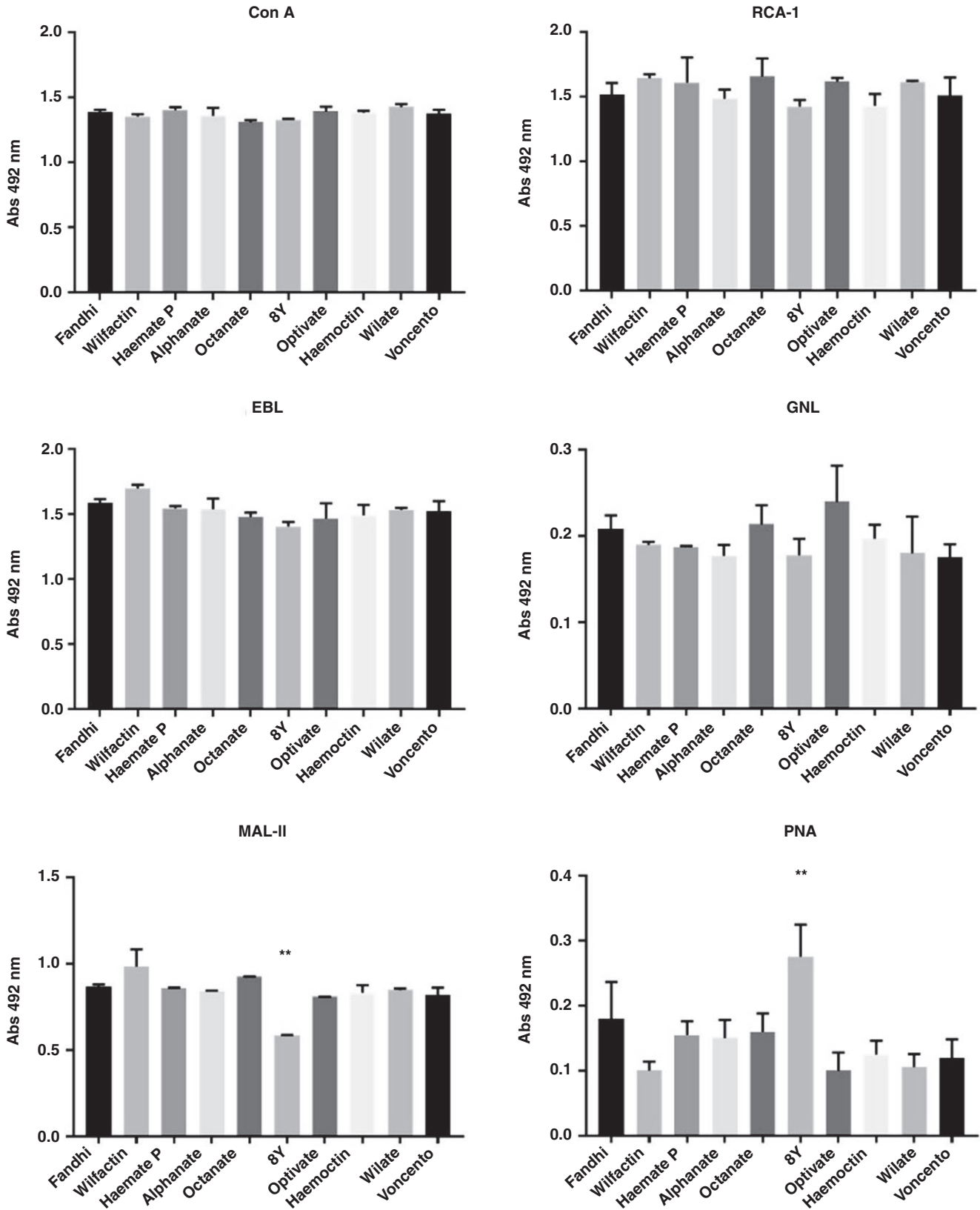


FIGURE 1 Lectin analysis of VWF concentrates. Maxisorp plates were coated overnight with polyclonal anti-VWF antibodies and then blocked with 20% PEG-5000. Wells were then incubated with 1 IU/mL of each VWF concentrate for 60 minutes at room temperature. Sugar structures were detected with a panel of biotinylated lectins diluted 1 in 1000 in TBS supplemented with divalent ions and subsequently with streptavidin-HRP. (mean \pm SD, n = 3). (** $P \leq 0.005$)

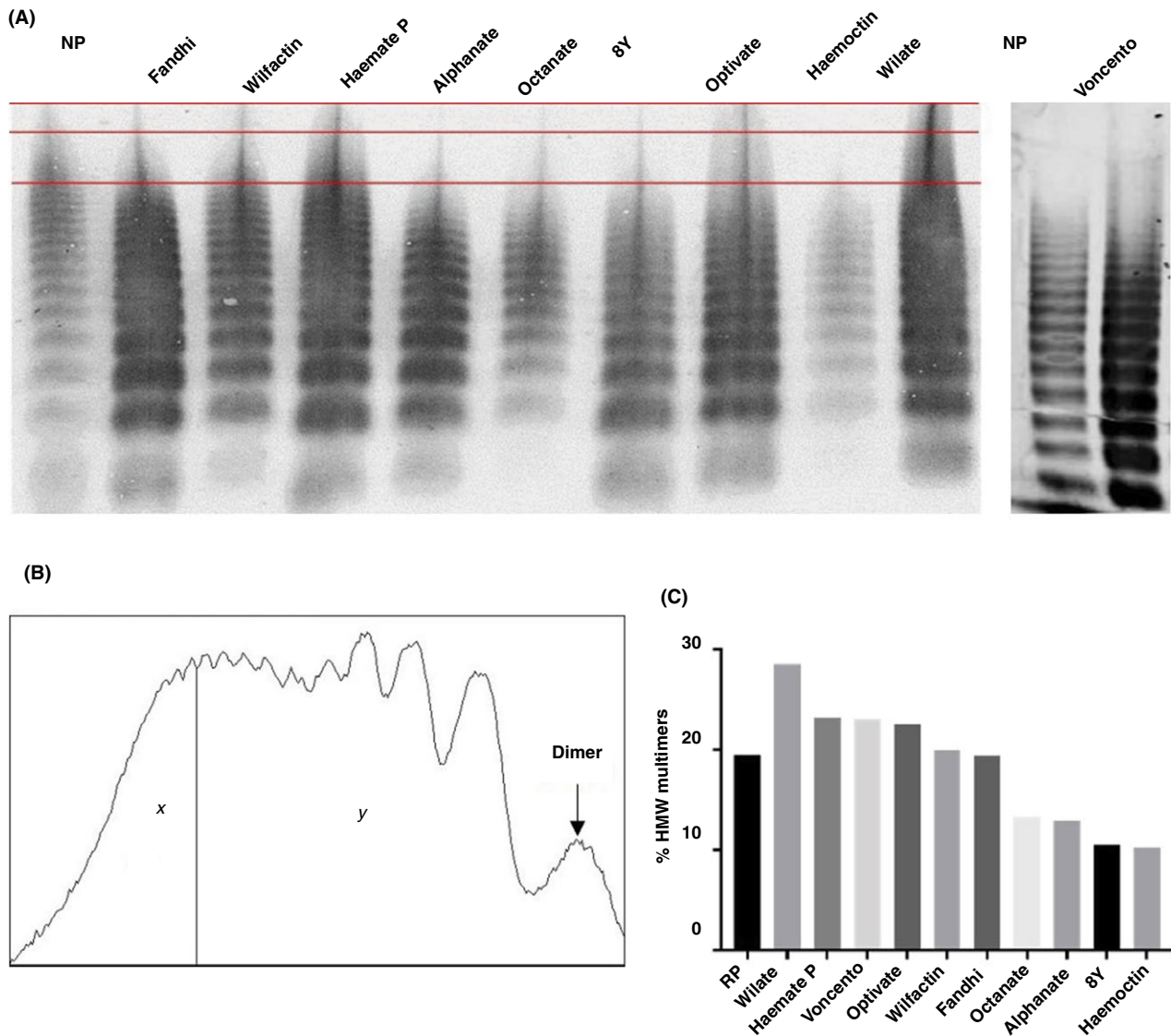


FIGURE 2 Multimeric analysis of VWF concentrates. (A) 1 IU/mL of each VWF concentrate was electrophoresed through 0.9% agarose gels under non-reducing conditions and subsequently transferred to nitrocellulose membranes. VWF multimers were detected with biotinylated rabbit anti-VWF polyclonal antibodies. LMW denotes low molecular weight multimers and HMW denotes high molecular weight multimers. (B) Densitometry analysis was performed by scanning the multimer gel lanes using ImageJ software. Ten peaks were counted from the dimer band peak, representing dimers to 10-mers (y). The remaining area under the curve was treated as one peak (x) representing high molecular weight multimers. The percentage of high multimers was determined by $x/(x + y) \times 100$. (C) Percentage of HMW multimers in the VWF concentrates compared to normal plasma

3.4 | Free-thiol content of VWF concentrates

Recent work has demonstrated that VWF contains a number of unpaired cysteine residues and that the resultant free-thiols are important for VWF function.^{20,23} Interestingly, differences were observed in the amount of MPB bound by the different concentrates indicating differing amounts of free-thiols. While Fandhi, Haemate P, Voncento, Optivate, 8Y, Haemoclin, and Wilate all had similar amounts of free-thiol; an ~2-fold decreased signal was seen with Wilfactin and Alphanate and the highest amounts of free-thiols were seen in Octanate with an one-to-two-fold increased MPB binding compared to the other concentrates (Figure 3).

3.5 | VWF-mediated platelet adhesion to collagen under shear conditions

Next, to provide a physiological measure of function, we investigated the ability of each of the VWF-containing concentrates to mediate platelet capture to type III collagen under high shear stress using a physiological flow assay. Three concentrates, Optivate, Haemate P, and Voncento demonstrated the high initial platelet adhesion, measured as percentage surface coverage, (defined as $\geq 35\%$ at 60 seconds) (Figure 4). Intermediate adhesion (21%–34% at 60 seconds post-perfusion) was observed with concentrates Fandhi, Wilfactin, 8Y, and Wilate with values of 30%, 23%, 25%, and 29%, respectively (Figure 4). Low adhesion at 60 seconds (<20%) was observed

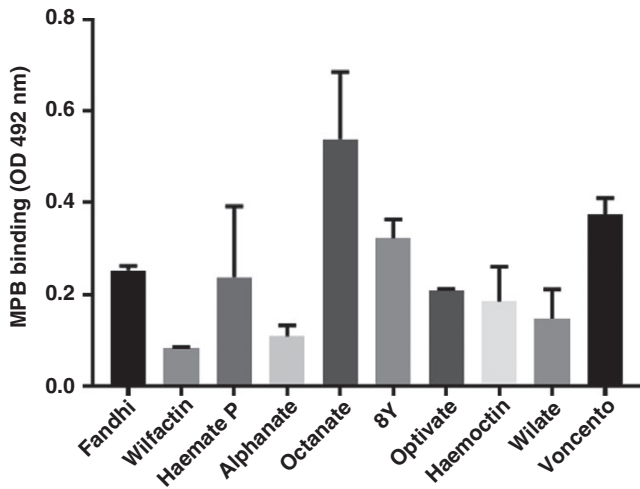


FIGURE 3 Free thiol content of VWF concentrates. 1 IU/mL of each concentrate was incubated with 100 μ mol/L MPB and the reaction quenched with 200 μ mol/L GSH. Samples were captured to anti-VWF coated Maxisorp plates and bound MPB detected with streptavidin-HRP. (mean \pm SD, n = 3)

with Alphanate, Octanate, and Haemocitin with 14%, 17%, and 18%, respectively (Figure 4). The difference in platelet capture between Haemate P and Voncento compared to Alphanate, Octanate, and Haemocitin was statistically significant ($P < 0.05$) as was the difference between Optivate and Alphanate ($P < 0.05$). Overall, the order of concentrates with the greatest platelet adhesion at 60 seconds was Haemate P = Voncento > Optivate > Wilate > Fandhi > 8Y = Wilfactin > Haemocitin = Octanate > Alphanate

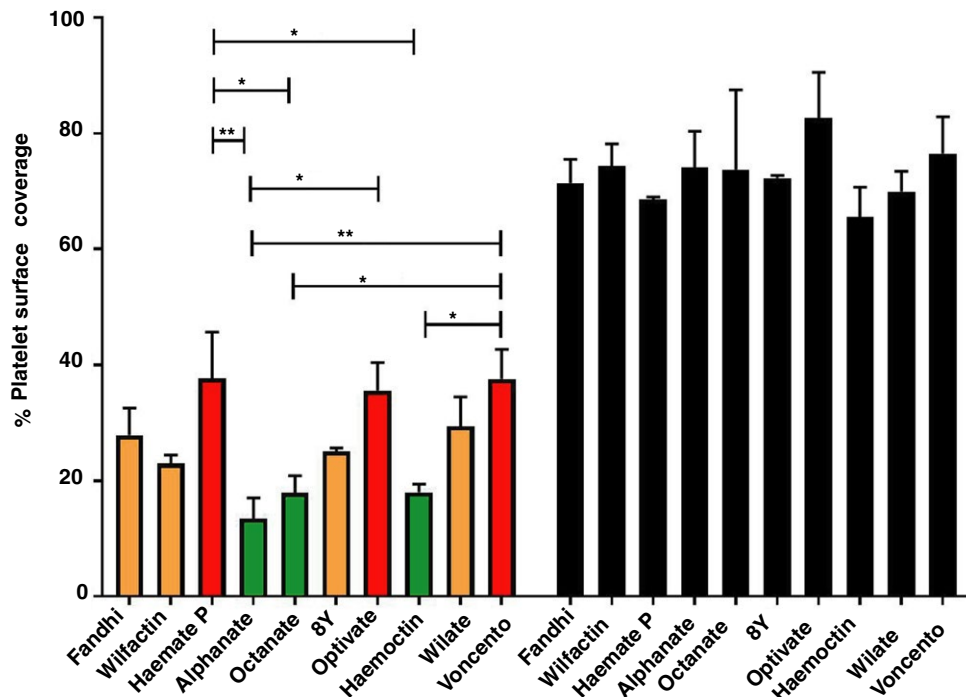


FIGURE 4 Analysis of VWF concentrate function under shear stress. Ibidi $VI^{0.1}$ flow chamber slides coated with 100 μ g/mL type III collagen were perfused with plasma-free blood supplemented with 1 IU/mL VWF for 5 minutes. Platelets were rendered fluorescent with DiOC6 and VWF-mediated platelet capture to collagen recorded in real-time. (mean \pm SD, n = 3). (* $P \leq 0.05$, ** $P \leq 0.005$). Red bars denote high platelet capture at 60secs (>35% platelet surface coverage). Orange bars denote intermediate surface coverage at 60secs (21-34%). Green bars denote low surface coverage at 60secs (<20%). Black bars are platelet surface coverage after 300secs perfusion

(Table 2). Adhesion when measured at 300 seconds showed little variability between the concentrates (Figure 4). These adhesion data, when taken as a whole, would suggest that only the initial rate of platelet adhesion is influenced by the type of VWF containing concentrate since surface coverage values at the end of the perfusion time do not differ greatly.

3.6 | ADAMTS13 concentration in the VWF concentrates

Since some of the concentrates had an absence of HMW multimers and reduced initial rates of platelet capture we hypothesized this could be due to the concentration of ADAMTS13 in the respective concentrate. Interestingly, the ADAMTS13 concentration varied between concentrates, with Haemocitin, 8Y and Alphanate having ~ 1.5 U/mL ADAMTS13, compared with almost an absence of ADAMTS13 in Wilate and Wilfactin. The three concentrates that had the highest initial rate of platelet capture (Haemate P, Voncento, and Optivate) had similar ADAMTS13 levels of ~ 0.7 U/mL (Table 1).

4 | DISCUSSION

Ten commercially available VWF-containing concentrates were assessed for their levels of FVIII:C, VWF antigen, and VWF activity (VWF:RCO and VWF:CB) using standard laboratory techniques as well as VWF multimer content, and also had their glycan and free-thiol status probed. Most importantly, we determined the ability of

TABLE 2 Rank order of platelet adhesion to pre-perfused collagen coat flow cells at 60 seconds comparing concentrate VWF multimer content, adhesion observed at 300 seconds, known albumin addition and viral contamination procedure during manufacturing

VWF concentrate name	Platelet adhesion 60 s (%)	Platelet adhesion 300 s (%)	Presence of high VWF multimers	Albumin presence ^a	Viral inactivation ^a
Haemate P	37.6	68.6	Yes	Yes	P (60°C) ^e
Voncento [®]	37.6	76.5	Yes	Yes	SDH ^l
Optivate [®]	35.6	82	Yes	No ^b	SDH (80°C) ^f
Wilate [®]	29.4	69	Yes	No ^{a,c}	SDH (100°C) ^g
Fandhi [®]	27.9	71.4	Yes	Unknown ^d	DH ^h
8Y [®]	25	72.3	Reduced	Yes	SDH (80°C) ⁱ
Wilfactin	23	74	Yes	Yes	SDH (80°C) ^f
Haemoctin	18	65.5	Reduced	No ^c	SDH (100°C) ^{j,k}
Octanate	17.9	73.8	Reduced	No	SDH (100°C) ^j
Alphanate	13.5	74.1	Reduced	Yes	SDH (80°C) ^f

Temperature of viral inactivation is given in brackets.

DH, dry heat; P, pasteurization; SDH, solvent detergent, dry heat.

^aData derived from Batlle et al (2009) unless otherwise stated.

^b<https://www.medicines.org.uk/emc/medicine/15945>.

^cClifton et al (2009).

^d<https://www.medicines.org.uk/emc/medicine/15509>, state "other plasma proteins" included as excipients.

^ePasteurization for 10 h at 60°C.

^fSolvent detergent, dry heat for 72 h at 80°C.

^gSolvent detergent, dry heat for 2 h at 100°C with controlled residual moisture.

^hDry heat for 72 h at 80°C.

ⁱSolvent detergent, dry heat for 72 h at 80°C plus 30 nm nanofiltration.

^jSolvent detergent, dry heat for 0.5 h at 100°C.

^khttp://mri.medagencies.org/download/DE_H_0478_003_PAR.pdf.

^lhttp://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/002493/WC500151259.pdf.

the concentrates to mediate platelet capture to collagen under shear stress using a flow-based assay system. Our findings show that the multimeric profile differed between concentrates and this correlated with the initial extent of platelet capture after 60 seconds, but ultimately did not affect the extent of platelet capture after 5 minutes. In addition, while there were some differences in free-thiol content and ADAMTS13 content between concentrates, this did not affect multimeric profile or function.

Of the 10 concentrates tested, only two are not labelled for use in VWD—Fandhi (to date there are no clinical trials of its use in VWD as stated; www.grifols.com/en/web/uk/bioscience/-/product/fandhi) and Haemoctin which does not contain VWF in pharmacologically effective quantities and is therefore not indicated in VWD (www.medicines.org.uk/emc/medicine/23194).

Each of the VWF-containing concentrates had VWF parameters assessed by VWF antigen, VWF ristocetin cofactor and VWF collagen binding assays and were found to be variable when the concentrates were reconstituted to their specified volumes according to manufacturer's instructions. However, there was a good correlation between VWF:Ag to VWF:RCO ratios and slightly less so for VWF:Ag to VWF:CB measurements (mean ratios ranged from 0.9 to 1.1 and 0.7 to 1, respectively) ($r^2 \geq 0.980$). Lectin analysis demonstrated that the VWF concentrates all broadly had a similar glycan structure.

One subtle yet significant difference was noted in that 8Y had less α 2-3 linked sialic acid and a corresponding increase in PNA binding potentially due to the loss of α 2-3 linked sialic acid capping the T-antigen structures. It is unclear why these differences would exist; since these concentrates are derived from plasma pools it is unlikely due to the starting material and is therefore likely to be a result of the manufacturing process. 8Y is reportedly treated with solvent detergent and dry heat at 80°C for 2 hours and previous data has suggested that heating can reduce glycan detection on glycoproteins, thus this treatment approach may result in loss of some sugar structures. However, none of the other concentrates that undergo similar processing present an altered glycan structure. A mass spectrometry approach would be required to prepare a detailed glycosylation profile of each concentrate.

To test the function of the concentrates in a physiological setting we determined their ability to mediate platelet capture to collagen under shear stress. Interestingly the concentrates varied in their ability to initially mediate platelet capture measured after 60 seconds, however by 300 seconds all the concentrates demonstrated a similar platelet capture profile. This suggests that the only initial adhesion of platelets was dependent upon VWF deposition onto the collagen. Further activation of platelets with the release of VWF and other constituents then enhanced adhesion in the latter part of the time course. It is estimated that, under normal conditions, platelets contribute about 15%-25% of the total

circulating VWF²⁴ and contain UL-MW forms which are therefore potentially more effective hemostatically than those of plasma.²⁵ Since the platelets used in this study were derived from healthy individuals, one may speculate that in a VWD patient where the platelet pool of VWF is either reduced or contains VWF with VWF function this effect would be more pronounced. Using rank order by platelet deposition at 60 seconds, in Table 2, the three concentrates demonstrating the greatest adhesion (defined as being $\geq 35\%$); Haemate P, Vonento, and Optivate, both contained the highest MW multimers. Conversely, the three concentrates demonstrating the lowest platelet adhesion ($\leq 20\%$), Haemoctin, Octanate, Alphanate, lacked the high MW forms, and to some extent intermediate MW multimers, suggesting that this may be decreasing the initial rate of platelet deposition. Of the four remaining concentrates that showed intermediate platelet adhesion at 60 seconds (21%-34%), Fandhi, Wilfactin, and 8Y had intermediate MW multimers similar to that of the normal plasma pool and little of the high forms. One concentrate Wilate, contained high MW forms of VWF. These data confirm that while the largest VWF multimers are required for platelet capture, a reduction in multimer size, at least to the extent seen with the VWF concentrates analyzed in this study, only affects the initial rate of platelet capture but results in normal platelet capture over a longer time frame.

As with the subtle differences in glycan structure, manufacturing differences of the concentrates as well as final formulation could theoretically contribute to qualitative differences in VWF multimeric distributions. Therefore, these were also contrasted with the rank order data in Table 2. At least four of the VWF-containing products, Wilate, Haemoctin, Optivate, and Octanate, are formulated without the addition of extrinsic albumin as a stabilizer the rest, with the exception of 8Y of whose status is unknown but is likely to have albumin as an excipient because of its relatively high level of total protein, contain albumin in their formulations.^{26,27} The presence or absence of albumin appears to have little influence on either the platelet adherence data or the presence of HMW multimeric forms of VWF. When contrasting known manufacturing practice that eliminates viral contamination, no trends were evident. Interestingly, the two concentrates also lacked the lowest MW VWF band, Octanate and Haemoctin, which might reflect manufacturing since both are heat treated at 100°C for 30 minutes.²⁶

An additional contributing factor to the loss of the high MW multimers compared to normal plasma in some of the concentrates could be their intrinsic ADAMTS13 activity. This metalloproteinase regulates the multimeric size of VWF in plasma by cleaving the protein at a site in the A2 domain which lies between the GPIb and collagen binding sites in the A1 and A3 domains, respectively.²⁸ It has been reported that Wilfactin and Wilate contain relatively low levels of ADAMTS13 and have a VWF triplet structure similar to that of normal plasma.²⁹ Additionally, this study also demonstrated that Fandhi and Haemate-P had higher ADAMTS13 activity with an altered triplet structure. Alphanate

is also reported to have high ADAMTS13 activity.³⁰ In keeping with these previously reports both Wilfactin and Wilate had the lowest ADAMTS13 concentration, while Alphanate, 8Y and Haemoctin had the highest ADAMTS13 concentration and indeed Alphanate and 8Y have been used to successfully to treat TTP.³⁰ While the higher ADAMTS13 concentration of Alphanate and Haemoctin may account for the lack of HMW multimers and thus reduced initial rates of platelet capture, Octanate had both lack of HMW multimers and low ADAMTS13 indicating that other currently unknown factors may also be involved. Another interesting observation from this study was that the free-thiol content of each of the concentrates studied differed. VWF free-thiols have recently been shown to be important for function, while no correlation between free-thiol content and function was seen, further studies will be required to determine how this may affect the function of the VWF concentrates. As with the subtle difference in glycan content it is not clear what would cause a difference in free-thiol content, but it is most likely attributed to the manufacturing process.

The differences seen in antigen and activity assays should be borne in mind when interpreting assays used for monitoring replacement therapy in the clinical setting. This may affect dosing and administration frequency which is generally guided by post treatment and trough levels. We do not expect that there would be any significant effect on efficacy with regard to prevention or treatment of bleeding symptoms in most situations, but this is a possibility in situations where von Willebrand disease is complicated by other hemostatic abnormalities.

In conclusion, use of flow assay analysis showed that all the VWF-containing concentrates studied were equally effective at mediating platelet adhesion to human placental collagen type III coated surfaces at 300 seconds. However, several of the VWF containing concentrates lacking the highest molecular weight VWF multimers demonstrated a reduced ability to initially mediate platelet capture to collagen surfaces at 60 seconds.

RELATIONSHIP DISCLOSURE

The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

AR designed the study, performed experiments, analyzed data, and wrote the paper. SV performed experiments, analyzed data, and wrote the paper. KG designed the study, analyzed data, and wrote the paper. ML analyzed data and wrote the paper. TM designed the study, performed experiments, analyzed data, and wrote the paper.

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