
Short title: Von Willebrand factor mutants W1745C and S1783A

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Abstract

Investigation of three families with bleeding symptoms demonstrated a defect in the collagen binding activity of von Willebrand factor in association with a normal VWF multimeric pattern and variable VWF levels. Genetic analysis showed affected individuals to be heterozygous for mutations in the A3 domain of VWF: S1731T, W1745C and S1783A respectively. One individual showed compound heterozygosity for W1745C and R760H. W1745C and S1783A have not been reported previously. The mutations were reproduced by site-directed mutagenesis and mutant VWF expressed in HEK293T cells. Collagen binding activity measured by immunosorbent assay varied according to collagen type: W1745C and S1783A were associated with a more pronounced binding defect to both type I and type III collagen, whereas the principal abnormality in S1731T patients was a reduction in binding to type I collagen only. The multimer pattern and distribution of mutant proteins was indistinguishable from wild-type recombinant VWF, confirming that the defect in collagen binding resulted from the loss of affinity at the binding site and not impairment of high-molecular weight multimer formation. Our findings demonstrate that mutations causing an abnormality in the binding of VWF to collagen may contribute to clinically significant bleeding symptoms. We propose that isolated collagen binding defects are classified as a distinct subtype of VWD.
Introduction

Von Willebrand factor (VWF) is a large multimeric glycoprotein that has two important roles in haemostasis. These are stabilizing factor VIII (FVIII), by acting as its carrier protein in the circulation, and attaching activated platelets to the sub-endothelium via binding to the GpIb receptor on platelets and to collagen in the sub-endothelial matrix. Enhancement of the platelet – subendothelium interaction is vital at sites of vascular injury during conditions of high shear stress. VWF binds to collagen via two sites: the A3 domain (residues 1683 – 1874) contains the main site for fibrillar collagen types I and III found within perivascular connective tissue, and a second site in the A1 domain (residues 1260 – 1471) binds non-fibrillar collagen type VI within the subendothelial matrix. However, the A1 domain can also bind collagen types I and III and the relative importance of the two domains has been extensively but inconclusively investigated. In all cases it appears that affinity of VWF for collagen is heavily dependent on the presence of VWF multimers of high or ultra-high molecular weight.

Type 2 von Willebrand disease (VWD) is characterized by a qualitative defect in VWF and is diagnosed by demonstration of a discrepancy between circulating plasma levels of VWF and its functional activity. In addition to measuring GpIbα-dependent function using ristocetin co-factor activity (VWF:RCo), it has been recommended that collagen binding activity (VWF:CB) is analyzed in the subclassification of type 2 VWD. A single defect in VWF collagen binding in association with normal multimeric structure has previously been reported.

In this report we describe the phenotypic and genotypic characterisation of three families with bleeding symptoms in whom the main laboratory abnormality was a reduction in VWF:CB. Analysis of the VWF coding sequence identified two novel mutations, W1745C and S1783A, and the previously described mutation S1731T, all within the A3 domain. The mutations have been characterized and shown to be responsible for the phenotype by expression studies. These studies illustrate the physiological importance of the A3 collagen binding site and the structural constraints on its function.

Materials and Methods

Phenotypic analysis

Bleeding scores were calculated retrospectively from the patient notes using the condensed MCMDM-1VWD questionnaire. Venous whole blood was taken into 0.106M sodium citrate (Sarstedt Monovette 9NC/3mL tubes, 9:1 v/v). Platelet-poor plasma (PPP) was prepared by double centrifugation at 2000g for 12 minutes at 4°C and stored in aliquots at -45°C until further testing. FVIII:C was analyzed by one-stage clotting assay. VWF antigen (VWF:Ag) was
measured by ELISA, and VWF:RCo was measured using lyophilized platelets as previously described. VWF:CB was performed by coating Covalink ELISA plates (Nunc, Loughborough, UK) with 2.7 μg/mL placental type III human collagen (Sigma, Poole, UK or Southern Biotechnology, Cambridge, UK) or 10 μg/mL placental type I human collagen (Sigma, Poole, UK) diluted in 0.14M NaCl, 0.006M Na₂HPO₄, 0.002M NaH₂PO₄ and 0.03M KCl (pH 7.3). Plates were blocked with 1.5% bovine serum albumin (BSA) in phosphate buffered saline (PBS). PPP and standard pooled normal plasma were diluted in PBS containing 3% polyethylene glycol. Collagen-bound VWF was detected with horse radish peroxidase (HRP)-conjugated rabbit anti-human VWF antibody (DakoPatts, Ely, UK). PFA-100 (Dade-Behring, Schwalbach, Germany) closure times were determined according to the manufacturer’s instructions. Platelet-rich plasma (PRP) was prepared by centrifugation at 130g for 15 minutes. Platelet function was assessed by optical aggregometry. Ristocetin-induced platelet aggregation (RIPA) was determined by adding ristocetin at concentrations of 0.5, 1.0, 1.25 and 1.5 mg/mL to PRP and monitoring light transmission in an aggregometer (Payton Associates, Ontario, Canada). VWF multimer composition was assessed by sodium dodecyl sulphate agarose gel electrophoresis followed by western blotting and detection with rabbit anti-human VWF antibody (DakoPatts, Ely, UK). The gel was then visualized using a Vectastain ABC-AP Kit and a BCIP/NBT Alkaline Phosphate Substrate Kit (Vector Laboratories, Peterborough, UK).

**Genetic analysis**

Genomic DNA was extracted from peripheral blood lymphocytes using the QIAamp® DNA Mini prep kit (Qiagen, West Sussex, UK) according to the manufacturer’s instructions. Fifty-one primer sets were used to amplify the essential regions of VWF (52 exons, flanking intronic regions, 5' promoter region and the 3' untranslated region). Primers were designed to optimally amplify sequences of interest at an annealing temperature of either 57 °C or 59 °C (sequences and PCR conditions available on request) using standard PCR methods. Amplicons were purified using microCLEAN (Web Scientific, Crewe, UK) prior to cycle sequencing using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK). Sequencing reaction products were purified using an in-house ethanol precipitation method and resuspended in formamide (Applied Biosystems, Warrington, UK) prior to direct sequencing on an Applied Biosystems AB3130xl DNA sequencer. Mutation detection was performed using the Mutation Surveyor® version 3.2 software package (BioGene Ltd, Kimbolton, UK). The numbering and description of mutations is according to the current ISTH SSC guidelines.
Molecular modelling
Mutated residues were modelled using the crystal structure (PDB Id: 1FE8) of the VWF A3 domain complexed with the RU5 antibody that inhibits collagen-binding. Structural images were generated using PyMOL (DeLano Scientific, Palo Alto, USA).

Expression of recombinant VWF
Generation of the expression vector pcDNA3.1 FL-VWF encoding for full length VWF has been previously described. A silent mutation has been previously introduced to mutate the KpnI restriction site located at base pairs 298-303, leaving a unique KpnI restriction site located in the VWF A2 domain. The VWF KpnI-AgeI fragment was cloned into pcDNA3.1 and used to generate the mutants, S1731T, W1745C, W1745A, S1783A, R760H and H1786A using the QuickChange XL site directed mutagenesis kit (Stratagene, Cambridge, UK). H1786A is a mutation that has not been described in patients but has been shown to abolish collagen binding in expression studies. Therefore this mutant was included as a control. Clones were digested with KpnI and AgeI and sub-cloned into pcDNA3.1 FL-VWF. All mutations and clones were verified by DNA sequencing.

VWF was expressed in HEK293T cells using 10mM PEI as a transfection reagent as previously described. At 3 days post-transfection media was collected and cells were lysed with lysis buffer (50mM Tris-HCl, 150mM NaCl, 1mM EDTA, 1% Triton X-100, pH 7.5). For the generation of collagen binding isotherms, media was collected and concentrated 10-fold using 100kDa cut-off spin filters (Amicon, Peterborough, United Kingdom) and dialysed into 20mM Tris-HCl, pH 7.4. Concentration of VWF samples did not alter multimeric structure, which was determined by ELISA and gel electrophoresis as previously described.

Collagen-binding activity of expressed proteins
The collagen-binding activity of VWF was measured by ELISA. Briefly, MaxiSorp plates (Nunc, Loughborough, United Kingdom) were coated with either human type III or human type I collagen derived from placenta (Sigma, Poole, UK) at final concentration 5µg/mL in carbonate buffer at room temperature. Wells were then blocked with 2.5% BSA in PBS. Following washing with PBS containing 0.1% Tween (PBS-T) serial dilutions of recombinant VWF samples and reference plasma diluted with PBS-T (1:10-1:320) were added to the wells and incubated for 2 hours at room temperature. To determine binding kinetics increasing concentrations of VWF were applied to the wells for 1 hour at room temperature. Wells were then washed with PBS-T and incubated with HRP-conjugated rabbit anti-human VWF antibody (DakoPatts, Ely, UK) diluted 1/1000 in PBS-T for 45mins at room temperature. Bound antibody was detected with Colour Fast O-phenylenediamine substrate (Sigma, Poole, UK) and the absorbance recorded at
492nm. Binding curves were generated using GraphPad Prism (Droitwich, United Kingdom) fitting the data to the one-site binding equation.

Results

Patient phenotype and genotype

Phenotypic and genetic results are shown in Figure 1. All patients had normal platelet counts, platelet aggregometry, RIPA, prothrombin time, activated partial thromboplastin time and thrombin time.

In family 1, the propositus (1A) was an elderly female suffering from lifelong bleeding episodes including epistaxis, ecchymosis, menorrhagia and prolonged bleeding following dental extractions, requiring multiple medical interventions. Her two offspring, 1B (son) and 1C (daughter) reported minor bleeding symptoms that had not required treatment. 1A demonstrated compound heterozygosity for two mutations in the VWF gene. The first is a novel mutation in exon 30: c.5235 G>T resulting in Trp1745Cys and the second is c.2279 G>A resulting in Arg760His. 1B was found to be heterozygous for Trp1745Cys only, while 1C was heterozygous for Arg760His only; confirming that these mutations are in trans in 1A. The co-existence of both mutations appears to produce a more significant bleeding disorder as the bleeding score in 1A (8) was higher than that in either of her children (4 and 2).

The position of Trp1745 in the A3 domain is shown in Fig. 2. Both individuals with this mutation (1A and 1B) had markedly reduced ratios of VWF collagen-binding activity to VWF antigen (CB:Ag) with type III collagen (0.1 and 0.3 respectively) and type I collagen (0.2 and 0.3 respectively). The ratio of VWF:RCo to VWF:Ag (RCo:Ag) was near unity in both individuals and VWF multimer analysis (see Fig. 1B) and RIPA were normal. Closure times in the PFA-100 were mildly prolonged with the ADP cartridge (1A = 173s, 1B = 189s, NR < 112s). With the epinephrine cartridge the closure time was prolonged in 1A only (1A = 271s, 1B = 90s, NR < 179s).

In contrast 1C demonstrated a concordant reduction in VWF:Ag, VWF:RCo and VWF:CB (to both collagen types) with normal multimer pattern. Thus Trp1745Cys is associated with a type 2 VWD phenotype with a specific defect in collagen binding while Arg760His in isolation produces a type 1 VWD phenotype with no additional effect on collagen binding. Administration of 0.3 µg/kg DDAVP to 1A resulted in correction of both VWF:Ag and VWF:CB into the normal range, but with a persistently discrepant CB:Ag ratio (samples taken 90 min post DDAVP administration showed VWF:Ag 162 IU/dL, VWF:RCo 140 IU/dL, VWF:CB 66 IU/dL, CB:Ag ratio 0.4).
The propositus (2D) in family 2 was investigated following a post-partum haemorrhage requiring blood transfusion. She gave a longstanding history of menorrhagia and her calculated bleeding score was 10, the highest of all the individuals in this study. Her son 2E suffered from mild bruising and epistaxis. Both were shown to be heterozygous for a novel mutation in exon 31: c.5347 T>G resulting in Ser1783Ala (Fig. 2). VWF:Ag, VWF:RCo and multimer analysis were all normal. VWF:CB was significantly reduced with both types I and III collagen, resulting in CB:Ag ratios of 0.2 – 0.4. Closure times in the PFA-100 were normal in 2D with both cassettes (Coll/Epi 111s, Coll/ADP 98s). The discrepant reduction in VWF:CB persisted following DDAVP administration to 2D despite normalisation of both VWF:Ag and VWF:CB (samples taken 90 mins post DDAVP administration showed VWF:Ag 191 IU/dL, VWF:CB 67 IU/dL, CB:Ag ratio 0.35).

In family 3, the female propositus (3G) presented in childhood with epistaxis and easy bruising. Her father, 3F, who reported mild bruising symptoms and both asymptomatic younger male siblings (3H and 3J) were also investigated. All family members were found to be heterozygous for a previously described mutation in exon 30: c.5191 T>A, resulting in Ser1731Thr 9. VWF:Ag and VWF:RCo were borderline low in 3F and 3G but normal in the asymptomatic brothers, 3H and 3J. Multimer analysis was normal in all family members tested (Fig. 1). VWF:CB binding to type I collagen was reduced in all family members (CB/Ag ratio 0.4 – 0.7), whereas only one (3G) showed a reduction in CB:Ag ratio with type III collagen.

**Expression of recombinant VWF mutants**

The recombinant proteins wt-rVWF, W1745C-rVWF, W1745A-rVWF, S1783A-rVWF, S1731T-rVWF, R760H-rVWF and H1786A-rVWF were all expressed in HEK293T cells. Co-transfections of W1745C-rVWF with wt-rVWF and R760H-rVWF were also expressed to mimic the heterozygous or compound heterozygous states. VWF ELISA demonstrated that R760H-rVWF was expressed at a significantly lower level than wt-rVWF. All the other recombinant proteins were expressed and secreted at similar levels to wt-rVWF (~2µg/mL) with a similar amount of VWF retained within the cell (~1.5µg/mL) (Fig. 3). Electrophoretic analysis demonstrated that all the mutant proteins had a similar multimer distribution to that of wt-rVWF (Fig. 3C).

**Collagen-binding function of recombinant VWF mutants**

In the diagnosis of type 2M VWD, a CB:Ag ratio >0.7 is taken to reflect normal collagen-binding function 16. The CB:Ag ratio for wt-rVWF binding to collagen type III is below this at 0.4 (Fig. 3B). This is most likely due to the difference in multimer distribution observed between plasma-derived and recombinant VWF (Fig. 3C). However, all recombinant VWF proteins had multimeric patterns that were indistinguishable from wt-rVWF, and we therefore attribute differences in collagen binding to differences in affinity at the collagen-binding site between the
molecules. H1786A-rVWF and S1783A-rVWF did not bind to type III collagen (Figs. 4B and 5B). W1745C-rVWF and W1745A-rVWF both produced CB:Ag ratios of <0.05 confirming that it is the loss of the large side chain of tryptophan, rather than the availability of an additional cysteine residue for disulphide bond formation, that affects collagen-binding (Fig. 4B). The defect in collagen binding was partly corrected when W1745C-rVWF was co-transfected with wt-rVWF. S1731T-rVWF had a CB:Ag ratio with type III collagen that was similar to wt-rVWF (0.43) (Fig. 2A).

As with type III collagen, the CB:Ag ratio for wt-rVWF with type I collagen (0.39) was below the normal plasma range. W1745C-rVWF, S1783A-rVWF, S1731T-rVWF and H1786A-rVWF did not bind to type I collagen, and so a ratio could not be determined. S1731T-rVWF had a reduced CB:Ag ratio of 0.12. This is consistent with the findings of Ribba et al. \( ^9 \) and demonstrates that mutation of S1731 affects binding to collagen type I but not type III.

R760H-rVWF showed similar CB:Ag ratio to wt-rVWF with both collagen types. Co-expression of W1745C-rVWF with R760H-rVWF showed the same results as for W1745C-rVWF / wt-rVWF (data not shown).

To further investigate the binding of VWF to collagen, binding isotherms were generated to calculate the \( K_{D,app} \) for the VWF-collagen interactions. As previously determined, wt-rVWF bound type III collagen with high affinity (\( K_{D,app} 2.0 \pm 0.1 \text{nM} \)) (Fig. 5B). In accordance with previous studies and the results obtained for the CB:Ag ratio the control H1786A-rVWF mutant and the S1783A-rVWF mutants did not bind to collagen type III. S1731T-rVWF bound type III collagen with similar affinity to wt-rVWF, \( K_{D,app} 3.7 \pm 0.5 \text{nM} \) (Fig. 5B). W1745C-rVWF and W1745A-rVWF both bound type III collagen with reduced affinity, \( K_{D,app} 16 \pm 2.6 \text{nM} \) and \( 21.3 \pm 6.3 \text{nM} \) respectively (Fig. 5B).

No binding of H1786A-rVWF, S1783A-rVWF, W1745C-rVWF or W1745A-rVWF was observed to type I collagen in keeping with the CB:Ag ratio results. S1731T-rVWF bound type I collagen with reduced affinity, \( K_{D,app} 27.1 \pm 5.2 \text{nM} \) compared to \( 7.3 \pm 0.8 \text{nM} \) for wt-rVWF (Fig. 4B), confirming that the S1731T mutation only affects binding to collagen type I.
Discussion

We have reported three unrelated families with bleeding symptoms in which the principal detectable abnormality was defective collagen binding, not attributable to loss of HMW multimers. Genetic analysis showed that affected individuals had mutations in the A3 domain of VWF, which contains the main binding site for fibrillar collagen. Mapping of the mutants onto the crystal structure of the A3 domain demonstrated their close proximity to the putative collagen-binding site. Experiments with recombinant mutant proteins confirmed that the defects in collagen binding were due to reduced affinity at the binding site rather than abnormalities of multimer formation.

Two novel A3 domain mutations have been described in this report in families with clinically significant bleeding: W1745C and S1783A. Both mutations result in reduced VWF affinity towards collagen types I and III. Previous studies have shown that S1783 is essential for collagen type III binding, but a mutation at this residue has not previously been reported in a patient 17, 18. In our study the W1745C kindred includes a member with milder bleeding symptoms than the affected members and who did not carry the W1745C mutation. This individual did however have a mild concordant reduction in VWF levels and functional activity (including collagen binding). The variation in VWF levels in this family results partly from the difference in ABO blood group and also from the presence of the R760H mutation. Expression studies showed that although R760H results in decreased VWF production it has no effect on collagen binding or multimer distribution. Therefore we conclude that its presence does not alter the interpretation of the effects of W1745C and confirmed this by co-expression studies. The effect of R760H is consistent with the position of this mutation in the VWF propeptide, a part of the protein that directs post-translational modification and secretion. R760H has previously been reported in the VWF database (http://www.vwf.group.shef.ac.uk/index.html) but without description of the phenotype. R760C is also reported in association with the type 2N subtype, indicating that mutation at this residue can affect the factor VIII binding function of VWF that is contained in the adjacent D’ domain. The mechanism underlying the abnormalities seen with R760C has been analysed in previous expression studies 19. It appears that R760 is part of the recognition motif for furin-dependent cleavage of the propeptide which is impaired by this substitution. However the presence of His at P4 in the furin cleavage site has been reported to result in satisfactory processing 20. There was no evidence that recombinant R760H-VWF retained the propeptide. This suggests that the effect of mutation at R760 is more complex than previously thought and requires further analysis that is beyond the scope of this study. The mixture of phenotypes in one pedigree emphasizes the difficulty in attributing phenotypic changes to mutations in VWF on a highly heterogeneous background. Nonetheless in family 1
the most significant bleeding symptoms (BS=8) were present in member 1A who had reduced collagen binding associated with normal VWF:Ag and a normal RCo:Ag ratio.

In family 2 both members carry the S1783A mutant, both are blood group O and both have normal levels of VWF:Ag and VWF:RCo but reduced collagen binding with an normal multimer pattern. As both have increased bleeding scores this indicates that a clinically significant phenotype can be associated with a pure defect in collagen binding that is not caused by loss of high molecular weight multimers.

The third mutation (S1731T) has previously been reported in two individuals with moderate bleeding symptoms similar to those reported here. In our study there was variation in the clinical and laboratory phenotypes between family members, all of whom had S1731T. Two of the offspring did not report bleeding symptoms, including following tonsiloadenoidectomy, indicating that this mutation may be clinically silent in some cases. The two symptomatic individuals in this family had lower levels of VWF:Ag, VWF:RCo and VWF:CB than their two asymptomatic relatives indicating that the phenotype is likely to have a complex composition. It is not possible to distinguish the contributions of VWF level and reduced collagen binding activity to the symptoms but the milder phenotype is in keeping with the milder defect seen with S1731T-rVWF in the collagen binding studies compared with the other recombinant mutants.

The clinical importance of these mutations can be assessed by comparison of the bleeding scores. With the bleeding questionnaire used, a positive score is considered to be ≥ 4. All the individuals heterozygous for W1745C or S1783A had scores ≥ 4 with the highest values of 10 for 2D with S1783A and 8 for 1A with W1745C/R760H. In contrast the scores amongst those heterozygous for S1731T were 0-2. Although the numbers involved are too small for statistical analysis there does appear to be a correlation between bleeding score and laboratory phenotype, with the lowest ratios for collagen binding (CB:Ag 0.1 and 0.2 in 1A and 0.2 and 0.3 in 2D, see Fig. 1A) found in the individuals with the greater bleeding symptoms. Similarly the near normality of the laboratory phenotype in some of the affected individuals in family 3 correlates with the normality of the bleeding score.

In the original report the S1731T mutation was associated with reduced binding to type I collagen (CB:Ag ratio 0.12) Binding to type III collagen was not assessed but the results reported here show that it is similar to wild type rVWF (CB:Ag ratio 0.45). This finding implies that mutations affecting binding to both types of fibrillar collagen may be clinically more significant than those affecting binding to type I collagen only. It may also indicate more stringent structural requirements for binding type I compared to type III collagen. This may be of importance when considering the type of collagen that should be used in binding assays.
The A3 domain is considered to be the major binding site for types I and III collagen. However, a recent study demonstrated that A3-deficient VWF mutants could bind normally to collagen via the A1 domain under conditions of flow. Subsequently it has been shown that mutations in the A3 domain abolishing collagen binding did not impair the ability of VWF to correct the bleeding time in VWF-/- mice, although they did result in delayed thrombus formation after FeCl3 injury. Similarly, antibody-mediated inhibition of the interaction between VWF and collagen abolished thrombus formation in a baboon model of mechanical arterial injury. Our results indicate that isolated defects in the A3 domain are associated with abnormal haemostatic function in vivo in the presence of a normal A1 domain.

Identification of these mutations in patients with VWD can give useful insights into the structural requirements for the interaction between VWF and collagen. Fig. 2 shows the positions of the three mutations identified in the families on a crystal structure of the VWF A3 domain complexed with an antibody that prevents the binding of collagen. This model indicates that the A3 domain is a rectangular hexahedron with the stabilising C1686 – C1872 disulphide bond on one surface. The putative collagen binding surface is located on an adjacent side of the domain formed by three loops. It appears that the residues at the surface of the binding site form a shallow groove that can accommodate the triple helix of collagen microfibrils.

W1745 is in loop β3α2 in the centre of the RU5 antibody binding surface. The replacement of the large hydrophobic residue W1745 by a much smaller cysteine residue is likely to influence domain structure by affecting local packing and topology. The similar defect in collagen binding seen with W1745A indicates that it is not due to the provision of an alternative position for aberrant disulphide bond formation. Further consideration of the crystal structure of the VWF A3 domain showed that W1745 may participate in a hydrophobic interaction with Y1780, and we note that a previous study demonstrated that the mutation Y1780A also significantly reduced collagen binding. To test this hypothesis we expressed rVWF with the mutation W1745F and as predicted this did not significantly affect collagen binding (data not shown), supporting the hypothesis that W1745 and Y1780 participate in an internal aromatic interaction that helps to maintain the structural configuration of the A3 domain. The effect of W1745 on collagen binding was assessed by Vanhoorelbeke and colleagues with a separate monoclonal antibody (82D6A3) that also binds to the A3 domain and inhibits collagen binding. In their study it did not appear that W1745C formed part of the epitope recognised by the antibody but interacted with residues in that epitope via its side chain. Thus it appears that mutation at W1745 might affect collagen binding by both direct and indirect mechanisms.
S1783 is in loop α3β4, which also contains H1786. Mutation of the residues in these loops to alanine (S1783A and H1786A), has previously shown them to be critical residues that abolish collagen binding\(^{17}\). H1786 is expected to make direct contact with collagen but S1783 is not (Fig.2)\(^{18}\), so its effect is likely to be via perturbation of the loop as a whole. As serine and alanine differ only in the replacement of a hydroxyl group by a hydrogen in the side chain, this demonstrates that even minor changes in conformation of the loop cannot be easily tolerated. S1731 is part of a β-sheet that runs through the centre of the domain and is not solvent exposed. This residue is unlikely to make direct contact with collagen and any effects on collagen binding are probably via a secondary effect on the surface of the A3 domain. Secondary effects of this nature might well be more subtle and less deleterious than changes in residues directly involved in ligand binding, particularly as serine and the residue to which it is mutated in this case, threonine, have similar side chains.

The defects observed in this study would have been missed had collagen-binding function not been analyzed. Without the collagen binding assay these patients would have been misclassified with type 1 VWD (subjects 1A and 1B) or the diagnosis of VWD might have been missed altogether (subjects 2D and 2E). In our laboratory the PFA-100 is not used in the diagnosis of VWD as previous studies have demonstrated its limitations as a screening tool\(^{25,\ 26}\). It may be used to monitor the response to therapy in selected patients as was seen in this study. The requirement for collagen in the PFA-100 cassettes suggests that this could be a sensitive test for defects of collagen binding. However, the normal closure times obtained in subjects 1B and 2D indicate that this is not the case. Thus, correct identification of these mutations requires the use of a collagen binding assay in the initial diagnostic assessment of all patients with possible VWD. Furthermore, the preferential binding to specific collagen types shown with some of the defects raises the question as to what type(s) of collagen should be used in the laboratory measurement of collagen binding. Previous studies have shown that laboratories use a wide variety of different collagen types in the VWF:CB assay and that this has a significant effect on the result\(^{27}\). Collagen binding assays are useful in the detection of HMW multimers and it has been suggested that this is best achieved using a mixture of type I (95%) and type III (5%) collagen\(^{28}\). While the use of a single type or source of collagen may be sufficient for detecting abnormalities in multimer distribution, this approach may miss clinically important defects affecting affinity at the collagen-binding site. The issues of which collagen type is the most important in the clinical setting, and whether mixing collagen types in a single assay is appropriate for detecting loss of affinity at the collagen-binding site, remain unclear. Further evaluation of naturally-occurring mutations with correlation to clinical phenotype is therefore required.
Collagen binding assays are sometimes used as an alternative to multimer analysis because they are particularly sensitive to the absence of high-molecular weight multimers. In this study, multimer pattern was normal both in plasma samples and in expressed proteins. ELISA studies with expressed proteins showed that the defective collagen binding was caused by reduced affinity to collagen. An explanation for this observation was provided by modelling of the mutants in the crystal structure of the A3 domain which showed that they were involved in the putative collagen-binding site. These findings demonstrate that abnormalities in collagen binding require further investigation with multimer and genetic analysis.

In the recently updated ISTH-SSC VWD classification, isolated collagen binding defects are included within the type 2M subgroup.\textsuperscript{29} The impairment of the VWF-dependent adhesion of platelets to the endothelium in type 2M VWD occurs in the presence of a full range of VWF multimers. This is a key characteristic of the 2M subtype which is also a feature of the mutants described here. However, there are three important differences with the 2M group. Firstly, type 2M mutations are clustered in the A1 domain (residues 1260 – 1471) of VWF whereas these mutations affecting collagen binding are found in A3. Secondly, 2M is characterised by impaired binding to platelet GpIb\(\alpha\) as shown by a reduced ratio of RCo:Ag. All eight of the individuals in this study with collagen-binding mutations had normal RCo:Ag ratios with no evidence of a qualitative defect in GpIb-binding. Finally, one of the aims of the revised ISTH recommendations was to correlate VWD phenotype with clinical observations. An important feature of the 2M phenotype for clinical practice is that it tends to be associated with a poor functional response to DDAVP. The two patients in this study who received DDAVP demonstrated good functional responses. This included a rise in VWF:CB which was due to an overall increase in the amount of circulating VWF. The VWF secreted in response to DDAVP continued to show a qualitative defect in collagen binding as shown by a persistence of the abnormal CB:Ag ratio. Thus, there are important genotypic, phenotypic and therapeutic differences between the mutations described here and those previously included in the type 2M subgroup. The logical conclusion of these findings is to recommend that the classification of VWD is altered to acknowledge these differences. However, before doing so it is important to consider whether these mutations have a significant impact in clinical practice. It is clear from the increased bleeding scores in two of the pedigrees that defects at the collagen binding site are associated with clinically significant bleeding symptoms. Therefore we propose that these defects are classified as a distinct subtype, which may be designated type 2CB and characterised by defective collagen binding in the presence of normal multimer formation and normal GpIb binding. Recognition of this subtype will require the incorporation of collagen-binding assays in the first-line diagnostic assessment of patients with suspected VWD.
In conclusion this study demonstrates that mutations in the A3 domain of VWF causing reduction in collagen binding, independent of multimer composition, may give rise to clinically significant bleeding symptoms. The severity of the defect varies according to the specific mutation and is probably modified by other factors. This type of VWD is currently included in the 2M subtype but has important differences when compared with other mutations in this group. The use of VWF:RCo as the only functional assay will not detect this abnormality. It is therefore likely that defects at the collagen binding site are under-diagnosed and we propose that these defects are classified as a distinct subtype of VWD.

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Authorship and Conflict of Interest Statements

AFR, TAJM, GM, SG, and MS obtained laboratory data. KG, CM and SAB obtained clinical data. All authors were involved in analyses and interpretation of the results. KG, AFR, MAL, CM and TAJM wrote the report. All authors read, commented on, and approved the final version of the manuscript. All authors had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict-of-interest disclosure: The authors declare no competing financial interests.
Reference List


(9) Ribba AS, Loisel I, Lavergne JM et al. Ser968Thr mutation within the A3 domain of von Willebrand factor (VWF) in two related patients leads to a defective binding of VWF to collagen. *Thromb Haemost* 2001 September;86(3):848-54.


Figure legends

Figure 1

**Phenotypic data for patients and family members.** A) Table showing laboratory results. Reference values are given for each assay. Ratios of antigen to activity are shown alongside activity columns. VWF – von Willebrand factor; Ag – antigen; RCo – Ristocetin co-factor activity; CB – collagen binding activity; WT – wild type. Bleeding scores were calculated using the condensed MCMDM-1VWD questionnaire. B) Gels showing VWF multimer pattern from plasma samples. Grey bars are placed to maintain patient confidentiality. Patient ID or type of control is shown at the bottom of each lane. A sample from 3J was not available for electrophoresis.

Figure 2

**Ribbon diagram of the VWF A3 domain (grey) complexed with the RU5 antibody (green) that inhibits collagen binding (PDB Id: 1FE8).** The putative collagen binding surface is toward the lower left of the A3 domain in this view. The three naturally mutated residues are shown as blue sticks. H1786 is shown as a yellow stick.

Figure 3

**Expression of recombinant VWF mutants.** Recombinant wt and mutant VWF were transiently expressed in HEK293T cells and VWF ELISA was used to determine the concentration of secreted VWF (A) and VWF retained within the cell lysate (B). Error bars represent mean and SD of 3 separate experiments each performed in duplicate. (C) The multimer composition of recombinant VWF was analyzed in 1.3% agarose gels and visualized with HRP-conjugated anti-VWF polyclonal antibodies. RP indicates reference plasma. Lane 1, wt-rVWF; lane 2, S1731T; lane 3, W1745C; lane 4, W1745A; lane 5, wt/W1745C; lane 6, S1783A. All mutants are expressed at similar levels to wild type rVWF, with similar supernatant to lysate ratios and normal multimer distribution.

Figure 4

**CB:Ag ratio of recombinant VWF mutants.** Collagen binding assays to (A) type I collagen and (B) type III collagen were performed in parallel with VWF:Ag ELISA. Error bars represent mean and SD of 3 separate experiments performed in duplicate. Wild type recombinant VWF and H1786A, (a mutation previously shown to abolish collagen binding), are shown as controls. Only S1731T shows significant binding which is greater with collagen type III. Co-expression of wild type VWF with W1745C significantly improved binding to type III collagen.
Figure 5

**Binding isotherms of the collagen-VWF interactions.** Microtitre plates were coated with either type I (A) or type III (B) human collagen at a final concentration of 5µg/mL and incubated with decreasing concentrations of wild type or mutant recombinant VWF. As previously reported, H1786A shows no binding to either collagen type and only S1731T binds significantly to type 1 collagen.
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<th>Blood Group</th>
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**Figure 1B**
Figure 4

A

B

CB (I):Ag Ratio

CB (III):Ag Ratio

wtVWF
S1745C
S1745A
S1745C
S1731T
H1786A

wtVWF
S1745C
S1745A
S1745C
S1731T
H1786A
Figure 5

A

Abs $492\text{nm}$

$[\text{VWF}]\text{nM}$

- $\text{wtVWF}$
- $\text{S1731T}$
- $\text{W1745C}$
- $\text{W1745A}$
- $\text{S1783A}$
- $\text{H1786A}$

B

Abs $492\text{nm}$

$[\text{VWF}]\text{nM}$

- $\text{wtVWF}$
- $\text{wtW1745C}$
- $\text{S1731T}$
- $\text{W1745C}$
- $\text{W1745A}$
- $\text{S1783A}$
- $\text{H1786A}$
Characterisation of W1745C and S1783A: two novel mutations causing defective collagen binding in the A3 domain of von Willebrand factor

Anne F. Riddell, Keith Gomez, Carolyn M. Millar, Gillian Mellars, Saher Gill, Simon A. Brown, Megan Sutherland, Mike A. Laffan and Thomas A.J. McKinnon