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## ORIGINAL ARTICLE

# Identification of von Willebrand factor D4 domain mutations in patients of Afro-Caribbean descent: In vitro characterization

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## Abstract

**Background:** Von Willebrand disease was diagnosed in two Afro-Caribbean patients and sequencing of the VWF gene (VWF) revealed the presence of multiple variants located throughout the gene, including variants located in the D4 domain of VWF: p.(Pro2145Thrfs\*5) in one patient and p.(Cys2216Phefs\*9) in the other patient. Interestingly, D4 variants have not been studied often.

**Objectives:** Our goal was to characterize how the D4 variants p.(Pro2145Thrfs\*5) and p.(Cys2216Phefs\*9) influenced VWF biosynthesis/secretion and functions using in vitro assays.

**Methods:** Recombinant VWF (rVWF), mutant or wild-type, was produced via transient transfection of the human embryonic kidney cell line 293T. The use of different tags for the wild-type and the mutant allele allowed us to distinguish between the two forms when measuring VWF antigen in medium and cell lysates. Binding of rVWF to its ligands, collagen, factor VIII, ADAMTS13, and platelet receptors was also investigated.

**Results:** Homozygous expression of the p.(Cys2216Phefs\*9)-rVWF mutation resulted in an almost complete intracellular retention of the protein. Heterozygous expression led to secretion of almost exclusively wild-type-rVWF, logically capable of normal interaction with the different ligands. In contrast, the p.(Pro2145Thrfs\*5)-rVWF exhibited reduced binding to type III collagen and  $\alpha$ IIb $\beta$ 3 integrin compared to wild-type-rVWF.

**Conclusions:** We report two mutations of the D4 domains that induced combined qualitative and quantitative defects.

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**KEYWORDS**

binding assays, intracellular retention, recombinant proteins, von Willebrand disease, von Willebrand factor

**Essentials**

- Gene variants identified in von Willebrand factor/D4 domain have been rarely duly characterized.
- Two D4 domain VWF variants display different effects on protein synthesis.
- The p.(Cys2216Phefs\*9) variant results in strongly reduced, barely detectable expression levels.
- The p.(Pro2145Thrfs\*5) variant displays dominant-negative effects, affecting ligand binding.

**1 | INTRODUCTION**

Von Willebrand factor (VWF) is a plasmatic multimeric glycoprotein exclusively produced by endothelial cells and megakaryocytes.<sup>1</sup> VWF plays an important role in primary hemostasis, mediating platelet adhesion and aggregation at the site of vascular lesions. In the circulation, VWF is also important for the protection of coagulation factor VIII (FVIII) from premature clearance.<sup>2,3</sup> The architecture of the VWF protein consists of a succession of different structural domains according to the sequence D1-D2-D'-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK, in which D1-D2 domains represent the VWF propeptide and the remaining domains (D' to CK), the mature VWF subunit.<sup>4</sup> Over the years, a number of functional binding sites have been identified on the VWF subunit. These include the binding sites for factor VIII (FVIII) (domains D'-D3), for platelet glycoprotein (Gp) Iba and collagen type VI (A1 domain), for collagens types I and III (A3 domain), for A Disintegrin And Metalloprotease with ThromboSpondin motif repeats 13 (ADAMTS13) (D4 domain) and for platelet GpIb/IIIa (C4 domain).<sup>5,6</sup> A cleavage site for the metalloprotease ADAMTS13 is also present on the A2 domain of VWF and is crucial for the regulation of the multimer size.<sup>7,8</sup>

Von Willebrand disease (VWD) is characterized by a bleeding tendency marked by either qualitative or quantitative defects of VWF and caused by mutations on the VWF gene (VWF) sequence.<sup>9,10</sup> VWD-type 1 (60%–80% of all VWD) and VWD-type 3 patients are characterized by a partial or complete deficiency in plasma VWF respectively. VWD-type 3 is transmitted as either a recessive trait or through the inheritance of 2 mutant codominant alleles. VWD-type 2 represents qualitative disorders and can be divided into four subtypes: 2A, 2B, 2M, and 2N, according to the VWF function perturbed by the mutation.

Because VWD is a heterogeneous disease, accurate identification and characterization of disease-causing variants through Sanger sequencing and next generation sequencing is important to formally diagnose VWD and to understand the molecular mechanisms contributing to pathogenesis.<sup>11,12</sup> In addition, functional analyses using recombinant proteins are also key to establish the causal relationships between sequence variants and patients' phenotype and to guide clinical decisions. Such studies have largely contributed to improve our knowledge about VWF

structure-function relationships and recombinant VWF (rVWF) variants in virtually every domain have been produced and studied.<sup>13</sup> However, most studies have focused on the N-terminal half of the VWF subunit (D' to A3 domains) and relatively few on the C-terminal half (D4 to CK domain), resulting in a relatively poor knowledge of these domains. In the D4 domain for example, numerous VWF sequence variations have been identified, leading to VWD-type 1, 2A, 2M, or even type 3.<sup>14–17</sup> However, very few of these variants have been expressed in *in vitro* systems and duly characterized.<sup>13</sup>

In the present study, taking into account the geographic specificities of VWF mutations,<sup>18</sup> we set out to investigate the effect of two D4 mutations, the c.6647del deletion and the c.6432dup duplication, both resulting in premature termination of protein synthesis and respectively identified in unrelated Afro-Caribbean patients with VWD-type 1 and 3, on VWF function and biosynthesis.

**2 | MATERIAL AND METHODS****2.1 | Patients**

Two patients carrying a mutation located in VWF and responsible for VWD were enrolled in this study after informed written consent in accordance with the Declaration of Helsinki. The study was approved by our institutional review board. The patients (P1 and P2) are two women respectively aged 90 and 40 years, both with bleeding symptoms.

**2.2 | Plasma samples**

Peripheral blood samples were collected in tubes containing 3.2% sodium citrate. After leaving the samples at room temperature for 30 min, they were centrifuged (15 min, 2500g) and plasma was collected and frozen at  $-80^{\circ}\text{C}$ . Plasma was analyzed for factor VIII (FVIII) procoagulant activity (FVIII:C) using a chromogenic assay. VWF antigen (VWF:Ag) levels were determined using a particle-based turbidimetric assay, and VWF ristocetin cofactor activity by platelet aggregometry.

## 2.3 | Plasmid construction

The expression vectors pcDNA3.1-hVWF-Myc and pcDNA3.1-hVWF-HA encoding full-length VWF cDNA were kindly provided by Dr. Eikenboom (Leiden University Medical Center, Leiden, The Netherlands) and have been previously described.<sup>19</sup> A HiFi DNA assembly strategy (Kit E5520; New England Biolabs) was used to modify the C-terminal region of hVWF. Mutagenic primers were used to generate the hVWF variants according to the modifications induced by the p.(Cys2216Phefs\*9) and the p.(Pro2145Thrfs\*5) mutations (Figure 1). In the mutated hVWF, the premature stop codon was relocated downstream of the HA-tag sequence, allowing for the distinction between the wild-type (WT) form (Myc-tagged) and the mutated forms (HA-tagged). The correct modifications of the expression vectors were verified by sequencing. Plasmid purification was performed using the NucleoBond PC 500 EF kit (Macherey-Nagel).

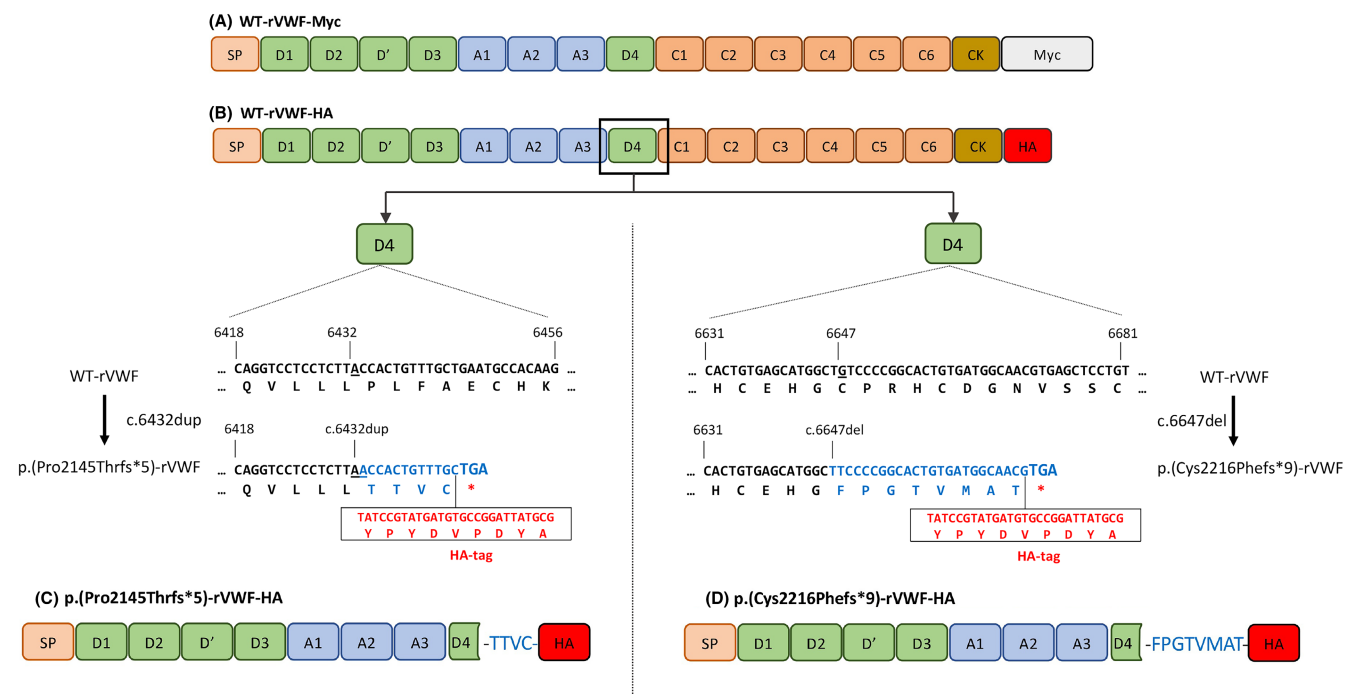
## 2.4 | Transfection experiments

Human embryonic kidney 293T (HEK293T) cells were grown in complete medium (Dulbecco's Modified Eagle's medium [DMEM]:F12 supplemented with 10% fetal bovine serum and 1% antibiotic cocktail at 37°C and 5% CO<sub>2</sub>).

A total of 3 µg of mutant or WT full-length VWF cDNA was used for transient transfection of HEK293T cells ( $2 \times 10^6$ ) by liposomal transfer using Lipofectamine 2000 kit (Thermo Fisher Scientific) according to the manufacturer's instructions. For WT and mutant co-transfections (heterozygous), 1.5 µg of each vector was used. Cell media and lysate were collected after 72 h. Cells were lysed with lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% 21 Nonidet, pH 8). Total rVWF antigen levels (VWF:Ag) from cellular supernatants and intracellular lysates were determined by enzyme-linked immunosorbent assays (ELISA) as described.<sup>20</sup> To determine respective quantities of Myc-tagged or HA-tagged rVWF, we used horseradish peroxidase (HRP) anti-Myc tag antibody (Ab1326; Abcam) or anti HA tag antibody (Ab1190, Abcam) as secondary antibodies. In all cases, bound antibody was detected with TMB (3,3', 5,5'-tetramethylbenzidine [Tebu-bio]). Absorbance was measured at 450 and 570 nm.

## 2.5 | Binding assays

For VWF binding tests, rVWF secreted in the supernatant was concentrated 4.5- to 21-fold, using Aquacide II (Calbiochem) and dialyzed against 25 mM Tris-HCl containing 150 mM NaCl, pH 7.4. The different binding assays were performed as described.<sup>21</sup> Briefly, for collagen assays, human collagen type III (Tebu-Bio) was coated on



**FIGURE 1** Overview of the VWF variants used in the study. Domain organization for WT-rVWF-Myc (A), WT-rVWF-HA (B), p.(Pro2145Thrfs\*5)-rVWF-HA (C), and p.(Cys2216Phefs\*9)-rVWF-HA (D). Nucleotide insertion (position 6432) or deletion (position 6647) is underlined in the nucleotide sequence. Effects of the nucleotide insertion (c.6432dup) or deletion (c.6647del) on the amino-acid sequence are depicted in blue. The WT sequence of human HA-tagged VWF contained in the pcDNA3.1 vector was used as template. Mutagenic primers were designed to generate the two constructs p.(Pro2145Thrfs\*5)-rVWF and p.(Cys2216Phefs\*9)-rVWF in pcDNA3.1 using a HiFi cloning strategy. To allow for the specific detection of the truncated VWF chain, the HA tag sequence (in red) was located upstream of the premature stop codon resulting from the frameshift

microtiter plates at 2 µg/ml and serial dilutions of rVWF samples (0–1 µg/ml) were added.

For Gplbα binding assays, 5 µg/ml of recombinant human (rhG-plbα) (Bio-Techne) was immobilized via a homemade monoclonal antibody to Gplbα. rVWF proteins diluted 0–1 µg/ml and containing 10 µg/ml of laboratory-purified botrocetin, were added to the wells.

For GplIbIIIa binding assays, 10 µg/ml human platelet-purified GplIbIIIa (Enzyme Research Laboratories, Swansea, UK) was immobilized via a homemade monoclonal antibody to GplIbIIIa. Serial dilutions or rVWF (0–2.5 µg/ml) were added.

For binding to ADAMTS13, recombinant human ADAMTS13 (Bio-Techne) was coated on microtiter wells at a concentration of 2 µg/ml and serial dilutions or rVWF (0–2.5 µg/ml) were added. In all of these tests, bound-rVWF was detected using rabbit anti-VWF-HRP antibodies (Dako, Agilent).

For FVIII binding, rVWF preparations (0–1 µg/ml) were coated using a polyclonal rabbit anti-VWF antibody (Dako). After the washes, 0.35 M CaCl<sub>2</sub> was added to the wells. This step was repeated twice for 10 min and was followed by six washes. Then, 60 mU/well of diluted rFVIII (Kogenate-FS, Bayer Healthcare) was added and incubated for 2 h at 37°C. After washing, the amount of bound FVIII was measured with an HRP-coupled polyclonal anti-FVIII antibody (Diagnostica Stago). Absorbance was measured at 450 nm. After carrying out several successive washes, the relative amount of rVWF immobilized in each well was also detected using rabbit polyclonal anti-VWF-HRP antibodies. The results are expressed as the ratio of the slope of the mutated rVWF-FVIII binding curve to the WT-rVWF binding slope.

## 2.6 | Multimers

VWF multimeric profiles were analyzed by 2% SDS agarose gel electrophoresis followed by immunoblotting. The membrane was probed by incubation with a homemade polyclonal goat antibody to VWF coupled to alkaline phosphatase followed by staining with alkaline phosphatase conjugate substrate kit (Bio-rad).

## 2.7 | Statistical analysis

Data are expressed as mean plus or minus standard deviation (SD) values ( $n = 3$  independent experiments) using Prism 7 software (GraphPad). Statistical analyses were also performed using Prism 7 software. A one-way ANOVA followed by a post-hoc Dunnett correction test was performed to make comparisons between the mutants and wild-type rVWF.  $p$  values  $<0.05$  were considered significant.

## 3 | RESULTS

Clinical and laboratory phenotypic data are summarized in Table 1. Both patients are of the O<sup>+</sup> blood group.

**TABLE 1** Phenotypic and genotypic data of two VWD subjects

| Patient | Nucleotide change | Amino acid change  | Mode of inheritance           | Mutation pathogenicities | Blood group | FVIII:C (%) | VWF:Ag (%) | VWF:Rco (%) | VWFpp (%) | VWF:FVIIIIB | Multimers                            |
|---------|-------------------|--------------------|-------------------------------|--------------------------|-------------|-------------|------------|-------------|-----------|-------------|--------------------------------------|
| 1       | c.2220G>A         | p.(Met740Ile)      | Heterozygous <sup>22</sup>    | Benign                   | O+          | 33          | 35–50      | 30          | NT        | 56%         | All present but moderately decreased |
|         | c.2451T>A         | p.(His817Gln)      | Heterozygous <sup>24,40</sup> | Benign                   |             |             |            |             |           |             |                                      |
|         | c.3258C>T         | p.(Tyr1086=)       | Heterozygous <sup>ND</sup>    | ND                       |             |             |            |             |           |             |                                      |
|         | c.4414G>C         | p.(Asp1472His)     | Homozygous <sup>41</sup>      | Benign                   |             |             |            |             |           |             |                                      |
|         | c.6554G>A         | p.(Arg2185Gln)     | Heterozygous <sup>23</sup>    | Benign                   |             |             |            |             |           |             |                                      |
|         | c.6647del         | p.(Cys2216Phefs*9) | Heterozygous <sup>ND</sup>    | ND                       |             |             |            |             |           |             |                                      |
|         | c.6859C>T         | p.(Arg2287Trp)     | Heterozygous <sup>42</sup>    | Benign                   |             |             |            |             |           |             |                                      |
|         | c.1530G>A         | p.(Val510=)        | Heterozygous <sup>18</sup>    | Damaging                 | O+          | 2–3         | 1–5        | 5           | 6         | NT          | Total absence                        |
| 2       | c.1892C>T         | p.(Ala631Val)      | Heterozygous <sup>23</sup>    | Benign                   |             |             |            |             |           |             |                                      |
|         | c.2220G>A         | p.(Met740Ile)      | Heterozygous <sup>22</sup>    | Benign                   |             |             |            |             |           |             |                                      |
|         | c.2451T>A         | p.(His817Gln)      | Heterozygous <sup>24,40</sup> | Benign                   |             |             |            |             |           |             |                                      |
|         | c.3445T>C         | p.(Cys1149Arg)     | Heterozygous <sup>35,43</sup> | Damaging                 |             |             |            |             |           |             |                                      |
|         | c.4414G>C         | p.(Asp1472His)     | Homozygous <sup>41</sup>      | Benign                   |             |             |            |             |           |             |                                      |
|         | c.6432dup         | p.(Pro2145Thrfs*5) | Heterozygous <sup>18</sup>    | Damaging                 |             |             |            |             |           |             |                                      |
|         | c.6554G>A         | p.(Arg2185Gln)     | Heterozygous <sup>23</sup>    | Benign                   |             |             |            |             |           |             |                                      |

Abbreviations: FVIII:C, factor VIII coagulant activity; ND, not described; NT, not tested; VWF:Ag, von Willebrand factor antigen; VWF:FVIIIIB, VWF binding to FVIII; VWF:Rco, ristocetin cofactor activity.

Patient 1 showed reduced VWF levels (VWF:Ag = 35%, VWF:ristocetin cofactor activity = 30%) as well as a reduced FVIII:C (33%). Such parallel decreases were evocative of VWD-type 1. The reduced binding to FVIII (VWF: FVIIIb) (56%) may suggest a 2N heterozygous status, resulting from the presence of the p.(His817Gln) variant.<sup>22</sup> However, and although this variant is known to decrease FVIII:C levels by 18 IU/dl per allele, it has been identified in a significant proportion of healthy individuals from African ancestry, allowing its classification as a benign variant in this population as indicated in Table 1.<sup>23,24</sup> After VWF sequencing, seven sequence variations (six heterozygous and one homozygous) were identified in the coding region, with two new variants: (1) the synonymous variant p.(Tyr1086 =) and (2) p.(Cys2216Phefs\*9), a mutation causing a shift of the reading frame by nine amino acids and the appearance of a premature stop codon, resulting in a truncated VWF protein missing part of the D4 domain and the C-terminal end of the molecule. The five other variants p.(Met740Ile), p.(His817Gln), p.(Asp1472His), and p.(Arg2185Gln), p.(Arg2287Trp) previously described as no or little deleterious and identified in healthy populations were also detected in this patient. This patient was classified as having VWD-type 1 with status of transmitter of VWD-type 3. Her bleeding score was 0 but she required Helixate (recombinant FVIII) treatment before and after receiving surgery. The patient is now deceased.

Patient 2 had severely reduced VWF levels, with a total absence of multimers evaluated by electrophoresis. Her biological profile was reminiscent of VWD-type 3. Three heterozygous sequence variations potentially deleterious (p.(Val510=), p.(Cys1149Arg) and p.(Pro2145Thrfs\*5)) were identified upon sequencing of VWF. The family study showed that the sequence variations p.(Cys1149Arg) and p.(Pro2145Thrfs\*5) are on different alleles. In addition to these three variants, five not detrimental variants (p.(Ala631Val), p.(Met740Ile), p.(His817Gln), p.(Asp1472His), and p.(Arg2185Gln)) were also detected. Her bleeding score was 32 with numerous bleeding episodes: epistaxis, bruising, oral cavity bleeding, prolonged bleeding from minor wounds, menorrhagia, hemarthrosis, and ankle arthropathy.

Although both patients were unrelated and exhibiting different phenotypes/severity, they were both from Afro-Caribbean origin and both exhibiting a frameshift mutation p.(Cys2216Phefs\*9) for patient 1 and p.(Pro2145Thrfs\*5) for patient 2 leading to a truncated VWF protein from the D4 domain onward. This similarity prompted us to study these two mutations in *in vitro* systems.

### 3.1 | Characterization of rVWF

The influence of the D4 mutations on VWF synthesis and/or secretion was measured *in vitro* after transient transfection of c.6647del (leading to the p.(Cys2216Phefs\*9) variant) and the c.6432dup (leading to the p.(Pro2145Thrfs\*5) variant) into HEK293T cells. Co-transfections were also performed to see the impact of these variants in a heterozygous status (transfections were performed with equimolar amounts of mutant VWF cDNA and WT-VWF cDNA). Of note, the HEK293T cells that we used throughout the study are

not making Weibel-Palade bodies, in contrast to HEK293 cells. The HEK293T cells have been previously used to examine secretion and/or intracellular retention of VWF<sup>25</sup> but cannot provide information on regulated storage of VWF.

Analysis of the culture supernatant collected 72 h after transfection showed a concentration of 1.2 µg/ml, in cells expressing WT-rVWF. In contrast, the p.(Pro2145Thrfs\*5)-rVWF variant exhibited strongly reduced secretion with VWF:Ag 0.12 µg/ml ( $p < 0.0001$  vs. WT), whereas the p.(Cys2216Phefs\*9)-rVWF variant was virtually absent ( $p < 0.0001$  vs. WT) (Figure 2A). After co-transfection of the VWF mutants with WT-VWF, a partial restoration of antigen levels was obtained: we respectively measured 0.53 µg/ml of VWF:Ag for p.(Pro2145Thrfs\*5)-rVWF and 0.28 µg/ml for p.(Cys2216Phefs\*9)-rVWF (Figure 2A). These values remained significantly lower than WT-rVWF ( $p < 0.0001$  vs. WT for both variants in the heterozygous state).

The intracellular concentration of rVWF obtained from cell lysates was also quantified (Figure 2B). In the homozygous transfections, both mutants exhibited a significant reduction of the intracellular VWF:Ag. After co-transfection with WT-VWF, the heterozygous variant p.(Pro2145Thrfs\*5)-rVWF was no longer different from WT-rVWF (0.23 µg/ml for both,  $p = 0.99$ ), whereas the heterozygous p.(Cys2216Phefs\*9)-rVWF was still significantly reduced (0.16 µg/ml,  $p = 0.02$ ) compared with WT-rVWF.

Measurement of the ratio supernatant/lysate (Figure 2C) of all mutants, both homozygous and heterozygous showed values lower than that of WT indicating some intracellular retention of the rVWF mutants.

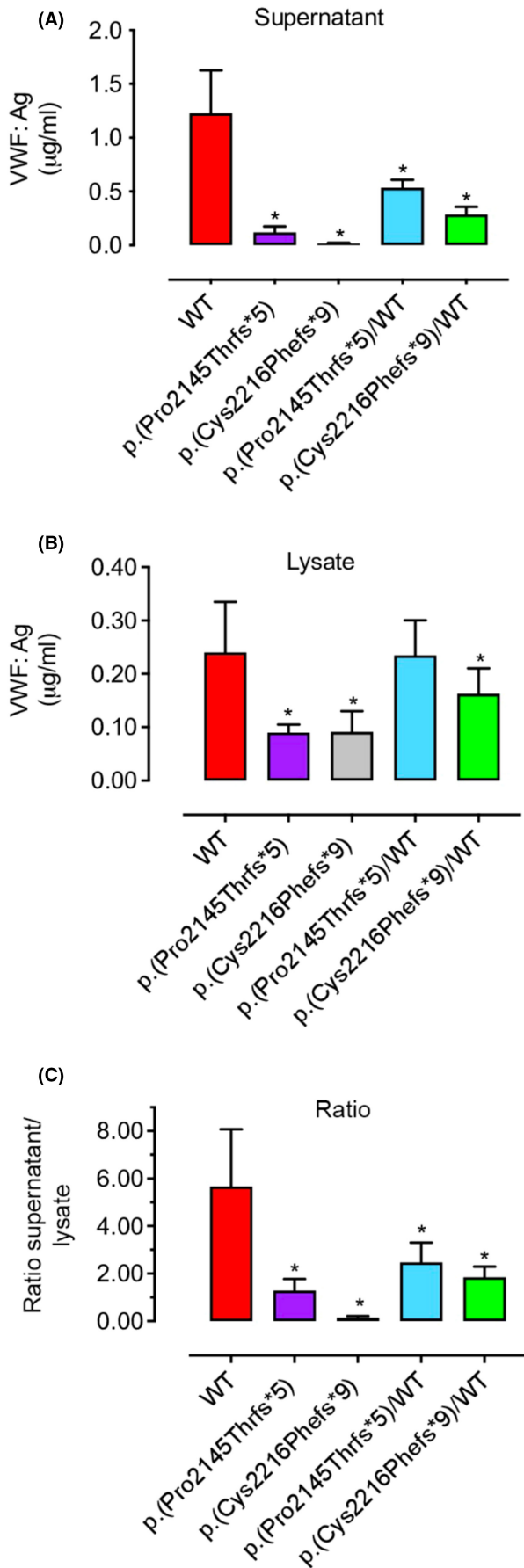
### 3.2 | Specific detection of WT and mutant VWF

Next, we aimed to measure the relative quantities of WT and mutant VWF in the total amount of secreted VWF. For this, we used specific ELISA assays. The Myc-tag was used to detect WT-rVWF, whereas the HA-tag was used to detect the mutant forms. As control, a co-transfection performed with WT-VWF-HA cDNA and WT-VWF-Myc cDNA was performed. Using this control, a perfect distribution of 50% of each form was obtained (Figure 3).

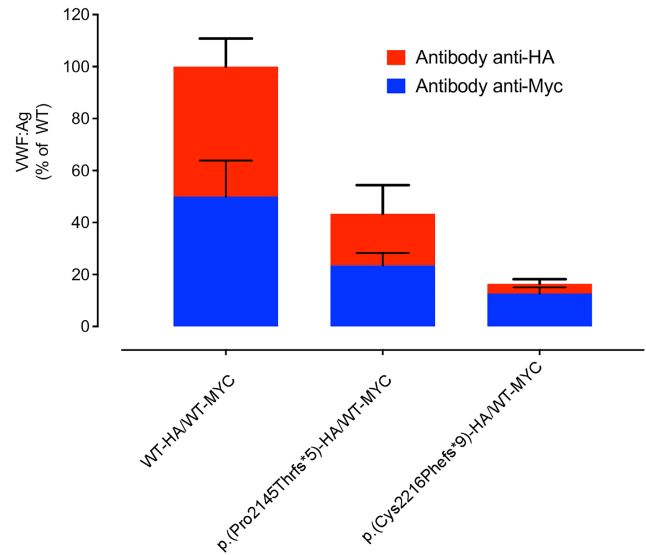
For the heterozygous mutant p.(Pro2145Thrfs\*5)-rVWF, we also observed a similar distribution with equal proportions of the WT and mutant forms, but the total amount of VWF secreted was reduced compared with WT-rVWF. For the heterozygous p.(Cys2216Phefs\*9)-rVWF variant, we observed a distribution of 75% and 25% between the WT-rVWF form and the mutated form, indicating specific intracellular retention of the mutant protein. Furthermore, the total amount of secreted VWF:Ag was strongly reduced, confirming results reported in Figure 2.

### 3.3 | Multimeric profiles

Secreted rVWF multimers were analyzed by electrophoresis to determine whether the coexpressed mutants induced alterations in



**FIGURE 2** In vitro expression study of rVWF. VWF:Ag levels were quantified by ELISA in the cell media (A) and lysate (B). Homozygous transfections with mutant VWF or heterozygous transfections mutant/WT-VWF were performed in HEK293T cells and medium or lysate was collected 72 h after transfection. (C) VWF:Ag ratio in supernatants over lysates. Results represent three different experiments performed in triplicates. The mean  $\pm$  SD values were compared using a one-way ANOVA and Dunnett posttest; \* $p < 0.05$



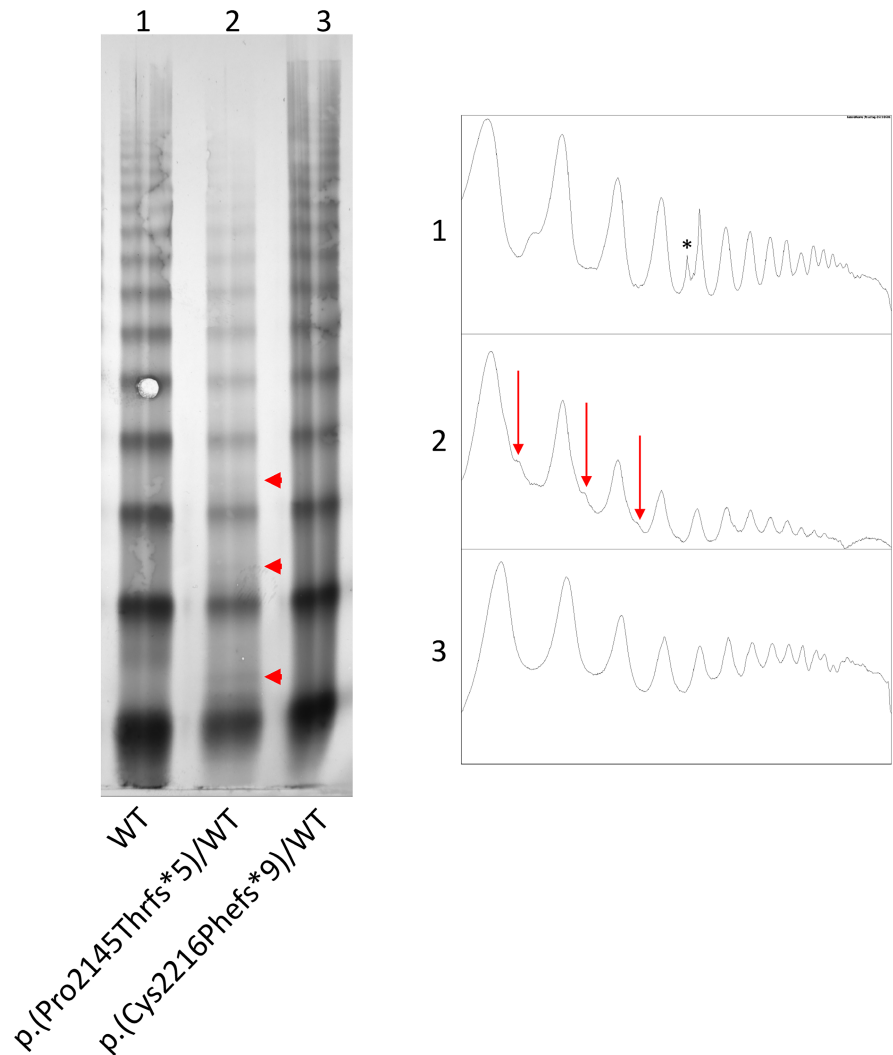
**FIGURE 3** Relative expression of each rVWF allele in cell medium after co-transfection. Co-transfections of HEK293T cells were performed using pcDNA3.1-VWF-Myc and pcDNA3.1-VWF-HA plasmids with the HA-tagged plasmid encoding mutant rVWF. Seventy-two hours after transfection, cell medium was collected and tag-specific ELISA assays were performed. Results represent three different experiments performed in triplicates. The mean  $\pm$  SD values were compared using a one-way ANOVA and Dunnett posttest; \* $p < 0.05$

the structure of VWF (Figure 4). The multimeric profile of mutant p.(Cys2216Phefs\*9)-rVWF was normal. For the p.(Pro2145Thrfs\*5)-rVWF variant, the apparent loss of the highest molecular weight multimers may be attributed to a lower quantity of antigen loaded on the gel. The most striking feature is the presence of a small additional band above the dimers and tetramers for the p.(Pro2145Thrfs\*5)-rVWF mutant (arrowheads), suggesting that odd multimers formed by WT and shorter polymers are indeed produced and released, as indicated by the tag-specific quantification method. A small shoulder on the densitometry analysis is also confirming the presence of these additional bands (red arrows) (Figure 4).

### 3.4 | Binding of recombinant mutant VWF to various ligands

To allow functional analysis of mutants, the transfection supernatants containing rVWF were concentrated 4.5-fold for WT, 10-fold

**FIGURE 4** Multimer pattern of mutants and WT-rVWF obtained from heterozygous expression in HEK293T. Red arrowheads indicate odd bands visible in heterozygous transfections of p.(Pro2145Thrfs\*5)-rVWF/WT-rVWF. Densitometric analysis was performed using the Gels tool of ImageJ software. Red arrows indicate odd peaks corresponding to uneven bands. Of note, the extra band that is visible in the densitometric analysis of sample 1 corresponds to a bubble artefact (asterisk)



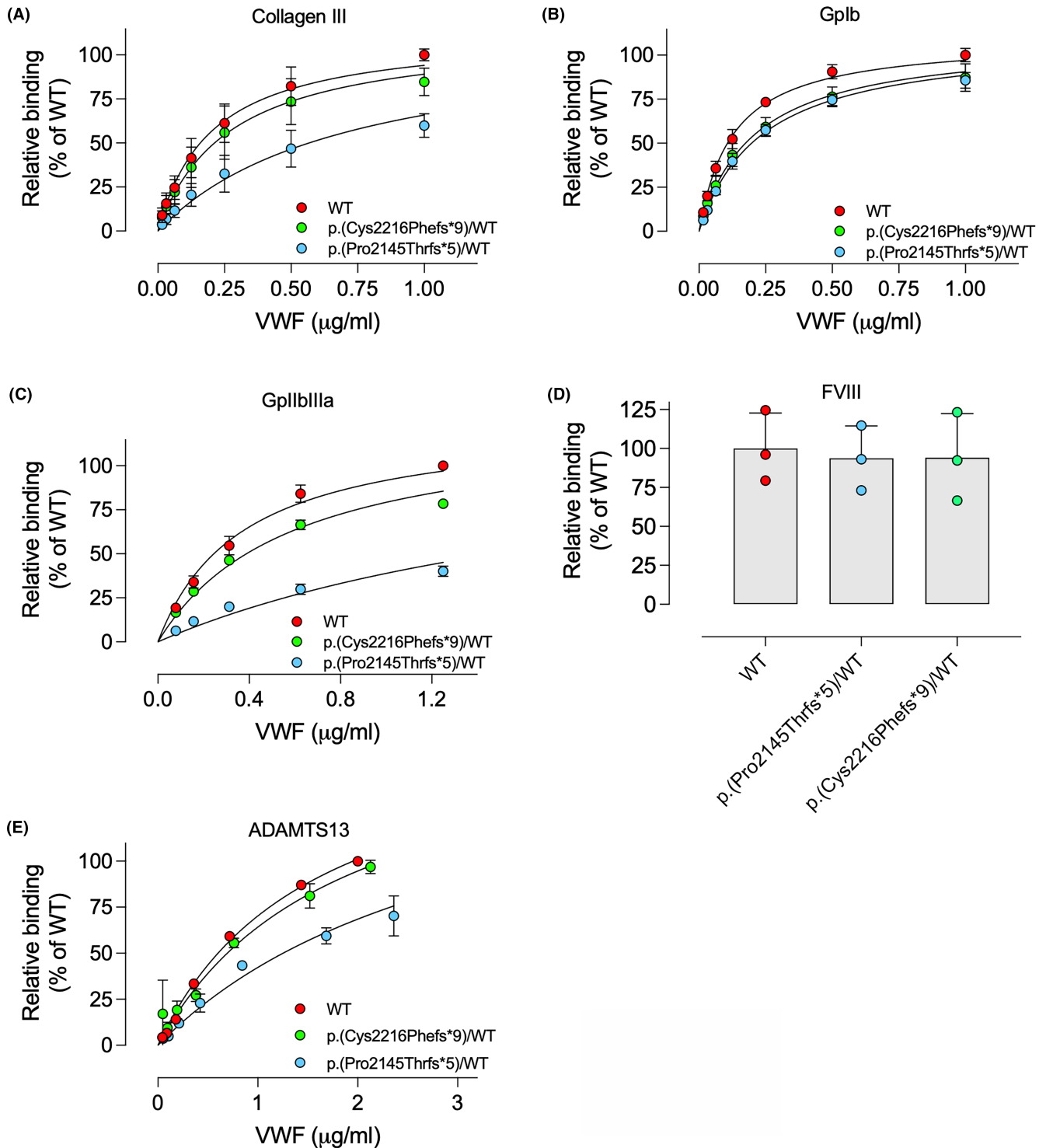
for p.(Pro2145Thrfs\*5)-rVWF and 21-fold for p.(Cys2216Phefs\*9)-rVWF to obtain similar rVWF concentrations in all samples. Following concentration and dialysis, rVWF:Ag were 2.87  $\mu\text{g/ml}$  for WT-rVWF, 3.04  $\mu\text{g/ml}$  for p.(Cys2216Phefs\*9)-rVWF and 3.37  $\mu\text{g/ml}$  for p.(Pro2145Thrfs\*5)-rVWF. Of note, binding tests were carried out only for heterozygous rVWF, better reflecting the patients' situation. We first measured the ability of mutant rVWF to bind to collagen type III and normalized our results to WT-rVWF. While the p.(Cys2216Phefs\*9)-rVWF variant exhibited normal binding to collagen type III, the p.(Pro2145Thrfs\*5)-rVWF variant displayed significantly reduced binding (half-maximum binding 0.71  $\pm$  0.13  $\mu\text{g/ml}$ ) (Figure 5A and Table 2) compared with WT-rVWF (half-maximum binding 0.20  $\pm$  0.04  $\mu\text{g/ml}$ ;  $p = 0.0006$ ). To examine the effect of mutations on platelet GpIb $\alpha$  binding, increasing concentrations of rVWF were applied to immobilized rhGpIb $\alpha$  in the presence of 10  $\mu\text{g/ml}$  botrocetin (Figure 5B). Both heterozygous variants displayed slightly but significantly decreased affinity for GpIb $\alpha$  (Table 2). Binding to GpIIb/IIIa revealed a strong reduction in binding for the p.(Pro2145Thrfs\*5)-rVWF variant (half-maximum binding 2.23  $\pm$  0.24  $\mu\text{g/ml}$ ) compared with WT-rVWF (half-maximum binding 0.37  $\pm$  0.04  $\mu\text{g/ml}$ ;  $p < 0.0001$ )

(Figure 5C). Binding of the p.(Cys2216Phefs\*9)-rVWF variant was only slightly decreased and no different compared with WT-rVWF ( $p = 0.191$ ). No defect in FVIII binding was detected for either variant (Figure 5D). Finally, binding to ADAMTS13 was tested and the p.(Pro2145Thrfs\*5)-rVWF displayed significantly reduced binding (half-maximum binding of 3.26  $\mu\text{g/ml}$ ) compared to WT-rVWF (half-maximum binding of 1.56  $\mu\text{g/ml}$ ) ( $p = 0.0001$ ) (Figure 5E). In contrast, the p.(Cys2216Phefs\*9)-rVWF variant did not display any reduced binding to ADAMTS13 (half-maximum binding of 1.80  $\mu\text{g/ml}$ ) compared with WT-rVWF ( $p = 0.32$ ) (Table 2).

## 4 | DISCUSSION

In this study, we describe a frameshift gene variant within the VWF D4 domain completely preventing VWF secretion potentially because of mRNA degradation and protein retention. Alternatively, for a second such variant resulting in residual protein secretion, a dominant-negative effect of VWF can be observed and leads to a more severe phenotype.





**FIGURE 5** Binding of heterozygous mutants and WT-rVWF to different VWF ligands. Binding of VWF mutants to collagen III (A), Gplb (B), GplIbIIIa (C), FVIII (D), and ADAMTS13 (E). Results are normalized and represent three different experiments performed in triplicates. Means  $\pm$  SD values are presented

Since the cloning of VWF in 1985,<sup>26</sup> hundreds of sequence variations of VWF have been identified in VWD patients.<sup>14-17</sup> Expression of VWF variants in heterologous cellular systems have also greatly contributed to elucidating pathological mechanisms as well as improving our knowledge on the roles of VWF domains. Although missense mutations have represented the bulk of published expression

findings,<sup>13</sup> a few studies have undertaken analysis of other VWF genetic alterations such as frameshift mutations,<sup>27</sup> splice site mutations,<sup>28-30</sup> or deletions mutants.<sup>31-34</sup> Even fewer studies have focused on genetic alterations identified in the D4 domain of VWF. We therefore decided to characterize two such genetic defects present in two patients of Afro-Caribbean origin, a deletion of a nucleotide

**TABLE 2** Half-maximum binding values ( $\mu\text{g}/\text{ml}$ ) for binding of WT-VWF and heterozygous mutants to the different ligands

|               | WT              | p.(Cys2216Phefs*9)/WT            | p.(Pro2145Thrfs*5)/WT            |
|---------------|-----------------|----------------------------------|----------------------------------|
| Collagen      | 0.20 $\pm$ 0.04 | 0.27 $\pm$ 0.05 ( $p = 0.524$ )  | 0.71 $\pm$ 0.13 ( $p = 0.0006$ ) |
| Gplb $\alpha$ | 0.13 $\pm$ 0.01 | 0.21 $\pm$ 0.02 ( $p = 0.0024$ ) | 0.23 $\pm$ 0.02 ( $p = 0.0007$ ) |
| GplIbIIIa     | 0.37 $\pm$ 0.04 | 0.59 $\pm$ 0.07 ( $p = 0.191$ )  | 2.23 $\pm$ 0.24 ( $p < 0.0001$ ) |
| ADAMTS13      | 1.56 $\pm$ 0.06 | 1.80 $\pm$ 0.23 ( $p = 0.320$ )  | 3.26 $\pm$ 0.26 ( $p = 0.0001$ ) |

in position 6647 leading to a frameshift variant p.(Cys2216Phefs\*9) and a duplication of a nucleotide in position 6432, also leading to a frameshift alteration p.(Pro2145Thrfs\*5). These patients display a number of other amino acid changes previously reported (Table 1); some little or no deleterious (for patient 1), whereas some potentially pathogenic such as p.(Val510=) and p.(Cys1149Arg) for patient 2.<sup>18,35</sup>

Because of the presence of a premature termination codon (PTC), the mutations p.(Cys2216Phefs\*9) and p.(Pro2145Thrfs\*5) can potentially lead to nonsense-mediated mRNA decay (NMD) and therefore the mutant allele would not be expressed in the patient. Indeed, PTC-introducing mutations in VWF were shown to be associated with NMD but this mechanism was found to be PTC position-dependent and the extent of degradation was not 100%.<sup>36</sup> Therefore, it is almost impossible to predict if and how the NMD is associated with a specific VWF variant, without performing cellular experiments.

This aspect prompted us to express WT and mutant cDNA with a specific tag for both variants to quantify the relative amount of both proteins. Before performing co-transfections, we first tested whether any homozygous mutant protein could be produced and secreted. Some protein expression was measured in cell lysates suggesting that at least part of the mRNAs were not degraded. However, virtually no VWF was secreted in the medium (Figure 2A-B), suggesting that protein retention was also involved. Co-transfection with WT cDNA led to a partial correction of protein expression in both cell medium and cell lysate; however, some differences could still be observed. Tag-specific ELISA assays were then used to specifically assess which VWF-form was being expressed preferentially. To validate this approach, we first used two differentially tagged WT-cDNA, and we obtained a perfect 50:50 repartition of rVWF in cell culture medium, showing that both plasmids allow similar expression level. Considering that the same cells were very likely transfected with both plasmids as shown previously,<sup>14</sup> this result indicates that multimers are made of an equal repartition of HA-tagged subunits and Myc-tagged subunits.

Knowing the relative expression of the mutant forms in the co-transfections (ie, 46% for p.(Pro2145Thrfs\*5) and 23% for p.(Cys2216Phefs\*9)) is of critical importance for interpretation of the binding assays. Indeed, for p.(Cys2216Phefs\*9), most binding assays were comparable to WT-VWF. Considering that mostly WT-VWF was secreted in the medium during the co-transfections with p.(Cys2216Phefs\*9), the result is not really surprising. For this mutant, the detrimental effect appears mostly at the expression level which is strongly reduced even in the co-transfections, preventing protein synthesis and secretion. The multimeric profile is not affected. For mutant p.(Pro2145Thrfs\*5), the situation is different.

Because the altered protein is synthesized and can interact with WT subunits, this translates in a dominant-negative effect of the mutation. Indeed, although expression level is low, 50% of the protein being expressed is of mutant origin. The dominant negative effect can be seen on the multimer gel with the odd band representing multimers that have incorporated the truncated mutant protein, bringing a stop to any subsequent polymerization. The effect of the mutation on binding of rVWF to its ligands is therefore clearly visible because binding to collagen III, GplIbIIIa, and ADAMTS13 is significantly reduced. For GplIbIIIa, considering that the binding site is located in the C4 domain, downstream of the termination of the mutant subunits, this decrease is expected and can be explained by the fact that only 50% of the subunits would still harbor this binding site. The decrease in binding to collagen III (A3 domain) or to ADAMTS13 (D4 domain) can also be logically explained. For ADAMTS13, because part of the D4 domain is missing, binding is likely suboptimal. As for collagen III, the truncation of the D4 domain is possibly affecting the structure of the neighboring A3 domain, resulting in a modification in the exposure of the collagen-binding site. Binding to FVIII is unaffected as expected for the binding to a ligand interacting with the N-terminal part of the molecule. As for Gplb $\alpha$ , binding is slightly affected, suggesting that the A1 domain structure may also be modified by mutations occurring in the D4 domain.

This tag-specific allele approach, developed and previously used by the team of Eikenboom,<sup>19</sup> thus appears as absolutely essential for interpretation of expression studies of rVWF. Indeed, without knowing the respective amounts of WT and mutant rVWF in the expression medium, no relevant conclusion of the effect of mutations can really be drawn.

In conclusion, our study about these two D4 mutations identified in patients from Afro-Caribbean origin highlights the difficulty of in vitro expression of genetic alterations leading to the occurrence of premature stop codons.

Although the purpose of this study was not to fully reproduce the patients' phenotype because of the presence of multiple genetic variations, what we have seen is that lack of expression of a mutant (eg, p.(Cys2216Phefs\*9)) is less deleterious than residual expression (eg, p.(Pro2145Thrfs\*5)), mostly because of the multimeric nature of VWF and the subsequent dominant negative effect. In the first case, because protein secretion is prevented, probably because of a combination of mRNA degradation and protein retention, multimers will be formed only by normal subunits, consistent with a VWD-type 1 phenotype. In the second case, multimers will be abnormal, as binding of an altered monomer would prevent further multimerization.<sup>37</sup> In patient 2, a known pathogenic variant associated with a dominant

negative effect preventing multimerization (p.(Cys1149Arg)) is present.<sup>38</sup> Here, we describe a second dominant-negative variant, (p.(Pro2145Thrfs\*5)), preventing dimerization. Because these mutations are located on different alleles, their association is absolutely deleterious for the patient, completely preventing multimerization and leading to a VWD-type 3 phenotype. In contrast, we can speculate that if the two mutations would have occurred in the same allele, a VWD-type 1 phenotype would have occurred.

Finally, each frameshift mutation is potentially associated with the disruption of a cysteine-bridge (p. Cys2139-p. Cys2163 and p. Cys2203-p. Cys2235, respectively), both being located in the D4-domain. To the best of our knowledge, no reports exist on the structural or functional consequences when either cysteine-bridge is absent in the VWF molecule. However, the notion that both cysteine-bridges are conserved in the D1, D2, and the D3 domain<sup>39</sup> suggests that they are pertinent to the integrity of the D-domain structure. It seems therefore possible that the disappearance of either cysteine-bridge affects correct folding, secretion, and/or function of the mutated VWF protein.

#### AUTHOR CONTRIBUTIONS

M.D. Dubois, I. Peyron, C. Casari, and P.J. Lenting performed experiments; C.V. Denis, C. Casari, P.J. Lenting, O.D. Christophe, and I. Peyron conceived the study; C.V. Denis, C. Casari, P.J. Lenting, and O.D. Christophe supervised the study; M.D. Dubois, C.V. Denis, C. Casari, and O.N. Pierre-Louis wrote the manuscript; C.V. Denis and A. de Jong provided essential reagents; M.D. Dubois, O.N. Pierre-Louis, S. Pierre-Louis, and J. Rabout recruited the patients and collected clinical data; S. Pierre-Louis, P.J. Lenting, O.D. Christophe, P. Boisseau, J. Goudemand, A. de Jong, and I. Peyron revised the paper; and all authors contributed to the editing of the final manuscript.

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#### RELATIONSHIP DISCLOSURE

All authors declare no conflict of interest.

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