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Angiopoetin-2 and IGFBP-7 as novel biomarkers of abnormal angiogenesis in von Willebrand disease.

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Abstract

von Willebrand factor (VWF) is recognized as an active regulator of angiogenesis, particularly through control of the endothelial storage and release of angiopoietin-2 (Ang-2). This offers a plausible explanation for the poorly understood association between von Willebrand disease (VWD) and gastrointestinal (GI) angiodysplasia. However, the circulating angiogenic biomarker profile of patients with VWD remains partially characterized. This study was designed to comprehensively explore angiogenic mediators across VWD types/subtypes and determine their relationship with the VWF multimeric profile and GI bleeding/angiodysplasia phenotype.

Seventy-six genetically confirmed patients with VWD and 30 healthy controls were included. A large panel of over 12 angiogenic mediators was assessed by multiplexed immunoassay.

Peripheral levels of Ang-2, IGFBP-7 and angiostatin were significantly higher in type 2A and 2B VWD compared to controls and type 1 VWD. IGFBP-7 was significantly higher in patients with an abnormal multimeric profile. These markers did not correlate with VWF antigen or activity. Soluble TIE-2 was significantly lower and PDGF-AB significantly higher in patients with angiodysplasia. TRAIL and soluble VEGF-R2 were lower in patients with angiodysplasia or GI bleeding. No differences were observed for VEGF-A, VEGF-C/D, BMP-10 or endoglin.

The structural integrity of VWF, rather than its plasma concentration, was associated with a distinct proangiogenic signature combining elevated Ang-2 and IGFBP-7 and reduced soluble TIE-2, supporting a role for VWF in the regulation of angiogenesis independent from its hemostatic function. Ang-2 and IGFBP-7 emerge as promising biomarkers and candidate therapeutic targets for VWD-associated angiodysplasia and GI bleeding, warranting validation in larger cohorts.

Key Words: von Willebrand disease, von Willebrand factor, high molecular weight multimers, angiogenesis, angiopoietin-2, insulin like growth factor-binding protein 7.

Introduction

von Willebrand disease (VWD) is the most common inherited bleeding disorder, resulting from a quantitative or qualitative deficiency of von Willebrand factor (VWF). VWF is a large multimeric glycoprotein that mediates platelet adhesion to the subendothelium and stabilizes circulating factor VIII (FVIII). Clinical classification distinguishes partial quantitative deficiency (type 1), qualitative defects (type 2A, 2B, 2M, 2N), and complete deficiency (type 3) [1].

Beyond its central role in primary hemostasis, VWF is increasingly recognized as an active regulator of vascular homeostasis and angiogenesis [2-5]. VWF is synthesized by endothelial cells and stored, together with several angiogenic mediators, in Weibel-Palade bodies [6,7]. Recent work has shown that VWF multimers control the endothelial storage and regulated release of angiopoietin-2 (Ang-2), a key modulator of endothelial quiescence and blood vessel destabilization, thereby directly linking VWF structure to the regulation of angiogenesis [8,9]. Lack of high-molecular-weight (HMW) multimers, whether constitutional (type 2A, type 2B and type 3 VWD) or acquired (Heyde syndrome, high-shear states such as generated by left ventricular assist devices) has therefore been proposed to favor uncontrolled and disorganized angiogenesis [2, 10, 11].

This mechanistic link provides a plausible explanation for one of the most intriguing clinical features of VWD, i.e. the frequent association between severe or qualitative forms of the disease and gastrointestinal (GI) angiodysplasia, a source of chronic and sometimes severe GI bleeding often refractory to standard replacement therapy. While this clinical picture has long been recognized, particularly in type 2A and type 3 VWD, the underlying biological mechanisms remain incompletely understood. Moreover, the profile of circulating biomarkers associated with these vascular abnormalities has only been partially explored [12,13]. A broader characterization of circulating angiogenic mediators could help clarify the pathophysiology of VWD-related angiodysplasia and identify candidate biomarkers for therapeutic targets.

The aim of this study was to comprehensively characterize circulating angiogenic mediators in a cohort of genetically confirmed patients with VWD across the different disease subtypes, and to determine whether their concentrations were associated with the VWF multimeric profile and the GI bleeding phenotype (angiodysplasia and GI bleeding). By combining a systematic phenotypic and genetic characterization of VWD with a broad angiogenic biomarker panel, this study aimed to provide new insight into the pathophysiology of angiogenesis abnormalities in VWD and to identify potential therapeutic targets for the management of VWD-associated angiodysplasia.

Material and methods

Patients and controls

This retrospective study included patients with constitutional VWD, as confirmed by genetic analysis of the VWF gene. Control subjects had normal coagulation parameters, in particular normal FVIII and VWF activity (VWF:Act) and antigen level (VWF:Ag), with a VWF:Act/VWF:Ag ratio >0.7, the absence of abnormal hemorrhagic symptoms (ISTH bleeding assessment tool score <3 in minors, <4 in men and <6 in women) and no history of angiodysplasia [14]. Exclusion criteria included pregnancy and breastfeeding, treatment likely to interfere with angiogenesis, blood transfusion or VWF concentrate infusion within 10 days prior to sampling.

This study was approved by the institutional ethics committee of Nantes University Hospital and was conducted in accordance with the Declaration of Helsinki. Patient records were pseudonymized using an in-house identification system. The study was registered at www.clinicaltrials.gov under the number NCT04810702 (March 2021).

Bleeding evaluation

Bleeding assessment included the occurrence of gastrointestinal bleeding within the five years prior to inclusion, as well as the detection of gastrointestinal angiodysplasia by endoscopy.

Laboratory evaluation

Venous blood was collected via standard phlebotomy into a Vacuette® tube saturated with 3.8% buffered sodium citrate (Greiner Bio-One, Courtaboeuf, France). Plasma was obtained by double centrifugation (2000 to 2500 g, 10-15 minutes) within 4 hours of collection and stored at -80°C until analysis. Samples were thawed for 4 minutes at 37°C immediately before testing.

Phenotypic assays included measurement of FVIII coagulant activity (FVIII:C) by a one-stage clotting assay using the SynthASil® reagent (Werfen, Barcelona, Spain) on an ACL TOP 750 analyzer (Werfen). VWF:Act was assessed by ristocetin cofactor activity using the VWF:GPIbR HemosIL® reagent on an AcuStar analyzer (Werfen). VWF:Ag was measured by a chemiluminescent immunoassay (CLIA) using the VWF:Ag HemosIL® reagent on an AcuStar instrument (Werfen).

VWF multimeric distribution by gel electrophoresis on 1.5% SDS-agarose was assessed as previously described [15].

Angiogenic mediators were measured using Luminex multiplexed immunoassays (Luminex xMAP technology, Luminex 200™ System, Bio-Techne, Minneapolis, MN), including Ang2, angiostatin, Galectin-3 (Gal3), soluble tyrosine kinase with immunoglobulin and EGF homology domains (sTIE-2), tumor necrosis factor-related

apoptosis-inducing ligand (TRAIL), endoglin, platelet-derived growth factor (PDGF)-AA, -AB, -BB, -CC and -DD, bone morphogenetic protein-10 (BMP-10), insulin-like growth factor binding protein-7 (IGFBP-7), vascular endothelial growth factor (VEGF)-A, -C, -D and VEGF receptor 2 (VEGF-R2).

VWF gene sequencing

Genomic DNA was extracted from whole blood collected on EDTA using the Quikgene 610 instrument (Fujifilm, Tokyo, Japan) according to manufacturer instructions. All patients provided consent to undergo full-length VWF gene sequencing. Raw data were processed using a bioinformatics pipeline developed by SOPHiA Genetics (Lausanne, Switzerland), enabling the identification of single nucleotide polymorphisms (SNPs) and copy number variations (CNVs).

Statistical analyses

Angiogenic biomarkers values falling below the detection limit were imputed using half the threshold when available, or half the minimum observed value otherwise. Values reported as <0.000 were imputed as 0, and non-interpretable measurements were coded as missing.

Comparisons between groups of respectively patients with VWD without angiodysplasia *vs* patients with angiodysplasia and patients with VWD without gastrointestinal bleeding *vs* patients with gastrointestinal bleeding were performed using the Wilcoxon rank-sum test. Comparisons involving more than two groups (VWD types, VWD subtypes and multimer profile) were conducted using Kruskal-Wallis test followed by Dunn's post-hoc test when the global test was significant, with unadjusted p-values reported due to the observational nature of the study. Simple linear regression analyses were used to explore potential linear relationships between relevant angiogenic biomarkers VWF:Act and VWF:Ag.

Statistical analyses were performed using R version 4.4.1. Figures were created using the Prism 11 software.

Results

Patient characteristics

A total of 76 patients with VWD (45 women and 31 men) along with 30 controls (16 women and 14 men), were included. The median age at inclusion was 40 years (range 3–83) for patients and 43 years (range 4-76) for controls. Overall, 11 patients had type 1 VWD and 56 type 2 VWD, including 21 type 2A, 14 type 2B and 21 type 2M. The remaining 9 patients had type 3 VWD. The VWF multimeric profile was normal in all controls, in 54% of patients with type 1 VWD, 14% of patients with type 2B VWD and 24% of patients with type 2M VWD. It was abnormal in all but one patient with type 2A. Multimers were consistently absent for patients with type 3 VWD. The main

baseline characteristics of the study population are summarized in Table 1. Data from *VWF* gene analysis, available for all 76 patients, are reported in Supplementary Table S1.

Table 1 : Baseline characteristics

Characteristics	Controls	Patients with VWD						
	(n = 30)	All VWD (n = 76)	Type 1 (n = 11)	Type 2 (all) (n=56)	Type 2A (n = 21)	Type 2B (n = 14)	Type 2M (n = 21)	Type 3 (n = 9)
Age (y), median (range)	43 (4-76)	40 (3-83)	28 (7-73)	42 (3-83)	45 (6-76)	37 (3-72)	39 (4-83)	37 (20-61)
Male sex, n (%)	14 (47)	31 (41)	5 (45)	25 (45)	7 (33)	8 (57)	10 (48)	1 (11)
VWF:Ag (IU/dL), median [IQR]	72 [60-93]	28 [18-40]	26 [19-35]	31 [22-42]	38 [27-53]	38 [30-45]	24 [17-29]	1 [1-1]
VWF:Act (IU/dL), median [IQR]	77 [65-102]	16 [8-26]	22 [18-28]	17 [10-27]	20 [14-28]	22 [10-30]	12 [8-17]	1 [1-1]
FVIII:C (IU/dL), median [IQR]	112 [80-136]	53 [39-71]	65 [55-73]	53 [43-76]	66 [50-80]	53 [45-71]	46 [38-55]	3 [2-8]
Normal VWF multimer profil, n (%)	30 (100)	14 (18)	6 (54)	8 (14)	1 (5)	2 (14)	5 (24)	0 (0)
Gastrointestinal bleeding in last 5 years, n (%)	0 (0)	5 (7)	0 (0)	2 (4)	1 (5)	0 (0)	1 (5)	3 (33)
Presence of angiodysplasia, n (%)	0 (0)	7 (9)	0 (0)	3 (5)	1 (5)	1 (7)	1 (5)	4 (44)

Abbreviations: FVIII:C, factor VIII coagulant activity ; IQR: interquartile range ; VWD: von Willebrand disease ; VWF:Act von Willebrand factor activity ; VWF:Ag: von Willebrand factor antigen ; y: year.

Main affected angiogenic parameters

An overall trend towards higher levels of Ang-2 was observed in patients with VWD, whatever their multimeric profile, compared to controls, but without significant difference (p=0.09) (Figure 1). Considering VWF subtypes, however, Ang-2 levels were significantly higher in patients with type 2 VWD than in controls (p = 0.004) and in patients with type 1 VWD (p = 0.031); in particular, Ang 2 levels were significantly higher in patients with types 2A and 2B but not type 2M compared to controls and type 1 VWD.

IGFBP-7 levels were significantly higher in patients with type 2 VWD than in controls (p=0.008), a difference also observed in patients with an abnormal multimeric profile when compared to controls (p = 0.0125). An upward trend for IGFBP-7 levels was observed in patients with type 2A and type 2B VWD compared to controls and/or patients with type 1 VWD, although this did not reach statistical significance (p = 0.10) (Figure 1).

Angiostatin levels were significantly higher in patients with type 2 VWD compared to both controls (p = 0.019) and patients with type 1 VWD (p = 0.012). In particular, angiostatin levels were significantly higher in patients with type 2A and type 2M VWD when compared to controls and patients with type 1 VWD. Notably, no significant difference in angiostatin levels was observed according to the VWF multimeric profile (Figure 1). There was no evidence of a linear association between VWF:Ag or VWF:Act levels and Ang-2, IGFBP-7 or angiostatin levels (Supplementary Figure S1).

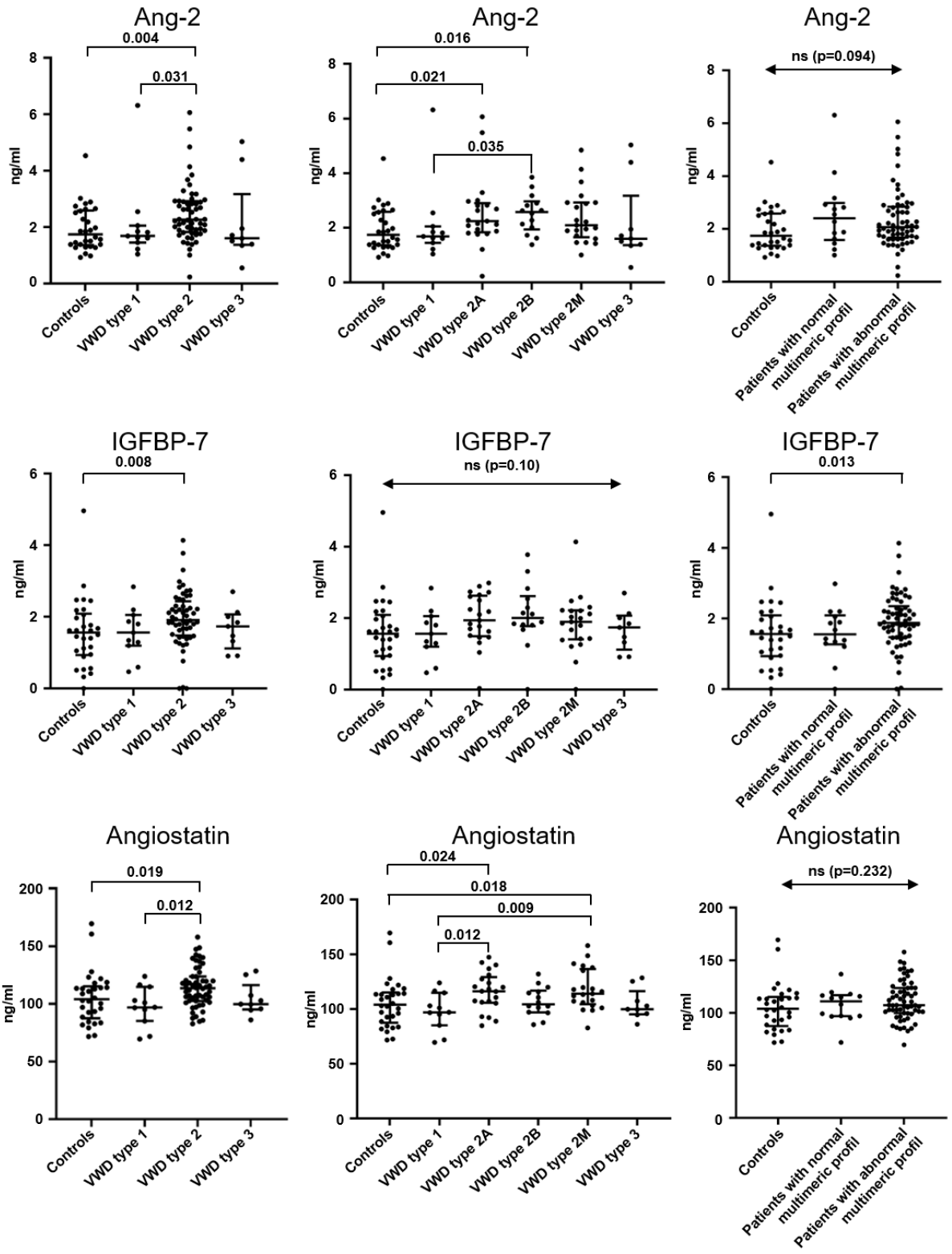


Figure 1: angiogenic markers that differ significantly depending on the type or subtype of VWD and on the VWF multimeric profile. Ang-2: angiopoietin 2, IGFBP-7: insulin like growth factor binding protein 7, VWD: von Willebrand disease

No significant difference was observed between patient and control groups for the other angiogenic markers based on VWD type (Table 2) and subtypes (Table 3) or VWF multimeric profile (Table 4).

Table 2: Angiogenic parameters according to VWD types.

Angiogenic mediator	Controls	Patients with VWD			
		Type 1	Type 2	Type 3	P value
	(n = 30)	(n = 11)	(n = 56)	(n = 9)	
Angiopoietin-2 (ng/mL)					
Median [IQR]	1.7 [1.4-2.6]	1.7 [1.5-2.1]	2.3 [1.8-2.9]	1.6 [1.4-2.0]	**0.008**
Mean (SD)	1.9 (0.8)	2.1 (1.4)	2.5 (1.0)	2.2 (1.5)	
Angiostatin (ng/mL)					
Median [IQR]	104 [88-114]	97 [85-115]	114 [103-124]	100 [96-107]	**0.016**
Mean (SD)	105 (22)	98 (17)	114 (18)	104 (14)	
IGFBP-7 (ng/mL)					
Median [IQR]	16 [10-21]	16 [12-21]	19 [15-24]	17 [13-20]	**0.039**
Mean (SD)	16 (10)	16 (7)	20 (8)	17 (6)	
Galectin-3 (ng/mL)					
Median [IQR]	0.2 [0.2-1.6]	0.2 [0.2-0.2]	0.2 [0.2-8.4]	2.2 [0.2-3.0]	0.052
Mean (SD)	1.0 (1.4)	0.3 (0.2)	1.3 (2.4)	2.3 (2.5)	
PDGF-AA (pg/mL)					
Median [IQR]	25 [12-48]	36 [4-64]	34 [17-61]	36 [29-116]	0.3
Mean (SD)	40 (50)	35 (28)	58 (80)	74 (79)	
PDGF-AB (pg/mL)					
Median [IQR]	36 [25-123]	81 [50-115]	66 [32-112]	81 [44-289]	0.3
Mean (SD)	91 (122)	79 (38)	102 (155)	151 (148)	
PDGF-BB (pg/mL)					
Median [IQR]	1.6 [1.6-3.8]	1.6 [1.6-3.1]	3.1 [1.6-4.1]	3.1 [1.6-3.1]	0.9
Mean (SD)	2.6 (2.2)	2.4 (1.4)	4.1 (5.9)	5.4 (9.6)	
PDGF-CC (pg/mL)					
Median [IQR]	71 [47-85]	84 [40-140]	79 [47-122]	96 [62-164]	0.5
Mean (SD)	75 (63)	86 (53)	103 (92)	127 (112)	
PDGF-DD (pg/mL)					
Median [IQR]	33 [25-45]	39 [29-48]	40 [27-48]	42 [35-105]	0.5
Mean (SD)	40 (26)	37 (17)	45 (34)	63 (42)	
VEGF-A (pg/mL)					
Median [IQR]	21 [2-101]	2 [2-2]	1.7 [1.7-58]	26 [2-60]	0.2
Mean (SD)	89 (160)	14 (42)	45 (92)	110 (255)	
VEGF-C (pg/mL)					
Median [IQR]	124 [71-141]	107 [71-151]	141 [98-171]	107 [71-151]	0.2
Mean (SD)	107 (63)	101 (58)	135 (69)	115 (64)	
VEGF-D (pg/mL)					

Median [IQR]	2.1 [1.4-2.4]	2.1 [0.8-2.7]	2.1 [1.0-2.7]	2.7 [1.5-3.3]	0.7
Mean (SD)	2.1 (1.5)	2.0 (0.9)	2.1 (1.3)	2.7 (1.9)	
sVEGF-R2 (ng/mL)					
Median [IQR]	3.3 [2.7-3.7]	3.6 [2.5-4.5]	3.1 [2.8-3.6]	2.5 [2.4-3.6]	0.4
Mean (SD)	3.3 (0.9)	3.5 (1.1)	3.2 (0.8)	2.8 (0.8)	
BMP-10 (pg/mL)					
Median [IQR]	61 [41-83]	56 [36-68]	58 [47-70]	53 [45-63]	0.8
Mean (SD)	64 (32)	54 (25)	59 (29)	55 (21)	
sTIE-2 (ng/mL)					
Median [IQR]	1.4 [1.2-1.8]	1.4 [1.2-1.6]	1.4 [1.1-1.8]	1.1 [1.0-1.7]	0.7
Mean (SD)	1.5 (0.4)	1.4 (0.4)	1.5 (0.5)	1.4 (0.6)	
MMP3 (ng/mL)					
Median [IQR]	3.7 [0.8-6.8]	4.2 [0.7-11]	4.2 [1.9-7.8]	4.2 [4.0-4.5]	0.8
Mean (SD)	4.7 (4.7)	5.6 (5.3)	5.8 (5.4)	4.4 (1.3)	
Endoglin (ng/mL)					
Median [IQR]	5.8 [4.7-7.1]	6.5 [5.6-7.6]	5.6 [4.7-7.4]	4.8 [4.7-5.8]	0.5
Mean (SD)	6.0 (1.6)	6.6 (1.4)	6.0 (1.9)	5.6 (1.7)	
TRAIL (pg/mL)					
Median [IQR]	0.2 [0.0-4.7]	0.0 [0.0-0.1]	0.0 [0.0-0.8]	0.0 [0.0-0.0]	0.11
Mean (SD)	3.0 (6.5)	0.7 (2.0)	1.6 (4.9)	3.1 (9.1)	

Abbreviations: BMP-10: bone morphogenic protein 10 ; IGFBP-7: insulin like growth factor binding protein 7 ; IQR: interquartile range ; MMP3: matrix metalloproteinase-3 : PDGF: platelet-derived growth factor ; SD: standard deviation ; TRAIL : tumor necrosis-factor-related apoptosis-inducing ligand ; VEGF: vascular endothelial growth factor ; VWD: von Willebrand disease.

Table 3: Angiogenic parameters according to VWD subtypes.

Angiogenic mediator	Controls	Patients with VWD					
		Type 1	Type 2A	Type 2B	Type 2M	Type 3	P value
	(n = 30)	(n = 11)	(n = 21)	(n = 14)	(n = 21)	(n = 9)	
Angiopoietin-2 (ng/mL)							
Median [IQR]	1.7 [1.4-2.6]	1.7 [1.5-2.1]	2.2 [1.9-2.8]	2.6 [2.0-2.9]	2.1 [1.7-2.9]	1.6 [1.4-2.0]	**0.032**
Mean (SD)	1.9 (0.8)	2.1 (1.4)	2.5 (1.3)	2.5 (0.7)	2.4 (1.0)	2.2 (1.5)	
Angiostatin (ng/mL)							
Median [IQR]	104 [88-114]	97 [85-115]	116 [106-127]	104 [97-116]	114 [106-135]	100 [96-107]	**0.023**
Mean (SD)	105 (22)	98 (17)	116 (18)	107 (13)	118 (19)	104 (14)	
IGFBP-7 (ng/mL)							
Median [IQR]	16 [10-21]	16 [12-21]	19 [15-26]	20 [18-25]	19 [14-22]	17 [13-20]	0.10
Mean (SD)	16 (10)	16 (7)	20 (7)	21 (9)	19 (8)	17 (6)	
Galectin-3 (ng/mL)							

Median [IQR]	0.2 [0.2-1.6]	0.2 [0.2-0.2]	0.2 [0.2-2.1]	0.2 [0.2-0.4]	0.2 [0.2-0.4]	2.2 [0.2-3.0]	0.12
Mean (SD)	1.0 (1.4)	0.3 (0.2)	1.4 (1.9)	1.3 (3.0)	1.3 (2.4)	2.3 (2.5)	
PDGF-AA (pg/mL)							
Median [IQR]	25 [12-48]	36 [4-64]	34 [23-58]	40 [25-64]	28 [13-53]	36 [29-116]	0.4
Mean (SD)	40 (50)	35 (28)	60 (71)	77 (123)	43 (46)	74 (79)	
PDGF-AB (pg/mL)							
Median [IQR]	36 [25-123]	81 [50-115]	76 [36-103]	61 [32-115]	66 [36-99]	81 [44-289]	0.6
Mean (SD)	91 (122)	79 (38)	84 (80)	151 (284)	87 (69)	151 (148)	
PDGF-BB (pg/mL)							
Median [IQR]	1.6 [1.6-3.8]	1.6 [1.6-3.1]	3.1 [1.6-4.6]	3.1 [1.67.5]	1.6 [0.4-3.1]	3.1 [1.6-3.1]	0.2
Mean (SD)	2.6 (2.2)	2.4 (1.4)	4.4 (4.7)	6.8 (9.8)	2.0 (1.6)	5.4 (9.6)	
PDGF-CC (pg/mL)							
Median [IQR]	71 [47-85]	84 [40-140]	79 [68-111]	99 [66-134]	62 [43-96]	96 [62-164]	0.4
Mean (SD)	75 (63)	86 (53)	108 (99)	124 (113)	84 (66)	127 (112)	
PDGF-DD (pg/mL)							
Median [IQR]	33 [25-45]	39 [29-48]	42 [28-52]	41 [36-48]	33 [19-44]	42 [35-105]	0.3
Mean (SD)	40 (26)	37 (17)	47 (32)	55 (46)	37 (25)	63 (42)	
VEGF-A (pg/mL)							
Median [IQR]	21 [2-101]	2 [2-2]	5 [2-64]	2 [2-12]	5 [2-66]	26 [2-60]	0.2
Mean (SD)	89 (160)	14 (42)	44 (66)	14 (26)	67 (132)	110 (255)	
VEGF-C (pg/mL)							
Median [IQR]	124 [71-141]	107 [71-151]	141 [84-190]	141 [107-157]	124 [107-151]	107 [71-151]	0.3
Mean (SD)	107 (63)	101 (58)	145 (73)	145 (80)	119 (56)	115 (64)	
VEGF-D (pg/mL)							
Median [IQR]	2.1 [1.4-2.4]	2.1 [0.8-2.7]	2.1 [0.8-2.7]	2.4 [2.1-2.7]	1.5 [1.1-2.7]	2.7 [1.5-3.3]	0.5
Mean (SD)	2.1 (1.5)	2.0 (0.9)	2.1 (1.5)	2.4 (0.9)	2.0 (1.3)	2.7 (1.9)	
sVEGF-R2 (ng/mL)							
Median [IQR]	3.3 [2.7-3.7]	3.6 [2.5-4.5]	3.0 [2.9-3.5]	3.2 [2.7-3.6]	3.1 [2.7-3.6]	2.5 [2.4-3.6]	0.7
Mean (SD)	3.3 (0.9)	3.5 (1.1)	3.3 (0.9)	3.1 (0.8)	3.2 (0.8)	2.8 (0.8)	
BMP-10 (pg/mL)							
Median [IQR]	61 [41-83]	56 [36-68]	61 [49-72]	63 [53-83]	49 [45-56]	53 [45-63]	0.14
Mean (SD)	64 (32)	54 (25)	59 (21)	75 (42)	48 (21)	55 (21)	
sTIE-2 (ng/mL)							
Median [IQR]	1.4 [1.2-1.8]	1.4 [1.2-1.6]	1.3 [1.1-1.7]	1.4 [1.3-1.7]	1.8 [1.1-2.0]	1.1 [1.0-1.7]	0.6
Mean (SD)	1.5 (0.4)	1.4 (0.4)	1.5 (0.6)	1.4 (0.3)	1.6 (0.6)	1.4 (0.6)	
MMP3 (ng/mL)							
Median [IQR]	3.7 [0.8-6.8]	4.2 [0.7-11]	4.4 [1.8-7.1]	4.2 [1.4-8.4]	3.7 [2.2-10.9]	4.2 [4.0-4.5]	>0.9
Mean (SD)	4.7 (4.7)	5.6 (5.3)	4.9 (3.9)	6.6 (7.5)	6.2 (5.1)	4.4 (1.3)	
Endoglin (ng/mL)							

Median [IQR]	5.8 [4.7-7.1]	6.5 [5.6-7.6]	5.6 [4.7-7.6]	6.1 [5.0-7.3]	5.3 [4.8-6.4]	4.8 [4.7-5.8]	0.7
Mean (SD)	6.0 (1.6)	6.6 (1.4)	6.2 (1.9)	5.8 (2.2)	5.8 (1.8)	5.6 (1.7)	
TRAIL (pg/mL)							
Median [IQR]	0.2 [0.0-4.7]	0.0 [0.0-0.1]	0.2 [0.0-1.1]	0.0 [0.0-0.0]	0.1 [0.0-0.7]	0.0 [0.0-0.0]	0.058
Mean (SD)	3.0 (6.5)	0.7 (2.0)	1.5 (3.3)	0.3 (0.6)	2.7 (7.2)	3.1 (9.1)	

Abbreviation: BMP-10: bone morphogenic protein 10 ; IGFBP-7: insulin like growth factor binding protein 7 ; IQR: interquartile range ; MMP3: matrix metalloproteinase-3 : PDGF: platelet-derived growth factor ; SD: standard deviation ; TRAIL : tumor necrosis-factor-related apoptosis-inducing ligand ; VEGF: vascular endothelial growth factor ; VWD: von Willebrand disease.

Table 4: Angiogenic parameters according to VWF multimers profile

Angiogenic mediator	Controls	Patients with VWD		p
		Normal VWF multimer profile	Abnormal VWF multimer profile	
	(n = 30)	(n = 14)	(n=62)	
Angiopoietin-2 (ng/mL)				
Median [IQR]	1.7 [1.4-2.6]	2.4 [1.6-2.9]	2.1 [1.7-2.8]	0.094
Mean (SD)	1.9 (0.8)	2.6 (1.4)	2.4 (1.1)	
Angiostatin (ng/mL)				
Median [IQR]	104 [88-114]	111 [97-116]	107 [100-124]	0.232
Mean (SD)	106 (22)	107 (16)	112 (19)	
IGFBP-7 (ng/mL)				
Median [IQR]	16 [10-21]	16 [13-21]	19 [15-23]	**0.029**
Mean (SD)	16 (10)	16 (7)	19 (8)	
Galectin-3 (ng/mL)				
Median [IQR]	0.2 [0.2-1.6]	0.2 [0.2-0.4]	0.2 [0.2-2.1]	0.741
Mean (SD)	1.0 (1.4)	0.6 (1.0)	1.4 (2.4)	
PDGF-AA (pg/mL)				
Median [IQR]	25 [12-48]	44 [14-64]	34 [16-64]	0.279
Mean (SD)	40 (50)	44 (34)	60 (81)	
PDGF-AB (pg/mL)				
Median [IQR]	36 [25-123]	64 [50-116]	72 [36-115]	0.227
Mean (SD)	91 (122)	84 (59)	109 (156)	
PDGF-BB (pg/mL)				
Median [IQR]	1.6 [1.6-3.8]	2.3 [1.6-4.6]	3.1 [1.6-3.1]	0.801
Mean (SD)	2.6 (2.2)	3.1 (2.3)	4.2 (6.6)	
PDGF-CC (pg/mL)				
Median [IQR]	71 [47-85]	87 [62-140]	79 [47-118]	0.281

Mean (SD)	75 (63)	99 (53)	104 (96)	
PDGF-DD (pg/mL)				
Median [IQR]	33 [25-45]	41 [29-44]	39 [28-49]	0.593
Mean (SD)	40 (26)	42 (23)	47 (35)	
VEGF-A (pg/mL)				
Median [IQR]	21 [2-101]	2 [2-15]	2 [2-60]	0.297
Mean (SD)	89 (160)	21 (40)	54 (129)	
VEGF-C (pg/mL)				
Median [IQR]	124 [71-141]	124 [89-171]	127 [89-157]	0.388
Mean (SD)	107 (63)	124 (63)	129 (69)	
VEGF-D (pg/mL)				
Median [IQR]	2.1 [1.4-2.4]	1.8 [1.4-2.7]	2.1 [0.8-2.7]	0.805
Mean (SD)	2.1 (1.5)	2.1 (1.2)	2.2 (1.4)	
VEGF-R2 (ng/mL)				
Median [IQR]	3.3 [2.7-3.7]	3.2 [2.8-4.3]	3.0 [2.6-3.7]	0.511
Mean (SD)	3.3 (0.9)	3.5 (1.0)	3.1 (0.8)	
BMP-10 (pg/mL)				
Median [IQR]	61 [41-83]	56 [49-61]	58 [45-72]	0.499
Mean (SD)	64 (32)	51 (23)	60 (29)	
TIE-2 (ng/mL)				
Median [IQR]	1.4 [1.2-1.8]	1.5 [1.0-1.9]	1.4 [1.1-1.7]	0.904
Mean (SD)	1.5 (0.4)	1.5 (0.4)	1.5 (0.5)	
MMP3 (ng/mL)				
Median [IQR]	3.7 [0.8-6.8]	6.4 [2.2-11.3]	4.2 [2.0-7.2]	0.351
Mean (SD)	4.7 (4.7)	6.8 (4.9)	5.3 (5.0)	
Endoglin (ng/mL)				
Median [IQR]	5.8 [4.7-7.1]	6.1 [4.7-7.3]	5.6 [4.8-7.4]	0.930
Mean (SD)	6.0 (1.6)	6.3 (1.8)	5.9 (1.8)	
TRAIL (pg/mL)				
Median [IQR]	0.2 [0.0-4.7]	0.0 [0.0-0.1]	0.0 [0.0-0.6]	0.102
Mean (SD)	3.0 (6.5)	0.8 (1.8)	1.9 (5.7)	

Abbreviation: BMP-10: bone morphogenic protein 10 ; IGFBP-7: insulin like growth factor binding protein 7 ; IQR: interquartile range ; MMP3: matrix metalloproteinase-3 : PDGF: platelet-derived growth factor ; SD: standard deviation ; TRAIL : tumor necrosis-factor-related apoptosis-inducing ligand ; VEGF: vascular endothelial growth factor ; VWD: von Willebrand disease.

The prevalence of angiodysplasia and GI bleeding was low in this cohort, with the sole exception of patients with type 3 VWD with 33% of angiodysplasia and 44% GI bleedings (Table 1). Plasma levels of Ang-2, IGFBP-7 and

angiostatin did not differ significantly between patients with angiodysplasia or GI bleeding and other patients, possibly due to the small sample size. Nevertheless, PDGF-AB was significantly higher in patients with angiodysplasia (p = 0.030), whereas soluble TIE-2 was significantly lower in patients with angiodysplasia and GI bleedings (p = 0.003 and 0.008, respectively). TRAIL and soluble VEGF-R2 (sVEGF-R2) levels were also significantly higher in patients without angiodysplasia (p 0.026 and 0.039, respectively) and there was a trend towards higher levels among patients with GI bleeding (p 0.086 and 0.064 respectively).

Multimer analysis revealed a loss of the HMW multimers in five patients, who were nevertheless classified as having type 1 VWD, as the mutations identified had previously been reported in the literature in association with this VWD type. Another patient with normal multimers was classified as having type 2A VWD, as the mutation identified was c.3301T>C, known to cause type 2A(IIE) VWD, in which the loss of HMW multimers is very moderate [16]. Finally, some of the patients with type 2M VWD exhibited a loss of HMW multimers. Indeed, of the 21 patients with type 2M VWD, 11 had the 2M-2A like subtype.

Discussion

The pathophysiology of angiodysplasia in VWD remains poorly understood. Yet, recent studies have highlighted a role for VWF multimers in the homeostasis of angiogenesis, particularly in cases of angiodysplasia with GI bleeding and tumor angiogenesis, suggesting that their decrease – or absence – may lead to a deregulation of this process [8,9,13,17-24].

In this study, several biomarkers related to angiogenesis in VWD patients were analyzed, taking in consideration VWD subtypes, VWF multimeric profiles, and GI bleeding manifestations associated with angiodysplasia.

The characteristics of this cohort align with expected phenotypes of the different VWD subtypes. Patients with type 3 VWD had the lowest VWF and FVIII levels, while those with type 2 VWD showed a discordance between VWF:Act and VWF:Ag levels [25,26]. Patients with type 3 VWD also had the highest prevalence of digestive angiodysplasias (44%) and Gl bleeding (33%), consistent with previously published data showing that severe forms of VWF deficiency are particularly associated with digestive angiodysplasia [27,28].

What makes this study stand out is the thoroughness of the VWF phenotypic analysis and the comprehensive systematic genetic profiling of the participants. VWD subtypes were homogeneously represented, supporting the representativeness of the study population.

Results of multimer analyses concurred with previous results. Of note, 11 patients had the 2M-2A like subtype. which is known to be associated with a loss of HMW multimers [29,30].

Yet, one of the most striking findings of this study is the elevation of Ang-2 levels in patients with type 2 VWD, particularly in subtypes 2A and 2B. This observation confirms *ex-vivo* recent work from Constantinescu-Bercu et al.[31], who demonstrated that VWF plays a major role in regulating angiogenesis by controlling the endothelial storage and release of Ang-2. Considering the context of VWF deficiency or dysfunction, increased endothelial release of Ang-2 and dysregulation of the Ang-2/TIE-2 receptor balance, promotes disordered angiogenesis and the formation of angiodysplasia and GI bleeding.

The particularly marked elevation of Ang-2 observed in forms associated with loss of HMW multimers is noteworthy. This is also supported within our cohort by reduced soluble TIE-2 in patients with angiodysplasia and GI bleedings since soluble TIE-2 acts as a decoy substrate for Ang-2 [32,33].

The findings in the present cohort also reveal a significant increase in IGFBP-7 among patients with abnormal multimeric VWF profile. IGFBP-7 is involved in vascular remodeling, cellular senescence, and endothelial adhesion and its elevation may reflect chronic endothelial activation or injury in qualitative forms of VWD [34,35]. Few studies have investigated the role of IGFBP-7 in VWD. In 2012, Van Breevoort et al. [36] identified IGFBP-7 as a component of Weibel-Palade bodies, attached to ultra large VWF strings and released upon exocytosis under flow conditions. IGFBP-7 may therefore be released in excess into the circulation in the absence of HMW multimers, as observed in this cohort of patients with type 2 VWD. IGFBP-7 has also been newly recognized as a regulator of angiogenesis through its interaction with CD93, and blockade of the IGFBP-7–CD93 axis has been shown to normalize tumor vascularization [37,38]. To the best of our knowledge, this study is the first to identify IGFBP-7 as a factor in neoangiogenesis in type 2 VWD.

Angiostatin is a proteolytic fragment of plasminogen and a potent inhibitor of angiogenesis and tumor metastasis, generated by limited proteolysis of plasminogen [39]. It inhibits angiogenesis through several complementary mechanisms, including inhibition of endothelial cell proliferation, induction of apoptosis in proliferating endothelial cells, inhibition of endothelial cell migration, and interaction with cell-surface receptors – notably ATP synthase - that disrupt the signalling pathways required for angiogenesis [40-43]. Physiological angiogenesis requires degradation of the vascular basement membrane and remodelling of the extracellular matrix so that endothelial cells can migrate and infiltrate surrounding tissues [44]. Matrix metalloproteinases (MMPs) are specifically recruited and activated for this purpose. MMPs also act on circulating plasminogen, generating angiostatin thereby restraining angiogenesis [45,46]. The elevated angiostatin levels observed in patients who also had high levels of Ang-2 and IGFBP-7 suggest that compensatory responses may be at work to counterbalance the

proangiogenic processes associated with VWF abnormalities. Figure 2 summarizes the mechanisms proposed to be involved.

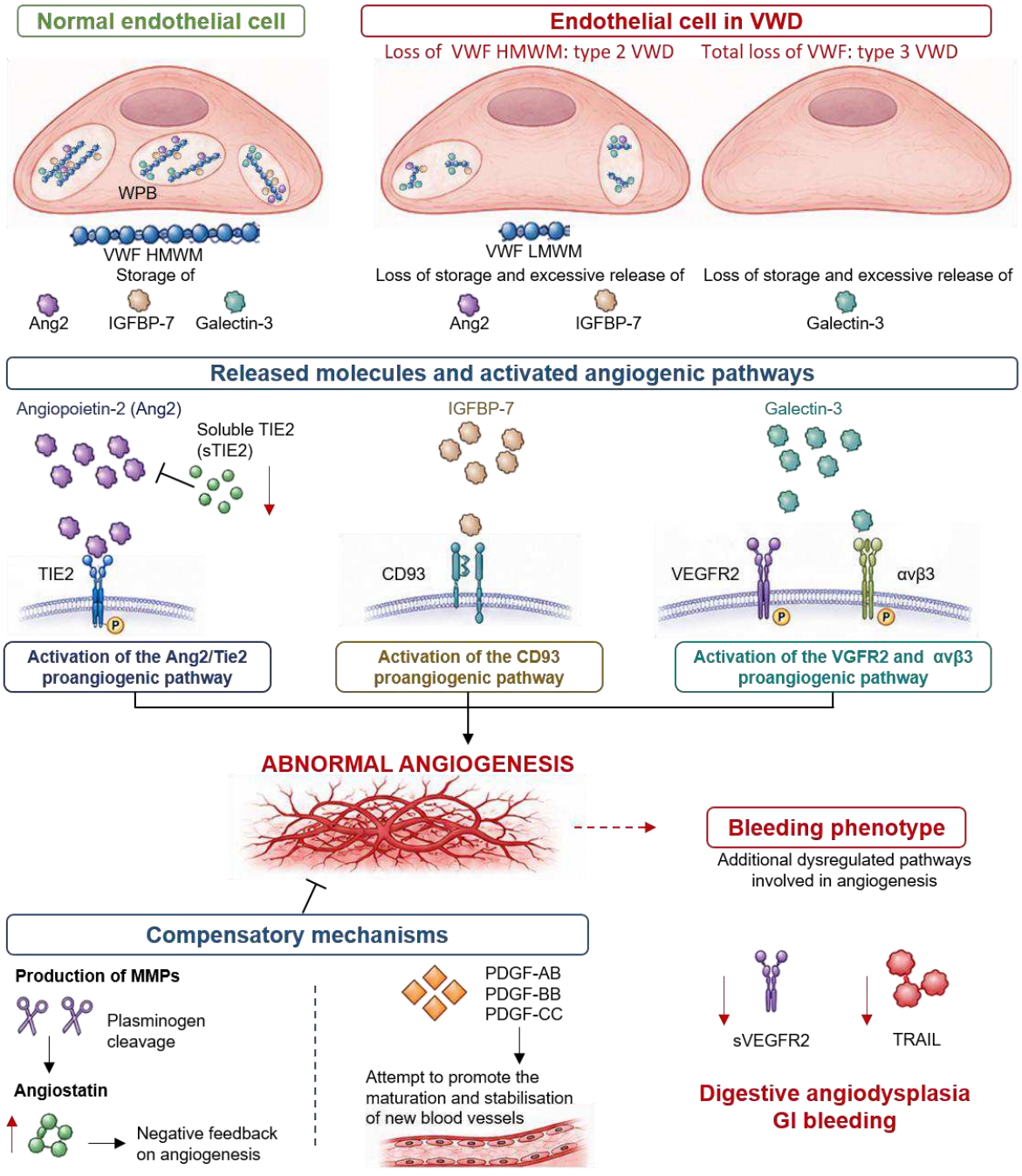


Figure 2: Possible pathophysiological mechanisms in the neoangiogenesis associated with VWD.
Normal endothelial cells, with WPB and VWF HMWM, allow for storage of Ang2, IGFBP-7 and galectin-3. Loss of VWF HMWM induces excessive release of Ang2 and IGFBP-7, while total VWF loss induces excessive release of galectin-3. Ang2 acts on angiogenesis via activation of the Ang2/Tie2 pathway and reduces the levels of soluble Tie2; IGFBP-7 probably acts on angiogenesis via the CD93 receptor; galectin-3 probably acts on angiogenesis through the VEGFR2 and αvβ3 receptors. These activations result in abnormal neoangiogenesis. This leads to the production of MMPs, which cleave plasminogen and produce angiostatin, thereby attempting to limit neoangiogenesis. Secretion of PDGF-AB, PDGF-BB and PDGF-CC attempts to trigger the maturation of the new blood vessels. Dysregulation of other angiogenic pathways, such as sVEGFR2 and TRAIL, appears necessary to trigger bleeding. Ang2: angiopoietin-2; PDGF: platelet derived growth factor; HMWM: high molecular weight multimers; LMW: low molecular weight multimers; IGFBP-7: insulin like growth factor binding protein 7; MMPs: matrix metalloproteinases; TIE2: tyrosine kinase with immunoglobulin and EGF homology domains 2; TRAIL: tumor necrosis-factor-related apoptosis-inducing ligand; VEGFR: vascular endothelial growth factor receptor VWF: von Willebrand Factor; WPB: Weibel Palade bodies.

Galectin-3 levels were also higher in patients with type 3 VWD, a difference that approached but did not reach statistical significance (p=0.052). Galectin-3 has previously been described as a component of Weibel-Palade bodies, storage of which is regulated by VWF. In the absence of VWF, galectin-3 is thought to be released in a constitutive rather than regulated manner. This excessive release is proposed to activate the VEGFR2 and αvβ3 integrin pathways, thereby promoting excessive, unregulated angiogenesis [13,16,47,48]. Galectin-3 levels are only slightly elevated in patients with type 2 VWD. It appears that it is the near-complete absence of VWF — rather than a partial quantitative or qualitative deficiency — that drives this increase, reinforcing the idea that the mechanisms of neoangiogenesis differ depending on the type of VWD.

Various other angiogenic biomarkers varied depending on the VWD subtype and the presence of angiodysplasia or GI bleedings, notably the different PDGF isoforms. Concentrations of PDGF-AB, PDGF-BB, and PDGF-CC were higher in certain type 2 subtypes, particularly types 2A and 2B. PDGFs are involved in the maturation and stabilization of new blood vessels [49,50]. Their elevation may also represent a compensatory mechanism secondary to suboptimal or disordered angiogenesis. However, the lack of statistically supported differences constrains the pathophysiological interpretation of the variations.

Regarding VEGFs, results appear more heterogeneous. No major and consistent increase in VEGF-A was observed across the different VWD subtypes, which is consistent with experimental data suggesting that the Ang-2/TIE-2 axis may play a more central role than the VEGF axis in the vascular abnormalities associated with VWF deficiency[4,5,8,16]. VEGF-C and VEGF-D levels were relatively comparable between groups, suggesting no proeminent lymphangiogenic mechanisms are present [51,52]. Similarly, concentrations of BMP-10 or endoglin showed no major change or alteration, indicating that not all angiogenic pathways are uniformly disrupted in VWD [53,54].

Remarkably, when patients were analyzed according to the presence of angiodysplasia or GI bleeding rather than VWD subtype, a distinct biomarker pattern emerged. Soluble TIE-2 was significantly lower in patients with angiodysplasia or gastrointestinal bleeding, while TRAIL and sVEGF-R2 were higher in patients without these conditions. VEGF-R2 is one of the main receptors for VEGF and has been extensively studied in angiogenesis [55]. Soluble form of VEGFR-2 exerts reducing effects on VEGF signaling by playing the role of a ligand-trap. Low levels of sVEGF-R2 have been associated with endothelial dysfunction and abnormal angiogenesis in various conditions [56-58], and this was observed here in patients with VWD and GI bleeding.

The findings regarding TRAIL are of particular interest, as its involvment in angiogenesis is both complex and context-dependent [59]. TRAIL is best known for inducing apoptosis in tumor cells, but several studies have shown that it can also inhibit VEGF-induced angiogenesis in endothelial cells by blocking VEGF-driven signaling [60-62], while other reports describe an opposite, pro-survival and proliferative effect of TRAIL on endothelial cells, comparable in magnitude to that of VEGF, through activation of the Akt and ERK pathways [63-65]. In the present cohort, the higher TRAIL levels observed in patients without angiodysplasia or GI bleeding are consistent with a predominantly anti-angiogenic, VEGF-restraining, role of TRAIL in this setting. Lower circulating TRAIL levels could remove a brake on VEGF-driven angiogenic signaling, thereby favoring the disorganized vascular proliferation underlying angiodysplasia. Combined with the concomitant decrease in soluble TIE-2, this pattern suggests that patients who develop angiodysplasia and GI bleedings may be losing simultaneously several independent inhibitory checkpoints on angiogenesis, rather than a single dominant pathway being affected. These findings should nonetheless be interpreted with caution due to the small number of patients with angiodysplasia or GI bleeding in this cohort.

The analysis based on the multimeric profiles provided particularly informative findings in that patients with an abnormal multimeric profile had higher IGFBP-7 peripheral levels and a trend toward higher Ang-2 levels, compared to patients with a normal multimeric profile. These findings suggest that the structural integrity of VWF may be more crucial for regulating neoangiogenesis than its plasma concentration. This aligns with clinical observations showing that patients with a loss of HMW multimers are more likely to develop digestive angiodysplasia [12,27,28].

This study has several limitations. First, it was a retrospective single-center pilot study, sample sizes in certain subgroups, particularly type 3 VWD, remaining limited. Second, biomarkers were measured at a single time point, without longitudinal follow-up or assessment of the influence of replacement therapy. Third, circulating concentrations may be influenced by inflammatory or cardiovascular factors not considered in this study. Finally, the absence of functional endothelial studies hinders the mechanistic insight and conclusions that can be derived.

However, this study highlights that Ang-2 and IGFBP-7 appear to be promising candidate targets for the treatment of patients with VWD, angiodysplasias and GI bleeding .

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

Authors contributed to the work as follows: A.B., N.D., M.T., C.T. and M.F. included patients, provided evaluation and collected samples. Y.R. performed the analysis of VWF multimers. P.B. performed the study on the *VWF* gene. C.G. performed statistical analyses. V.H., M.F., and A.B. coordinated the work. A.B. wrote the first draft manuscrit, which has been corrected and approved by all authors.

CONFLICT OF INTEREST

Marc Fouassier received research funding and hospitality from TAKEDA.The remaining authors declare no competing interests.

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