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ORIGINAL ARTICLE

Distinct mechanisms account for acquired von Willebrand syndrome in plasma cell dyscrasias

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Abstract Acquired von Willebrand syndrome (AVWS) is a rare bleeding disorder that may cause life-threatening hemorrhages in patients with plasma cell dyscrasias (PCDs). Early diagnosis and treatment require a thorough understanding of its underlying pathophysiology. Two patients with IgG MGUS presented with dramatically decreased plasma von Willebrand factor (VWF) and a severe type-1 pattern on multimer analysis. A prompt response to intravenous immunoglobulins (IVIG), but not to VWF/FVIII, was consistent with accelerated immunologic clearance of plasma VWF. Another IgG MGUS patient showed a type-2 pattern and a less pronounced response to IVIG, suggesting that additional mechanism(s) contributed to AVWS evolution. In a patient with Waldenström's macroglobulinemia and severe depletion of plasma VWF, multimer analysis indicated association of the IgM paraprotein with VWF before, but not after plasmapheresis, resulting in destruction of the agarose gel and a characteristically distorted band structure of VWF multimers. A type-2 pattern with highly abnormal VWF triplets and laboratory evidence of excessive fibrinolytic activity suggested that plasmin-mediated VWF degradation contributed to AVWS in a patient with multiple myeloma (MM) and AL amyloidosis. Finally, in a patient with IgG MM, maximally prolonged PFA-100® closure times and a specific defect in ristocetin-induced platelet agglutination, both of which resolved after remission induction, indicated interference of the paraprotein with VWF binding to platelet GPIb. Importantly, in none of the six patients, circulating autoantibodies to VWF were detected by a specific in-house ELISA. In summary, when evaluating PCD patients with severe bleeding symptoms, AVWS due to various pathogenic mechanisms should be considered.

Keywords Acquired von Willebrand syndrome · Monoclonal gammopathy of undetermined significance · Waldenström's macroglobulinemia · Multiple myeloma · Amyloid light-chain amyloidosis · Fibrinolysis

Introduction

Acquired von Willebrand syndrome (AVWS) is a rare, but potentially underdiagnosed bleeding disorder characterized by quantitative and/or qualitative abnormalities of plasma von Willebrand factor (VWF). AVWS may be associated with hypothyroidism or with a variety of lympho- or myeloproliferative, cardiovascular, and autoimmune diseases [1]. Depending on the underlying mechanism, reduced synthesis, increased adsorption (e.g., onto tumor cells or platelets), accelerated clearance (i.e., by autoantibodies), or enhanced degradation by the VWF cleaving metalloproteinase, ADAMTS13, or other enzymes may account for the bleeding tendency in previously asymptomatic patients [2].

Plasma cell dyscrasias (PCDs) are a heterogeneous group of hematological disorders characterized by abnormal proliferation of monoclonal plasma cells with or without production of a circulating paraprotein. Although various mechanisms have been suggested to cause AVWS in

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PCD patients, in many cases, the underlying pathophysiology remains obscure [3, 4].

Here, we describe six additional cases of PCD-associated AVWS and provide further insight into the distinct pathomechanisms with significant implications for diagnosis and treatment.

Materials and methods

Patients

All patients and controls provided informed consent to the conduction of experiments and to the publication of clinical and laboratory findings.

Assays of VWF

VWF antigen (VWF:Ag), VWF ristocetin cofactor activity (VWF:RCo), and VWF GPIb binding activity (VWF:Ac) were measured by turbidimetry using the VWF Ag® test, the BC von Willebrand Reagent, and the INNOVANCE® VWF Ac assay, respectively. Shear-stress-related VWF activity was determined by PFA-100® (all from Siemens Healthcare). VWF plasma multimers were analyzed by SDS-agarose gel electrophoresis with luminescent visualization [5]. When indicated, the nitrocellulose filter was first stained with horseradish peroxidase (HRP)-conjugated goat anti-human IgM antibody (Bio-Rad). Following HRP inactivation with sodium azide, VWF staining was carried out as previously described [5].

Detection of VWF autoantibodies

A previously described enzyme-linked immunosorbent assay (ELISA) was used to detect IgG, IgA, and IgM antibodies to VWF. This assay employs recombinant human VWF expressed in CHO cells that is devoid of ABO blood group antigens to avoid cross reactivity with isoagglutinins [6].

Measurement of u-PA and PAP complexes

Commercial ELISAs were used to quantify u-PA (Quantikine®; R&D Systems) and plasmin-α_2-antiplasmin (PAP) complexes (Technozym®; Technoclone) in plasma.

Detection of platelet autoantibodies

The PakPlus® ELISA (Immucor GTI Diagnostics) was used to detect circulating antibodies against GPIb/IX, GPIa/IIa, GPIIb/IIIa, GPIV, and HLA class 1.

Platelet flow cytometry

For analysis of IgG binding, fixed platelets were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit polyclonal anti-human IgG antibody (Sigma Aldrich) and analyzed on a FACSCalibur™ (BD Biosciences). As a positive control, platelets from a healthy donor were pre-incubated with 100 μg/mL abciximab (ReoPro®; Janssen Biotech). For analysis of P-selectin expression, platelets were incubated with buffer (PBS) or 20 μM adenosine diphosphate (ADP), fixed, and labeled with phycoerythrin (PE)-conjugated CD62P monoclonal (clone, CLB-Thromb/6) or isotype control antibody (Beckman Coulter).

Platelet aggregation

The Apact™ four-channel light transmittance aggregometer (Rolf Greiner BioChemica) was used to measure platelet aggregation in response to 20 μM ADP, 50 μM thrombin receptor activator peptide (TRAP-6), or 1.5 mg/mL ristocetin [7].

Results

AVWS in IgG MGUS

Patient 1 had a 3-year history of easy bruising and extensive soft-tissue hematomas. The activated partial thromboplastin time (APTT) was prolonged due to decreased factor VIII (FVIII) activity (Table 1). A FVIII inhibitor was excluded by Bethesda assay and ELISA. PFA-100® closure times were maximally prolonged, and VWF:Ag and VWF:RCo were severely decreased. There was no apparent loss of larger VWF plasma multimers, consistent with a severe type-1 pattern (Fig. 1a). No circulating autoantibodies to VWF were detected.

Serum immunofixation revealed an IgG-kappa paraprotein. Although a bone marrow biopsy was not performed, clinical and laboratory findings were compatible with IgG monoclonal gammopathy of undetermined significance (MGUS).

Following the administration of VWF/FVIII concentrate (Haemate® P; CSL Behring) at 125 IU VWF:RCo/kg, no appreciable increase in plasma VWF was observed (Fig. 1b). In contrast, administration of intravenous immunoglobulins (IVIG) at 1 g/kg on two consecutive days resulted in rapid normalization of plasma VWF (Fig. 1b). Because these findings suggested that AVWS was due to increased immunologic clearance of plasma VWF, the patient received 40 mg of dexamethasone per day on four consecutive days. However, this treatment neither affected the IgG-kappa paraprotein nor VWF parameters. Subsequent minor soft-tissue bleeds were successfully managed with oral tranexamic acid (TXA).

Table 1 Types of plasma cell dyscrasias and laboratory findings associated with acquired von Willebrand syndrome

	Normal	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age (years)		62	73	71	65	63	63
Gender		male	female	male	male	female	male
PCD		MGUS	MGUS	MGUS	WM	MM + AL amyloidosis	MM
Paraprotein		IgG-κ	IgG-λ	IgG-κ	IgM-κ	λ-LC	IgG-λ
Serum concentration of paraprotein (g/L)[a]		2.2	2.7	8.4	56.0	n. a.	51.4
Platelets (10^9/L)	150–400	299	333	211	159	187	275
APTT (sec)	25–38	50	48	32	67	46	37
FVIII:C (%)	60–160	11	9	70	20	61	173
PFA-100®							
coll/epi (sec)	84–160	>300	>300	>300	>300	>300	>300
coll/ADP (sec)	68–121	>300	>300	>300	>300	>300	>300
VWF:Ag (%)	60–200	12	5	58	12	126	384
VWF:RCo (%)	70–180	<10	n. d.	n. d	n. d.	n. d.	n. d.
VWF:Ac (%)	61–171	n. d.	<4	22	17	20	309
VWF plasma multimers		Severe type 1	Severe type 1	Type 2	Distorted	Type 2	Normal
Anti-VWF	negative	Negative	Negative	Negative	Negative	Negative	Negative
Proposed mechanism		Immunologic clearance	Immunologic clearance	Immunologic clearance, ADAMTS13-mediated degradation	Non-specific complex formation	Plasmin- and ADAMTS13-mediated degradation	Inhibition of GPIb binding

Abbreviations are as follows (in alphabetical order): *AL* amyloid light chain, *Anti-VWF* autoantibodies to von Willebrand factor (IgG, IgA, or IgM), *APTT* activated partial thromboplastin time, *coll/ADP* collagen/adenosine diphosphate, *coll/epi* collagen/epinephrine, *FVIII:C* factor VIII clotting activity, *LC* light chain, *MGUS* monoclonal gammopathy of undetermined significance, *MM* multiple myeloma, *n. a.* not applicable, *n. d.* not done, *PCD* plasma cell dyscrasia, *PFA* platelet function analyzer, *VWF* von Willebrand factor, *VWF:Ac* VWF GPIb binding activity, *VWF:Ag* VWF antigen, *VWF:RCo* VWF ristocetin cofactor activity, *WM* Waldenström's macroglobulinemia

[a] Paraprotein concentrations were assessed by serum electrophoresis using the following formula: M protein in % x total serum protein. No M protein was detected in patient 5

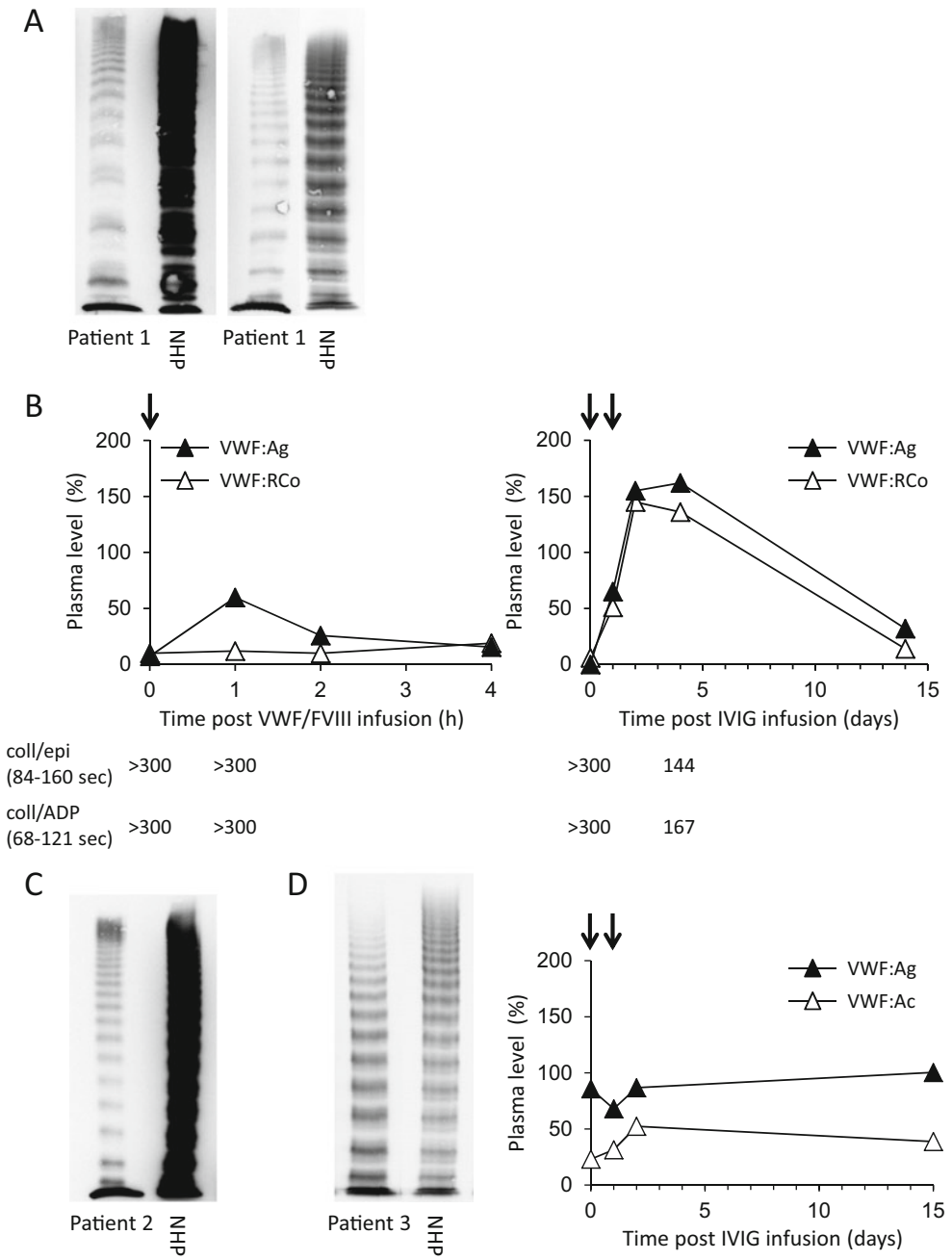


Fig. 1 IgG MGUS-associated AVWS. **a** Multimer analysis of plasma VWF in patient 1 using both a medium-resolution 1.6 % agarose gel (left two lanes) and a low-resolution 1.2 % agarose gel (*right two lanes*), which allows for better migration of the larger multimers into the gel. For illustration purposes only, a shorter exposure time was used for the NHP sample in the 1.2 % agarose gel. NHP denotes normal human plasma. **b** *Left panel*: Time course of plasma VWF:Ag and VWF:RCo in patient 1 after the administration of VWF/FVIII at a dose of 125 IU VWF:RCo/kg. *Right panel*: Time course of VWF:Ag and VWF:RCo in patient 1 after the administration of intravenous immunoglobulins (IVIG) at a dose of 1 g/kg on two consecutive days. Time points of VWF/FVIII and IVIG infusions are indicated by *arrows*. PFA-100® closure times, using both the collagen/epinephrine (coll/epi) and the collagen/ADP (coll/ADP) cartridges, were measured at baseline as well as 1 h and 4 days after the administration of VWF/FVIII (*left panel*) or IVIG (*right panel*), respectively. **c** Multimer analysis of plasma VWF in patient 2 using a medium-resolution 1.6 % agarose gel. NHP denotes normal human plasma. **d** *Left panel*: Multimer analysis of plasma VWF in patient 3 using a low-resolution 1.2 % agarose gel. NHP denotes normal human plasma. *Right panel*: Time course of VWF:Ag and VWF:Ac in patient 3 after the administration of IVIG at a dose of 1 g/kg on two consecutive days. Time points of IVIG infusions are indicated by *arrows*

Patient 2 presented with new-onset spontaneous cutaneous hematomas and gingival bleeding after a minor dental procedure. Laboratory findings were similar to those obtained in patient 1 (Table 1). Likewise, multimer analysis revealed a

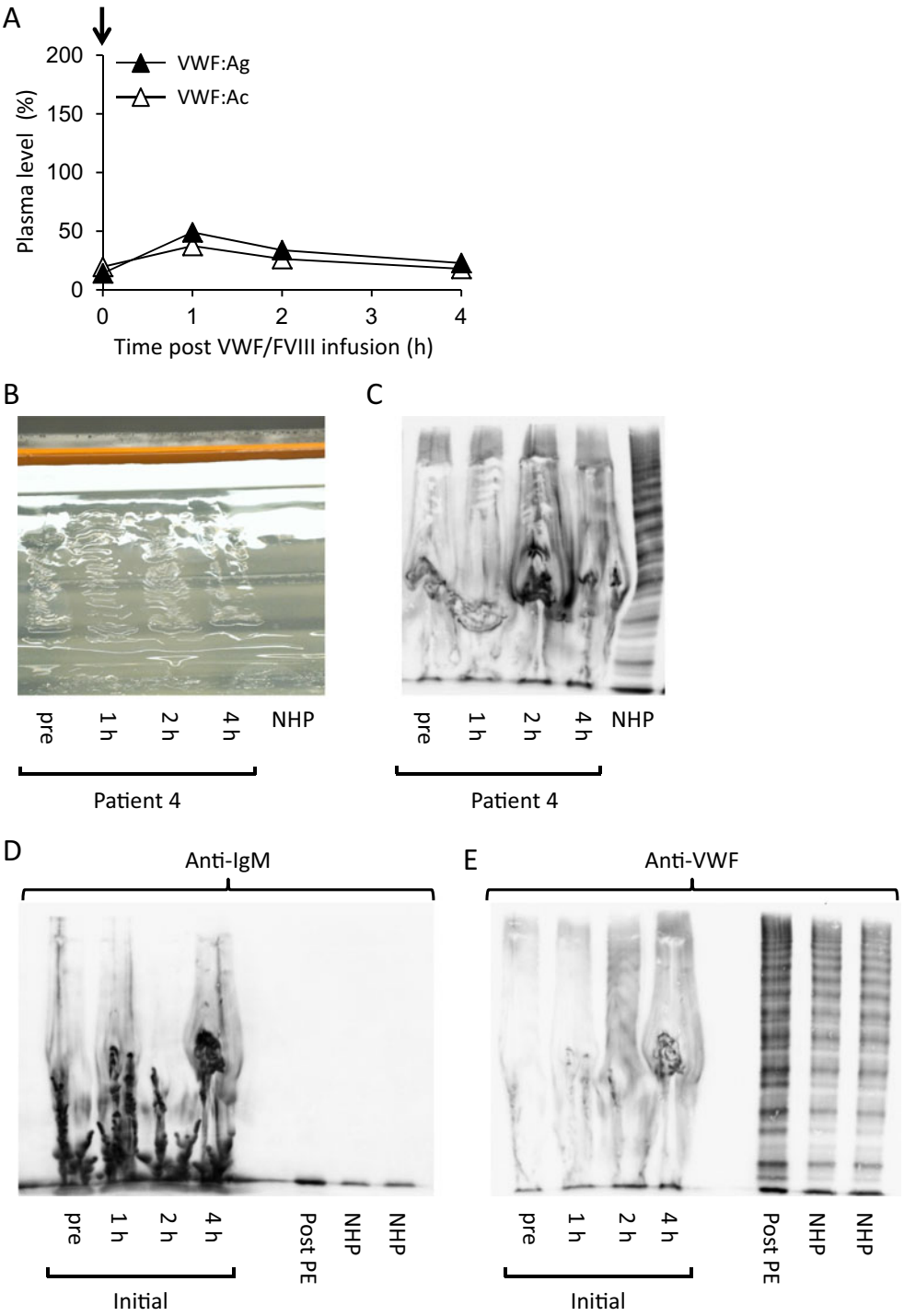


Fig. 2 AVWS in Waldenström's macroglobulinemia. **a** Time course of VWF:Ag and VWF:Ac in patient 4 after the administration of VWF/FVIII at a dose of 85 IU VWF:RCo/kg. Time point of VWF/FVIII infusion is indicated by *arrow*. **b** and **c** Multimer analysis performed on plasma samples obtained immediately before as well as 1, 2, and 4 h after VWF/FVIII infusion revealed a significantly altered 1.6 % agarose gel (**b**) and a severely distorted band structure of VWF multimers (**c**). NHP denotes normal human plasma. **d** and **e** To investigate the association of the IgM paraprotein with plasma VWF, initial plasma samples obtained before and after VWF/FVIII infusion (diluted 1:10) and the plasma sample obtained 2 days after the last of five plasma exchange (PE) therapies (diluted 1:100) were loaded on the same medium-resolution 1.6 % agarose gel. The nitrocellulose filter was then consecutively stained for IgM (**d**) and VWF (**e**), revealing co-localization only before, but not after plasmapheresis. IgM serum concentrations were 69 and 21 g/L in the initial and follow-up plasma samples, respectively. *NHP* denotes normal human plasma (diluted 1:20)

severe type-1 pattern (Fig. 1c), and tests for circulating autoantibodies to FVIII or VWF were negative. An IgG-lambda paraprotein was detected by serum immunofixation. In the absence of end-organ damage, a diagnosis of IgG MGUS was made. Following extensive counseling on her bleeding disorder, the patient was lost to follow-up.

Patient 3 experienced extensive bleeding following endoscopic resection of a colorectal adenoma, which required the transfusion of two units of packed red blood cells. PFA-100® closure times were maximally prolonged, and VWF:Ac and VWF:Ag showed an abnormal activity-to-antigen ratio of 0.38 (normal, >0.70) (Table 1). VWF multimer analysis confirmed the loss of larger plasma multimers, consistent with a type-2 pattern (Fig. 1d). Testing for VWF autoantibodies was negative. Infusion of VWF/FVIII concentrate (Haemate® P) at 55 IU VWF:RCo/kg had no appreciable effect on plasma

VWF (not shown). IVIG at 1 g/kg on two consecutive days slightly increased VWF:Ac, but did not normalize the pathological VWF activity-to-antigen ratio (Fig. 1d) or multimer pattern (not shown). Serum immunofixation revealed an IgG-kappa paraprotein (serum concentration, 8.4 g/L), and 9 % plasma cells were detected by bone marrow aspiration cytology. Compatible with a diagnosis of IgG MGUS, there was no evidence of hypercalcemia, renal insufficiency, anemia, or osteolytic bone lesions. Based on significantly elevated serum-free kappa-light chains of 755.6 mg/L (normal, 3.3–19.4 mg/L) and the patient's bleeding tendency, however, induction chemotherapy with bortezomib and dexamethasone was initiated [8]. After the first treatment cycle, the patient opted against further therapy of his PCD, but was readmitted 8 months later for elective endoscopic resection of multiple residual colorectal adenomas. At that time, the IgG-kappa paraprotein had increased to 11.0 g/L, and there was persistent evidence of AVWS with VWF:Ac and VWF:Ag plasma levels of 17 and 57 %, respectively. Following the infusion of IVIG at 1 g/kg on two consecutive days, the patient received VWF/FVIII (Haemate® P) at 80 IU VWF:RCo/kg immediately before the procedure in addition to TXA. Despite this prophylactic treatment, rectal bleeding occurred resulting in a drop of the hemoglobin level from 14.4 to 12.6 g/dL. Sufficient hemostasis was achieved under aggressive substitution therapy with VWF/FVIII at 120 IU VWF:RCo/kg every 8–12 h for 5 days. The patient did not require transfusion of packed red blood cells and was discharged home free of any bleeding symptoms 1 week after the procedure.

AVWS in Waldenström's macroglobulinemia (WM)

Patient 4 had a 1-week history of spontaneous gingival bleeding. PFA-100® closure times were maximally prolonged, and VWF:Ag and VWF:Ac were significantly decreased (Table 1).

Administration of VWF/FVIII (Haemate® P) at 85 IU VWF:RCo/kg resulted in a reduced incremental recovery and a shortened half-life of infused VWF (Fig. 2a). Multimer analysis performed on plasma samples that had been obtained before and after VWF/FVIII infusion revealed a destroyed agarose gel (Fig. 2b) and a severely distorted band structure of VWF multimers (Fig. 2c). No circulating autoantibodies to VWF were detected.

The patient was diagnosed with WM based on a massively elevated serum IgM of 69 g/L and the presence of 28 % clonal lymphoplasmacytic cells in a bone marrow aspirate.

Two weeks later, the patient presented with severe sepsis due to a superinfected gluteal hematoma in the vicinity of the bone marrow puncture site. Following surgical debridement, plasmapheresis was initiated, and the patient received VWF/FVIII (Haemate® P) at 160 IU VWF:RCo/kg every 8 h. After the last of five plasma exchanges, the IgM serum level had decreased to 21 g/L with normalization of FVIII and VWF levels. VWF/FVIII substitution was tapered off, and the patient continued treatment for his underlying PCD. At 3 months of follow-up, the IgM serum level was 32 g/L with no clinical or laboratory evidence for AVWS recurrence.

When the initial and follow-up plasma samples were loaded on the same gel, consecutive staining for IgM (Fig. 2d) and VWF (Fig. 2e) revealed co-localization only in the severely distorted lanes of the initial plasma samples, indicating that the IgM paraprotein associated with plasma VWF at presentation, but not after resolution of AVWS following plasmapheresis.

AVWS in AL amyloidosis

Patient 5 had a 6-month history of easy bruising and increased bleeding after a tooth extraction. The APTT was prolonged without reductions in plasma clotting factors VIII, IX, XI, and XII, while the thrombin time and the prothrombin time were normal. The severely decreased VWF activity-to-antigen ratio of 0.16 was indicative of AVWS type 2 (Table 1). Multimer analysis confirmed the loss of larger and intermediate-sized VWF plasma multimers and revealed an abnormal triplet structure of VWF oligomers (Fig. 3a). Testing for VWF autoantibodies was negative.

Significantly reduced plasma levels of plasminogen and α_2-antiplasmin indicated increased fibrinolytic activity (Table 2), but plasma levels of D-dimer and fibrinogen argued against overt disseminated intravascular coagulation (DIC). A 10-day course of oral TXA acid at 1000 mg every 8 h had no significant effect on VWF parameters (Table 2), and administration of VWF/FVIII (Haemate® P) at 80 IU VWF:RCo/kg resulted in an inadequate and only short-lasting response with no appreciable appearance of larger VWF plasma multimers (Fig. 3b).

A diagnosis of multiple myeloma with light-chain (AL) amyloidosis was made, and the patient received induction chemotherapy with bortezomib and dexamethasone followed by high-dose melphalan with autologous peripheral blood stem cell transplantation (PBSCT). Nine months after PBSCT, free lambda-light chains (λ-LCs) had declined to 30.8 mg/L, and plasminogen and α_2-antiplasmin levels had normalized (Table 2). However, markedly increased PAP complexes, as measured on two different occasions (i.e., 5 and 9 months after PBSCT), still indicated significant fibrinolytic activity (Fig. 3c, Table 2). Although there was persistence of AVWS, the loss of larger multimers and the atypical triplet structure of VWF oligomers appeared less pronounced at 9 months after PBSZT (Fig. 3d, Table 2). There was no evidence for congestive heart failure or aortic valve stenosis as alternative causes for AVWS. At 15 months after PBSCT, serum-free λ-LCs had increased to 84.0 mg/L, and a decline in the α_2–antiplasmin level to 47 % combined with a dramatic rise of PAP complexes to 19,446 ng/mL indicated massive plasmin generation. Progression of the patient's PCD was

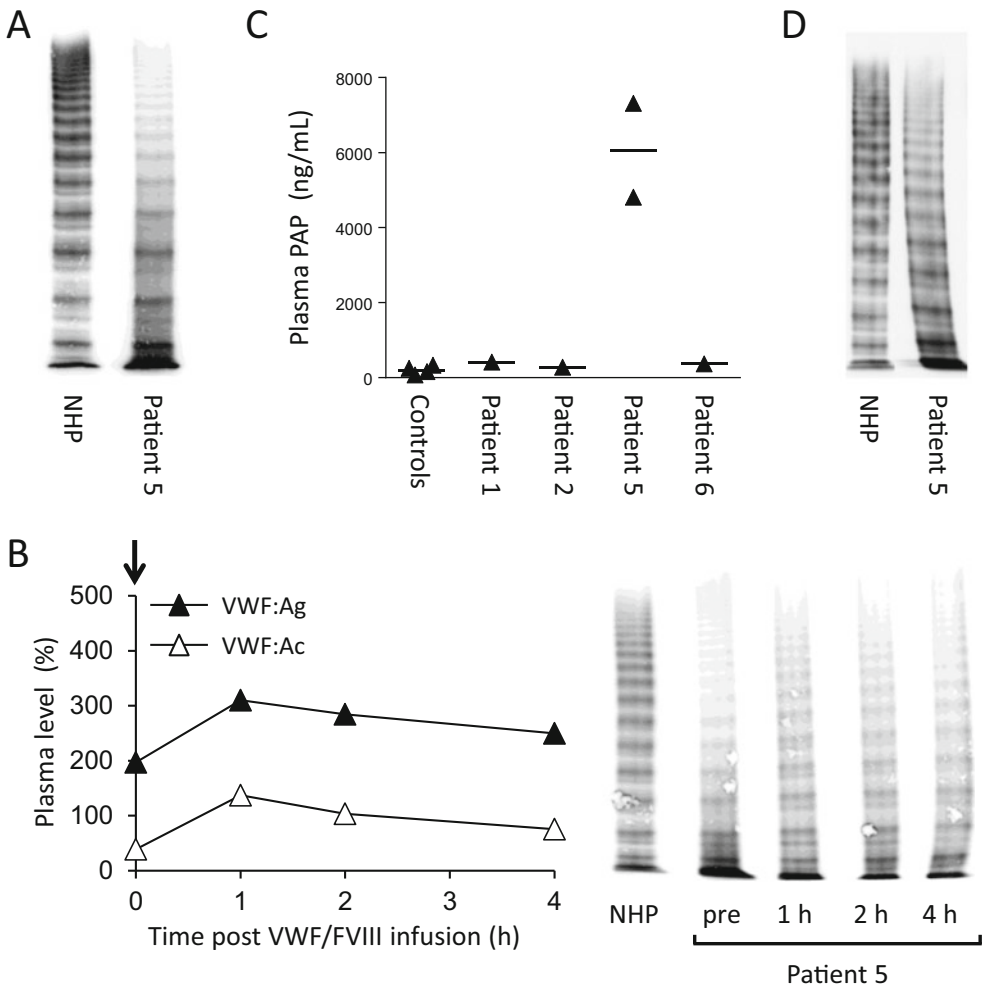


Fig. 3 AVWS in multiple myeloma with AL amyloidosis. **a** Multimer analysis of plasma VWF in patient 5 using a medium-resolution 1.6 % agarose gel. NHP denotes normal human plasma. **b** *Left panel*: Time course of VWF:Ag and VWF:Ac in patient 5 after the administration of VWF/FVIII at a dose of 80 IU VWF:RCo/kg. Time point of VWF/FVIII infusion is indicated by *arrow*. *Right panel*: Multimer analysis performed on plasma samples obtained immediately before as well as 1, 2, and 4 h after VWF/FVIII infusion shows no appreciable increase of larger multimers. Analysis was performed using a low-resolution 1.2 % agarose gel. *NHP* denotes normal human plasma. **c** Levels of PAP vcomplexes were measured in two plasma samples from patient 5 obtained 5 and 9 months after autologous PBSCT (also see Table 2). PAP levels measured in four healthy controls and patients 1, 2, and 6 are shown for comparison. **d** Multimer analysis of plasma VWF in patient 5 using a low-resolution 1.3 % agarose gel. Sample was obtained 9 months after autologous PBSCT. *NHP* denotes normal human plasma

confirmed 3 months later, when serum-free λ-LCs were measured at 91.0 mg/L. Importantly, this finding was paralleled by progressive plasminogen consumption, as indicated by a plasminogen plasma level of 59 %, and reappearance of highly atypical VWF triplets on multimer analysis (not shown). The patient continued to receive systemic therapy in a clinical trial.

AVWS in advanced myeloma

Patient 6 had IgG-lambda multiple myeloma (MM), stage III, and underwent spine surgery for an unstable vertebral fracture. The postoperative course was complicated by continuous thoracic bleeding requiring extensive blood product support and four surgical revisions.

At that time, serum IgG was 54 g/L and free λ-LCs were 3150 mg/L. Maximally prolonged PFA-100® closure times indicated a severe defect in primary hemostasis (Table 1). However, VWF parameters were not decreased, but actually above their respective reference ranges, and multimer analysis was normal (Fig. 4a). Routine light-transmission aggregometry revealed normal platelet aggregation in response to various agonists (e.g., ADP, collagen, TRAP-6), but virtually absent platelet agglutination in response to ristocetin at 1.2 or 1.5 mg/mL, a finding consistent with acquired (i.e., immune-mediated) Bernard-Soulier syndrome. However, testing the patient's serum for circulating antibodies to GPIb/IX or other platelet antigens and VWF was negative.

Table 2 PCD, fibrinolytic, and VWF parameters over the course of treatment in patient 5

	Normal	Initial	After TXA	After induction CTX	5 months after PBSCT	9 months after PBSCT	15 months after PBSCT
Free λ-LCs (mg/L)	5.7–26.3	217.8	n. d.	81.8	32.1	30.8	84.0
dFLC (mg/L)		216.8	n. d.	77.3	15.1	17.4	69.0
APTT (sec)	25–38	46	42	42	35	33	47
Fibrinogen (g/L)	1.8–4.0	1.8	2.2	4.6	3.9	5.3	3.5
D-dimer (mg/L)	<0.5	1.1	0.3	0.5	0.7	0.6	2.5
Plasminogen (%)	75–140	27	n. d.	76	88	86	83
α_2-antiplasmin (%)	80–120	24	38	25	42	89	47
PAP (ng/mL)	0–514	n. d.	n. d.	n. d.	7310	4809	19,446
VWF:Ag (%)	60–200	126	167	189	553	329	317
VWF:Ac (%)	61–179	20	19	52	52	70	44
Multimers		Type 2, atypical triplets	n. d.	Type 2, atypical triplets	Type 2, atypical triplets	Type 2, atypical triplets and loss of larger multimers less pronounced	n. d.

Abbreviations are as follows (in alphabetical order): *APTT* activated partial thromboplastin time, *CTX* chemotherapy, *dFLC* difference of serum free light chains, *LC* light chain, *n. d.* not done, *PAP* plasmin-α_2-antiplasmin, *PBSCT* peripheral blood stem cell transplantation, *TXA* tranexamic acid, *VWF* von Willebrand factor, *VWF:Ac* VWF GPIb binding activity, *VWF:Ag* VWF antigen

Flow cytometry indicated that the patient's platelets were not abundantly loaded with IgG (Fig. 4b). Furthermore, normal ADP-induced CD62P expression argued against an intrinsic platelet function defect (Fig. 4c). Mixing studies were performed to investigate whether abolished ristocetin-induced platelet agglutination (RIPA) was due to a plasma-borne or platelet-derived defect. First, we reproduced the finding that RIPA was selectively inhibited in the patient's (Fig. 4d, panel I), but not in a healthy control's platelet-rich plasma (PRP) (Fig. 4d, II). Second, we found that washed platelets from a healthy control suspended in the patient's plasma did not respond to ristocetin (Fig. 4d, III). Third, RIPA was restored when the patient's washed platelets were suspended in normal plasma (Fig. 4d, IV). Taken together, these findings indicated that abolished RIPA was due to a factor present in plasma, rather than on the platelet membrane.

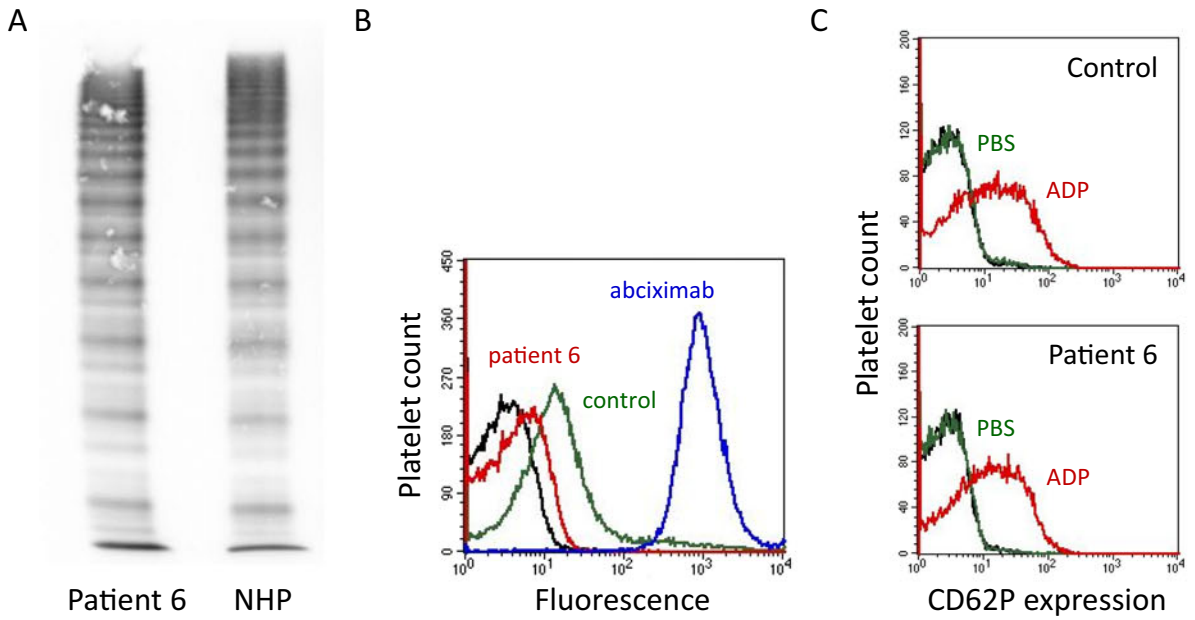
Induction and high-dose melphalan chemotherapy with autologous PBSCT resulted in normalization of serum IgG, free λ-LCs, and PFA-100® closure times. In addition, RIPA was completely restored in both the patient's PRP (Fig. 4d, V) and in a suspension of a healthy control's platelets in the patient's plasma (Fig. 4d, VI).

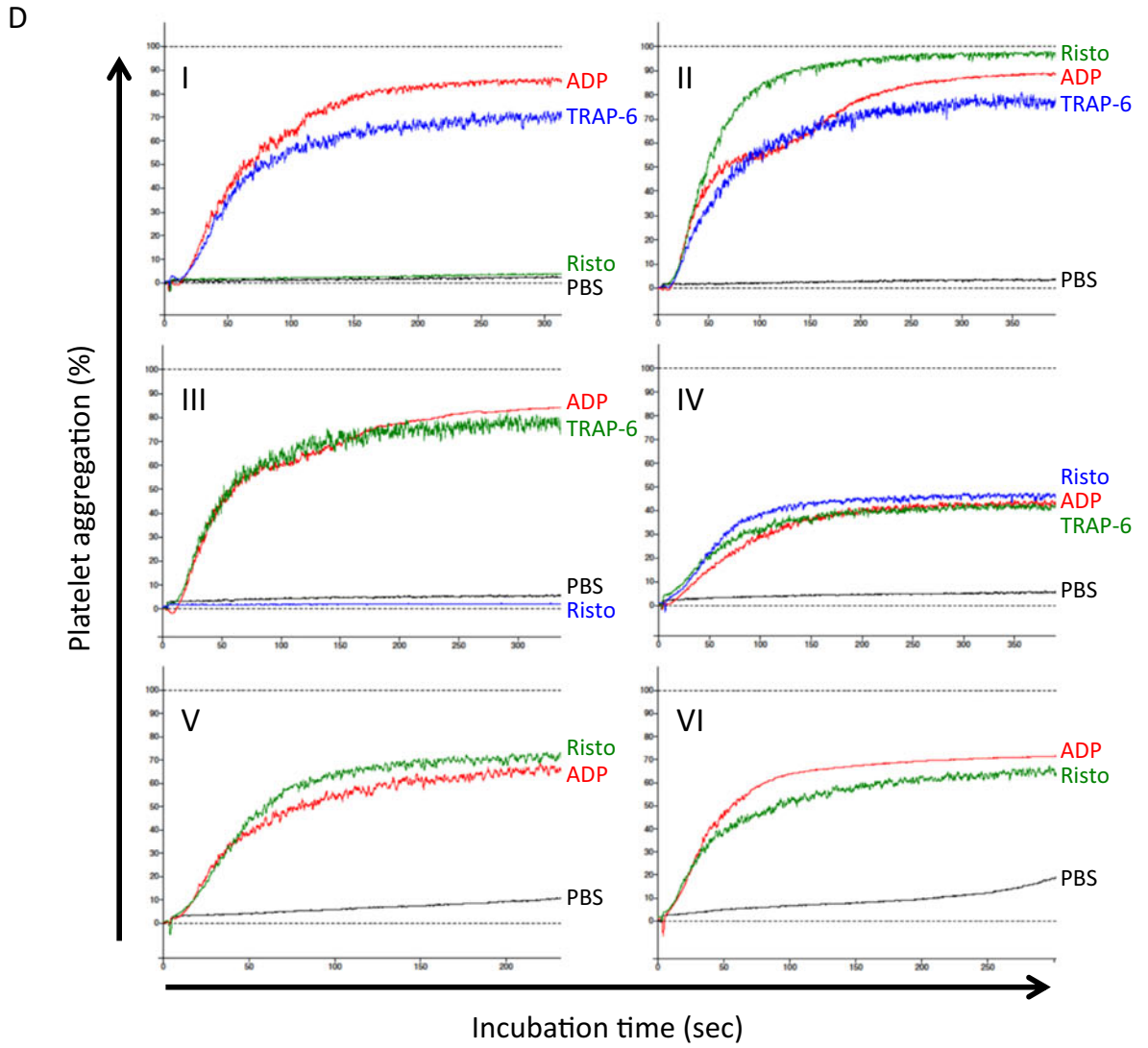
Discussion

In this report, we describe six additional cases of PCD-associated AVWS. Importantly, at least four different pathogenic mechanisms resulted in distinct structural or functional abnormalities of plasma VWF.

The pathophysiology of AVWS in IgG MGUS likely involves accelerated immunologic clearance of circulating VWF by cells of the reticuloendothelial system through an Fc-dependent mechanism [9, 10]. Consistent with immune-mediated VWF depletion, patients 1 and 2, both of whom had IgG MGUS, presented with markedly reduced plasma VWF, and multimer analysis indicated a quantitative rather than a qualitative defect (Fig. 1a, c). In contrast, a selective loss of larger VWF plasma multimers was evident in patient 3 (Fig. 1d). This type-2 pattern has previously been reported in

Fig. 4 Defective ristocetin-induced platelet agglutination in advanced multiple myeloma. **a** Multimer analysis of plasma VWF in patient 6 using a medium-resolution 1.6 % agarose gel. *NHP* denotes normal human plasma. **b** Flow cytometric analysis of IgG binding to platelets. Fixed platelets from patient 6 and a healthy control were incubated with FITC-conjugated rabbit polyclonal anti-human IgG antibody. As a positive control, platelets from a healthy donor were pre-incubated with abciximab, a humanized monoclonal Fab fragment against GPIIb/IIIa. The *black histogram* indicates autofluorescence of platelets (i.e., in the absence of FITC-conjugated anti-human IgG). **c** Platelets from a healthy control (*upper panel*) and patient 6 (*lower panel*) were incubated with buffer (PBS) or 20 μM adenosine diphosphate (ADP) and analyzed for CD62P (P-selectin) expression using a PE-conjugated monoclonal antibody. *Black histograms* indicate unstimulated platelets treated with PE-conjugated control IgG. **d** Results of light-transmission aggregometry using buffer (PBS), 1.5 μg/mL ristocetin (Risto), 20 μM ADP, or 50 μM thrombin receptor activator peptide (TRAP-6). Samples are as follows: I, platelet-rich plasma (PRP) from patient 6 at initial presentation; II, PRP from a healthy control; III, platelets from a healthy control suspended in the patient's plasma; IV, platelets from patient 6 suspended in a healthy control's plasma; V, PRP from patient 6 after remission induction; VI, platelets from a healthy control suspended in the patient's plasma after remission induction


A
Patient 6
NHP
B
Platelet count
abciximab
patient 6
control
Fluorescence
C
Platelet count
Control
PBS
ADP
Patient 6
PBS
ADP
CD62P expression


D
Platelet aggregation (%)
I
ADP
TRAP-6
Risto
PBS
II
Risto
ADP
TRAP-6
PBS
III
ADP
TRAP-6
PBS
Risto
IV
Risto
ADP
TRAP-6
PBS
V
Risto
ADP
PBS
VI
ADP
Risto
PBS
Incubation time (sec)

other patients with IgG MGUS [9] and may point to distinct mechanisms of AVWS within this PCD entity.

Our findings in patients 1, 3, and 4 are consistent with a lack of effect of desmopressin and VWF concentrate in patients with AVWS due to an IgG or IgM paraprotein [11, 12]. However, in contrast to patients with IgM MGUS or WM, those with IgG MGUS typically respond to IVIG, showing a transient correction of VWF/FVIII levels for up to 2–3 weeks [10]. The specific mechanisms by which IVIG diminishes clearance of circulating VWF in IgG MGUS is not completely understood, but may involve inhibition of pathogenic VWF antibodies or B-cell receptors by transfused anti-idiotype antibodies, blockage of macrophage Fc receptors, and/or activation of the complement system [13].

Inhibitory autoantibodies to VWF have rarely been detected using functional neutralization assays [14–16]. Although immunoassays with immobilized plasma-derived VWF provide the possibility to detect non-neutralizing antibodies, they are prone to false-positive results due to cross-reactivity of isoagglutinins with ABH antigen structures on N-linked oligosaccharide chains present on VWF [17].

Interestingly, we could not detect anti-VWF antibodies in plasmas from 23 patients with AVWS due to IgG ($n=14$) or IgM MGUS ($n=9$) using an in-house ELISA, while strongly positive results were obtained in two patients with autoimmune-mediated AVWS and one patient with congenital von Willebrand disease type 3 and VWF alloantibodies [6, 18]. It may be speculated that, in IgG MGUS, the paraprotein is saturated in complexes with VWF, thus preventing its detection by ELISA. Alternatively, the IgG paraprotein may not (always) function as a VWF autoantibody in MGUS-associated AVWS.

Both the magnitude and the kinetics of the VWF response to IVIG, which was administered at the same dose and schedule, were different in patients 1 and 3 (Fig. 1b, d). Based on this observation and the distinct multimer pattern, it is tempting to speculate that additional (i.e., non-immunologic) mechanisms, such as enhanced ADAMTS13-mediated VWF degradation upon binding of plasma VWF to GPIb aberrantly expressed on clonal plasma cells, contributed to the evolution of AVWS in patient 3 [19].

AVWS in IgM MGUS or WM is characterized by an unmistakable multimer pattern, similar to that observed in patient 4 (Fig. 2b, c), which is presumably due to migration of giant VWF-IgM complexes into the gel [20]. Consistently, consecutive staining for IgM and VWF indicated that the paraprotein indeed associated with plasma VWF and thus interfered with proper migration of multimers into the gel (Fig. 2d, e). Importantly, the multimer pattern normalized after plasmapheresis with no apparent co-localization of IgM and VWF. Although serum IgM was still elevated at 21–32 g/L during follow-up, plasma VWF parameters stayed normal with an unremarkable activity-to-antigen ratio. These findings pointed to a more loose interaction of the IgM paraprotein with plasma VWF (rather than specific high-affinity binding) and indicated that a much higher IgM threshold level was required to cause AVWS recurrence. Further arguing against a specific immune-mediated mechanism, we could not find a circulating VWF antibody by ELISA.

In patient 5, initial laboratory findings were consistent with activation of the fibrinolytic system (Table 2), which has previously been observed in patients with systemic amyloidosis [21]. An abnormal triplet structure of VWF oligomers further suggested that aberrant plasmin generation indeed contributed to AVWS (Fig. 3a).

AVWS with decreased VWF:RCo, but normal-to-increased VWF:Ag and FVIII:C levels has been reported in four patients with AL amyloidosis [22], two of whom had selective loss of larger multimers. Although fibrinolytic markers were not assessed in this study, activation of fibrinolysis contributing to AVWS was demonstrated in two other PCD patients, one with AL amyloidosis and one with IgM MGUS [23, 24]. Treatment with TXA over several months resulted in normalization of fibrinolytic parameters and plasma VWF. Our patient, however, did not respond to TXA, possibly due to the relatively short treatment duration.

Plasmin is a non-specific protease that, besides fibrin, also cleaves fibrinogen and VWF [25]. The conversion of plasminogen to plasmin is mediated by tissue- (t-PA) or urokinase-type plasminogen activator (u-PA). Binding to fibrin dramatically increases t-PA-, but not u-PA-mediated plasminogen activation [26, 27]. While t-PA is the principal activator of intravascular fibrinolysis, u-PA is predominantly involved in extracellular proteolysis relevant to cell migration and tissue remodeling. Importantly, u-PA expression may be upregulated in cancer cells [28, 29]. In this regard, it is noteworthy that we also measured increased plasma levels of u-PA (1178, 1589, and 1642 pg/mL) in the same three plasma samples that were used for analysis of PAP complexes (Fig. 3c, Table 2). In comparison, u-PA plasma levels were 280–413 pg/mL in patients 1, 2, and 6.

Excessive activation of the fibrinolytic system through overexpression of u-PA has been observed in patients with AL amyloidosis, but not in those with MM [30]. It is tempting to speculate that aberrant expression of u-PA by bone marrow plasma cells contributed to AVWS in our patient by promoting plasmin-mediated VWF degradation. At initial presentation, significantly decreased plasminogen and α_2-antiplasmin levels indicated exhaustion of the antifibrinolytic system. The subsequent course of laboratory parameters supports our hypothesis that the degree of plasmin generation was related to PCD activity: consistent with a very good partial, but not complete response to autologous PBSCT, plasminogen and α_2-antiplasmin levels normalized, while markedly increased PAP plasma levels still indicated significant fibrinolytic activity (Table 2). Under these conditions, plasmin-mediated VWF

proteolysis likely occurred on the surface of plasma cells rather than within the circulation, which might have been the case at initial presentation. Although AVWS was still present 9 months after PBSCT, the relative combined proportion of larger and intermediate-sized VWF plasma multimers had increased from 3.5 to 31.5 % (normal, 70–75 %) over the course of treatment (Fig. 3a, d). Taken together, upon binding of VWF to aberrantly expressed GPIb on the surface of clonal plasma cells, both ADAMTS13- and plasmin-mediated VWF proteolysis may have contributed to AVWS in our patient [19]. In this regard, the less prominent pathological triplet structure of VWF oligomers 9 months after PBSCT (i.e., during a very good partial response) (Fig. 3d) is consistent with decreased plasmin-, but continued ADAMTS13-mediated VWF degradation. Based on these findings, future studies should more systematically assess plasmin generation and ADAMTS13 activity in patients with PCD-associated AVWS to better characterize the various mechanisms of VWF proteolysis in this rare bleeding disorder.

Patient 5 presented with a prolonged APTT that was not due to a deficiency in one or more of the intrinsic clotting factors VIII, IX, XI, or XII. Although we cannot rule out the possibility of a lupus anticoagulant, normalization of the APTT following efficacious treatment of the underlying PCD suggested that circulating paraprotein components may have specifically interfered with proper fibrin generation and/or polymerization in the APTT assay [31]. In support of this hypothesis, PCD progression at 15 months after PBSCT was accompanied by the reoccurrence of APTT prolongation in patient 5 (Table 2).

The hemostatic defect in patient 6 was characterized by abolished RIPA. In combination with maximally prolonged PFA-100® closure times and normal platelet aggregation in response to other agonists, this finding initially prompted us to suspect acquired Bernard-Soulier syndrome. However, mixing experiments clearly indicated a functional defect in plasma VWF and not platelet GPIb (Fig. 4d). Normal findings on multimer analysis further suggested that abolished VWF function was due to an inhibitor present in plasma.

Under fluid shear stress, globular VWF unwinds to a string-like structure, which exposes binding sites for platelet GPIb within the VWF A1 domain [32]. This shear-induced conformational change can be mimicked by the bacterial glycopeptide ristocetin, which presumably interacts with two sites outside the A1 loop, allowing binding of VWF to platelet GPIb in the absence of high shear stress [33]. Intriguingly, VWF:Ac was actually increased in our patient. Similar to the VWF:RCo assay, the VWF:Ac test is based on the binding of VWF to GPIb, but it uses a recombinant GPIb fragment carrying two gain-of-function mutations that enables binding of VWF in the absence of ristocetin. Unfortunately, we were not able to test the patient's initial plasma sample in a "classical" VWF:RCo assay, which uses fixed platelets as a source for GPIb.

Inhibition of both ristocetin- and shear stress-induced binding of the VWF A1 domain to platelet GPIb due to a paraprotein has been shown in a patient with IgD-lambda MM [34]. Similar to our case, RIPA was dramatically reduced, while VWF:Ag and multimer analysis were normal. However, a VWF:RCo assay using formalin-fixed platelets revealed significantly decreased VWF activity. These findings indicate that a VWF activity assay dependent on either ristocetin- (i.e., VWF:RCo or RIPA) or shear stress-induced conformational changes within the VWF A1 domain (i.e., PFA-100®) is required to detect the specific hemostatic defect observed in the previous and our patient. In contrast, using only the INNOVANCE® VWF:Ac test as a functional readout might miss the correct diagnosis in a bleeding PCD patient.

The disappearance of paraprotein (components) following autologous PBSCT was accompanied by complete normalization of hemostatic parameters in patient 6, supporting our hypothesis that the circulating paraprotein inhibited ristocetin- and shear stress-induced binding of the VWF A1 domain to platelet GPIb.

In summary, our case series delineates distinct mechanisms of AVWS in patients with PCDs. All of these mechanisms should thus be considered in clinical practice when evaluating PCD patients with severe bleeding symptoms.

Author contribution CD gathered clinical data, designed and interpreted experiments, and wrote the first draft of the manuscript. SS, KH, CB, RD, and UB designed experiments, interpreted clinical and laboratory data, and critically revised the manuscript. BS conducted experiments and interpreted data. FL oversaw the study, designed and interpreted experiments, and co-wrote the manuscript.

Compliance with ethical standards All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

Conflict of interest The authors declare that they have no conflict of interest. This case series has not been published elsewhere nor is it under consideration for publication elsewhere.

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