Variations in glycosylation of von Willebrand factor with O-linked sialylated T antigen are associated with its plasma levels

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The glycosylation profile of von Willebrand factor (VWF) is known to strongly influence its plasma levels. VWF contains several carbohydrate structures, including O-linked glycans that primarily consist of sialylated T antigen (NeuAcα2-3Gal-[β1-3]-[NeuAcα2-6]GalNAc). It is not yet known whether O-linked carbohydrates affect VWF levels. We developed an immunosorbent assay based on neuraminidase incubation allowing subsequent binding of peanut agglutinin (PNA) to desialylated O-linked T antigen on VWF. An inverse relation was found between PNA binding and VWF antigen levels in healthy individuals (n = 111; Pearson rank = −0.43; P < .001). A similar inverse association was observed in randomly selected plasma samples from our diagnostic laboratory: 252% ± 125% for VWF levels less than 0.5 U/mL (n = 15); 131% ± 36% for VWF levels between 0.5 and 1.5 U/mL (n = 32); and 92% ± 40% for VWF levels more than 1.5 U/mL (n = 19). Reduced or increased PNA binding was also observed in patients with increased (liver cirrhosis) or reduced (von Willebrand disease [VWD] type 1) VWF antigen levels, respectively. VWD type 1 patients further displayed increased ratios of propeptide over mature VWF antigen levels (0.38 ± 0.18 versus 0.17 ± 0.03 for patients and controls, respectively; P < .001), which is indicative of reduced VWF survival in these patients. Of interest, a linear relation between PNA binding and propeptide/VWF ratio was observed (Spearman rank = 0.47), suggesting a potential association between O-linked glycosylation and VWF survival. Finally, we detected a marked decrease in PNA binding in post-DDAVP (1-deamino-8-D-arginine vasopressin) samples from various patients, indicating that the O-linked glycosylation profile of VWF stored in endothelial storage organelles may differ from circulating VWF. (Blood. 2007;109: 2430-2437)© 2007 by The American Society of Hematology

Introduction

von Willebrand factor (VWF) is a multimeric glycoprotein that plays a dual role in the hemostatic process. First, VWF forms a complex with coagulation factor VIII, which enhances factor VIII survival in vivo. Second, VWF contributes to platelet adhesion and aggregation by acting as a molecular bridge between subendothelial collagen and platelets.1,2 The relevance of VWF for the hemostatic system is apparent from the notion that deficiency or dysfunction of VWF is associated with von Willebrand disease (VWD), the commonest inherited bleeding disorder.3 VWD can be categorized in qualitative VWF defects (VWD type 2) or quantitative VWF deficiencies (VWD type 1 and type 3; partial and virtually complete deficiency, respectively). Partial deficiencies as defined for VWD type 1 may be due to decreased synthesis, impaired secretion, increased clearance, or combinations thereof.

The dominant source of circulating VWF is the endothelium, where VWF is synthesized as a single prepropeptide chain with a discrete domain structure: D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK.4 After removal of the signal peptide upon entering the endoplasmic reticulum (ER), disulphide bonding within the carboxy-terminal CK domains endows formation of pro-VWF dimers. Further processing proceeds within the Golgi, and involves multimerization of pro-VWF dimers through the formation of intermolecular cysteine bonds within the D'-D3 domains. Proteolytic processing in the trans-Golgi network separates the propeptide (D1-D2 domains) from the mature VWF multimer.3 Following synthesis in endothelial cells, VWF and its propeptide may be stored in endothelial-specific storage organelles, the Weibel-Palade bodies.6 While stored in these organelles, the multimerization process may continue, giving rise to VWF molecules that consist of higher molecular weight multimers compared with those that circulate in plasma. These high-molecular-weight multimers have a higher hemostatic potential, and can be released from the Weibel-Palade bodies into the circulation upon stimulation of endothelial cells by various agonists, for instance after desmopressin treatment.7

Analysis of the primary VWF sequence predicts the presence of 10 O-linked and 12 N-linked glycosylation sites,8 and biochemical analysis has demonstrated the presence of carbohydrate residues at

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these sites.\textsuperscript{9,10} Initial N-linked carbohydrate structures are coupled to VWF in the early stage of synthesis.\textsuperscript{11} Further processing into complex N-linked side chains proceeds within the Golgi, where O-linked glycosylation also occurs, as well as the attachment of sialyl groups to both O- and N-linked sugars. VWF is one of the rare plasma proteins for which it has been demonstrated that the N-linked carbohydrates contain ABO blood group determinants.\textsuperscript{9,10} These blood group antigens, however, are not present on the O-linked carbohydrates. Of interest, the nature of the blood group determinant strongly influences VWF levels: the average VWF levels are approximately 25% lower in persons with blood group O than those in non-O individuals.\textsuperscript{12} A second indication that the glycosylation profile of VWF is an important determinant of VWF plasma levels is illustrated by the finding that the half-life of endogenous VWF is reduced 2-fold in mice genetically deficient for the sialyl-transferase ST3Gal-IV.\textsuperscript{13} Moreover, in a patient group referred to the hospital for real or suspected bleeding disorder, reduced ST3Gal-IV-mediated sialylation was found to be associated with reduced VWF plasma levels.\textsuperscript{13}

As for the O-linked carbohydrates, no data have been reported about their correlation with VWF plasma levels, apart from one report by Stoddart et al who demonstrated that recombinant VWF lacking O-linked carbohydrates has a reduced half-life when administered in rats.\textsuperscript{14} In the 1980s, studies were performed regarding the analysis of O-linked carbohydrate structures using VWF purified from therapeutic preparations. Initial analysis revealed some heterogeneity of the O-linked carbohydrate moiety,\textsuperscript{15} whereas more detailed studies using a combination of methylation studies and nuclear magnetic resonance (NMR) spectroscopy demonstrated that the majority (at least 70%) of O-linked carbohydrates is composed of the sialylated tumor-associated T antigen.\textsuperscript{16} This structure consists of the disaccharide galactose-(\(\beta\)-1-3)-N-acetylgalactosamine and is sialylated through capping with 2 N-acetylneuramic acid residues (ie, NeuAc(\(\alpha\)-2,3)-Gal-(\(\beta\)-1-3)-[NeuAc(\(\alpha\)-2,6)-GalNAc). To investigate a potential relation between the occurrence of this glycan structure on circulating VWF and VWF levels, we have designed an assay in which VWF-linked carbohydrates are desialylated using neuraminidase, allowing subsequent detection of the remaining T-antigen structure using the lectin peanut agglutinin (PNA). We observed an inverse nonlinear association between PNA binding and VWF plasma levels, which was most pronounced in patients suffering from liver cirrhosis and VWD type 1.

### Patients, materials, and methods

#### Plasma samples

Plasma from healthy volunteers (n = 111) and patients (n = 66) was collected in 3.1% citrate using a Vacutainer system (Becton Dickinson, Lincoln Park, NJ). These patient samples were randomly provided in an anonymous manner by our diagnostic laboratory. Fifty-four patients in stable condition with biopsy-proven liver cirrhosis of various etiology (including alcohol abuse, viral hepatitis, autoimmune hepatitis, primary biliary cirrhosis, and cryptogenic cirrhosis) were included in this study.\textsuperscript{17} The patients were classified according to Pugh’s modification of the Child classification.\textsuperscript{18} Nineteen patients with Child A cirrhosis, 17 patients with Child B cirrhosis, and 18 with Child C cirrhosis were studied. VWD type 1 patients (n = 32) were classified as VWD type 1 on the basis of bleeding symptoms and VWF-related laboratory criteria (antigen, ristocetin cofactor activity, and/or multimeric pattern). For some VWD type 1 patients, the underlying genetic defect has been determined (Table 1). Plasma samples of unaffected family members (n = 15) were included as control. Other patients (VWD type 2A and 2N, hemophilia A, and primary platelet function disorder) were diagnosed using the institutional criteria for these diseases, and anonymous samples were provided for the present study. Treatment with 1-deamino-8-D-arginine vasopressin (DDAVP) was given as part of routine patient care. For normal pooled plasma (NPP), platelet-poor plasma from 40 healthy individuals was pooled and stored in aliquots at \(-80^\circ\)C. All patients gave informed consent for the sampling of blood for scientific purposes, per the Declaration of Helsinki. Approval was obtained from the institutional review boards of the University Medical Center Utrecht (Utrecht, the Netherlands), Erasmus Medical Center (Rotterdam, the Netherlands), Leiden University Medical Center (Leiden, the Netherlands), Medical University of Lille (Lille, France), and Centre Hospitalier Universitaire (Nantes, France).

#### Proteins and antibodies

Polyclonal antibodies against VWF, horseradish peroxidase (HRP)–conjugated antibodies against VWF, and HRP-conjugated streptavidin were obtained from DakoCytomation (Glostrup, Denmark). Recombinant llama-derived antibody fragment directed against the A1 domain of VWF (control nanobody) and in-house polyclonal antibodies against VWF D'-D3 domains have been described previously.\textsuperscript{21,22} Arthrobacter ureafaciens–derived neuraminidase was purchased from Calbiotech (San Diego, CA). Avidin/biotin blocking kit, biotinylated PNA (btPNA), GalNAc, and galactose were obtained from Vector Laboratories (Burlingame, CA). Recombinant O-glycosidase, GlcNAc, and glucose were from Sigma (St Louis, MO), while Gal-(\(\beta\)-1-3)-GalNAc was obtained from Dextra Laboratories (Berkshire, United Kingdom).

#### VWF

Plasma-derived (pd) VWF was purified from Haemate P (250 IU; Behringwerke, Marburg, Germany) as described.\textsuperscript{23} Expression and purification of recombinant wild-type (wt)–VWF and VWF/D'-D3 have been described previously.\textsuperscript{22,24} CDNA encoding VWF lacking the propeptide (D1-D2 domains) was constructed by fusion of the sequences encoding the VWF signal peptide (amino acids 1-22) and mature VWF (amino acids 764-2813)
using standard molecular biological techniques. The sequence of the resulting VWF/delta-pro product was verified, and the construct was subsequently cloned into the pNUT vector.24 cDNAs encoding A1 domain residues 1238 to 1494 and 1260 to 1481 (A1/1238-1494 and A1/1260-1481) were obtained by polymerase chain reaction using wt-VWF as a template. PCR products were sequenced and cloned into a pNUT expression vector encoding a C-terminal 6-histidine tag.21 Expression of VWF/delta-pro, A1/1238-1494, and A1/1260-1481 in serum-free medium using baby hamster kidney cells was performed as described for wt-VWF.24

VWF and propeptide antigen assays

VWF antigen levels were quantified as described before.25 Propeptide antigen levels were determined using in-house polyclonal rabbit antibodies raised against purified recombinant propeptide. In brief, protein G–Sepharose–purified polyclonal antibodies (5 

Microtiter wells (Maxisorb; Nunc, Roskilde, Denmark) were coated with polyclonal anti-VWF antibodies (1 

Wells were then blocked using the PBS to reach a VWF antigen concentration between 1 and 10 mU/mL, hour at 37°C. Samples (either plasma or purified proteins) were diluted in dilution-buffer. After washing 3 times with wash-buffer, plasma samples for 1 hour at 37°C. Serial dilutions of plasma samples were prepared using dilution-buffer. After washing 3 times with wash-buffer, plates were incubated with HRP-conjugated polyclonal antipropeptide antibodies (1 

bound propeptide was detected by measuring HRP activity using o-phenylenediamine (OPD; Merck, Darmstadt, Germany) as a substrate. NPP was used as a reference in both VWF and propeptide antigen assays.

Immunosorbent assay for VWF-linked T antigen

Microtiter wells (Maxisorb; Nunc) were coated with polyclonal anti-VWF antibodies (3 

PBS/0.1 mM CaCl2 overnight at 37°C. Wells were washed 3 times with PBS/0.1% (vol/vol) Tween-20, wells were blocked with the same buffer supplemented with 1% (wt/vol) bovine serum albumin and 0.2 M EDTA (dilution-buffer) for 1 hour at 37°C. Wells were washed 3 times with wash-buffer and incubated with plasma samples for 1 hour at 37°C. Serial dilutions of plasma samples were prepared using dilution-buffer. After washing 3 times with wash-buffer, plates were incubated with HRP-conjugated polyclonal antipropeptide antibodies (1 

wells were subsequently incubated with neuraminidase (5 mU/mL) in PBS/1 mM CaCl2 overnight at 37°C. Wells were then blocked using the avidin/biotin blocking kit according to the manufacturers' instructions. After washing with PBS/0.1% (vol/vol) Tween-20, biPNA (5 

HRP activity using OPD as a substrate. PNA binding was quantified by calculating the relative slopes of the initial linear parts of the curves. NPP was used as a reference and the slope for NPP was referred to as 100%. In control experiments, specificity was tested using VWF-deficient plasma, which revealed no PNA binding in the absence of VWF (not shown). To determine the intraexperimental variation of the assay, 10 randomly chosen wells were incubated with one sample (healthy volunteer no. 12). This sample was also tested in 10 different experiments to determine interexperimental variation. These tests revealed that the intraexperimental variation was 10% and the interexperimental variation was 16%. Throughout the paper, PNA binding refers to neuraminidase-treated samples, unless stated otherwise. In none of the samples tested was PNA binding detected when neuraminidase-incubation was omitted.

Data analysis and statistics

Analysis of data was performed using Graphpad Prism program (Graphpad Prism version 4.0 for Windows; GraphPad Software, San Diego, CA). All data are expressed as mean with SD. To address statistical differences, data were analyzed using Student unpaired 2-tailed t test, with the Welch correction being performed when necessary. P values less than .05 were considered significant.

Results

An immunosorbent assay to detect O-linked sugars on VWF

It has previously been shown that O-linked sugars on VWF primarily consist of the sialylated T antigen.16 To detect the presence of this glycan on circulating VWF, a PNA-based immunosorbent assay was developed, in which 3 main steps can be distinguished: (1) diluted samples are applied to microtiter wells coated with anti-VWF antibodies; (2) bound VWF is desialylated via incubation with neuraminidase; and (3) biPNA is added, and bound biPNA is detected using streptavidine-coupled peroxidase.

To optimize this assay, we first determined which amount of neuraminidase is needed to remove sialyl groups from VWF. Various concentrations of neuraminidase (0-4 mU/mL) were added to a fixed amount of antibody-bound pd-VWF (50 mU/mL). No binding of biPNA could be detected in the absence of neuraminidase (not shown), consistent with the fact that PNA binds only to desialylated Gal-(β1-3)-GalNAc. Increasing concentrations of neuraminidase progressively improved biPNA binding, and near maximal desialylation occurred at 2.5 mU/mL neuraminidase. As for biPNA, a dose-dependent binding could be observed when added to neuraminidase-treated VWF (not shown). Half-maximal binding was observed at 1.5 ± 0.2 µg/mL. Given these data, we decided to use 5 mU/mL neuraminidase and 5 µg/mL biPNA throughout the study.

Specificity of PNA binding to VWF

The specificity of the assay was then tested in 2 types of experiments. First, binding of biPNA (5 µg/mL) to neuraminidase-treated VWF (50 mU/mL) was assessed in the presence of various carbohydrate structures. Binding of biPNA to VWF was unaffected in the presence of glucose, GalNAc, GalNac, or galactose (IC50 at > 1.0 mM; Figure 1A), even when concentrations up to 10 mM were used (not shown). In contrast, biPNA binding was efficiently inhibited in the presence of the T-antigen structure Gal-(β1-3)-GalNAc (IC50, 0.3 mM; Figure 1A). In an alternative approach, the specificity of binding was examined using a number of recombinant VWF variants, including recombinant wt-VWF and VWF/delta-pro (representing fully multimerized and dimeric VWF, respectively). Both recombinant wt-VWF and VWF/delta-pro were similar to pd-VWF in their ability to bind biPNA (Figure 1B). Also, no difference in PNA binding was observed for VWF preparations enriched in either high- or low-molecular-weight multimers (not shown), indicating that biPNA binding is independent of the extent of multimerization. In addition, we observed little, if any (< 5% compared with wt-VWF) binding of biPNA to VWF/D′-D3, a dimeric construct that contains 3 N-linked but no O-linked carbohydrates. Moreover, PNA binding was reduced by more than 85% upon removal of O-linked glycans via incubation with O-glycosidase (Figure 1B). We further compared 2 distinct A1 domain constructs, encompassing residues 1238 to 1494 and 1260 to 1481; these constructs comprise 8 and 2 Ser/Thr residues, respectively, that are known to be O-glycosylated in pd-VWF (Figure 1C). To analyze biPNA binding in these experiments, proteins were immobilized using a recombinant llama-derived antibody fragment directed against the A1 domain instead of polyclonal anti-VWF antibodies. Binding of biPNA to the short A1/1260-1481 fragment was decreased by 66% ± 7% compared with the long A1/1238-1494 fragment (Figure 1C). Taken together, these data indicate that PNA binding is (1) specific to the...
of our institute (n = 66). In these samples, VWF levels varied to a larger extent compared with the healthy volunteers: 0.13-4.1 U/mL versus 0.44-2.9 U/mL, respectively. Again, an inverse relationship was found between PNA binding and VWF levels (Figure 2C), the data of which were best fitted using an equation describing a single exponential decay ($\tau^2 = 0.57$). This relationship became more apparent by arbitrarily dividing the samples into 3 subgroups: (1) increased VWF levels (>1.5 U/mL: mean ± SD = 2.2 ± 0.7 U/mL, n = 19); (2) intermediate VWF levels (between 0.5 and 1.5 U/mL: 1.1 ± 0.3 U/mL, n = 32); and (3) reduced VWF levels (<0.5 U/mL: 0.3 ± 0.1 U/mL, n = 15). PNA binding in the group with reduced VWF levels (252% ± 125%) was higher than in the other 2 groups ($P = .002$ and $P = .001$ for the intermediate and high group, respectively; Figure 2D). Moreover, a significant difference was found between the intermediate and high VWF-level groups (131% ± 36% and 92% ± 40%, respectively; $P < .001$; Figure 2D). Apparently, there seems to be an association between VWF levels and the presence of the sialylated O-linked Gal-(β1-3)-GalNAc structure on VWF.

### Reduced PNA binding to VWF in patients with liver cirrhosis

To investigate the relationship between VWF plasma levels and the presence of Gal-(β1-3)-GalNAc structures in more detail, we examined plasmas of patients with liver cirrhosis and elevated VWF levels. This patient group has been described previously, and 3 categories of patients can be distinguished according to Pugh’s modification of the Child classification. The VWF levels were 4.5 ± 2.3 U/mL (n = 19), 5.5 ± 2.8 U/mL (n = 17), and 7.8 ± 3.4 U/mL (n = 18) for Child A, Child B, and Child C–classified patients, respectively. When tested for PNA binding, desialylated O-linked T-antigen structure on VWF, (2) dependent on the number of O-linked glycans, and (3) independent of the extent of multimerization.

### Association between PNA binding and VWF plasma levels

The PNA-based assay was subsequently used to determine VWF molecules containing O-linked Gal-(β1-3)-GalNAc structures in plasma samples of healthy volunteers (n = 111). The average VWF antigen level in this group was 1.2 ± 0.5 U/mL. As expected, VWF levels were lower in blood group O individuals compared with non-O individuals (1.0 ± 0.3 U/mL versus 1.3 ± 0.5 U/mL for O [n = 38] and non-O [n = 73] individuals, respectively; $P < .001$). PNA binding was quantified by measuring absorbance at 490 nm versus concentration of VWF in diluted samples for NPP (X) and a representative sample (B). The slope found for NPP was set at 100%. The slope for this particular sample was calculated to be 177%. Relative PNA binding was determined for plasma samples from healthy volunteers (n = 111) and samples that were randomly provided by our diagnostic laboratory (n = 66; C). The dotted lines indicate VWF values of 0.5 and 1.5 U/mL. The drawn solid lines were obtained by fitting the data in a model for linear regression (Pearson rank = −0.43; $P < .001$; Figure 2B). PNA binding was higher in persons with blood group O than in non-O individuals (104.5% ± 15.9% versus 92.6% ± 20.3%, respectively; $P < .002$). A similar analysis was also performed in a series of plasma samples that were obtained via the diagnostic laboratory.
we could again observe an inverse nonlinear relationship between VWF and PNA binding (Figure 3A). In addition, a correlation between the severity of the disease and relative PNA binding was observed when the patients were subdivided according to the Child classification (Figure 3B). The more severe the disease, the lower the PNA reactivity toward VWF: 53% ± 21%, 41% ± 20%, and 31% ± 16% for Child A, Child B, and Child C-classified patients, respectively. These data indicate that increased VWF levels in liver cirrhosis patients are characterized by a suboptimal glycosylation pattern with O-linked Gal-(β1-3)-GalNAc structures on VWF and reduced survival of VWF.

**Increased PNA binding in patients with VWD type 1**

To study binding of PNA to VWF present in plasma with reduced VWF levels, we collected samples from patients with VWD type 1 (n = 32), the genetic defect of which was established in 20 patients (Table 1). As a control, plasma from healthy volunteers (n = 111) and nonaffected type 1 family members (n = 15) was used. The average VWF antigen levels were 1.2 ± 0.5 U/mL, 0.7 ± 0.2 U/mL, and 0.3 ± 0.2 U/mL for healthy volunteers, nonaffected family members, and type 1 patients, respectively (Figure 4A). PNA binding was significantly increased in nonaffected family members (119% ± 24%; P < .001) and to a larger extent in samples from VWD type 1 patients (171% ± 58%; P < .001; Figure 4B) when compared with healthy volunteers (96.7% ± 20.7%). Thus, low levels of VWF present in VWD type 1 patients and (to a lesser extent) their unaffected family members are associated with increased PNA binding upon neuraminidase incubation, suggesting the presence of a surplus of O-linked sialylated T antigen on their VWF molecules.

**Association between PNA binding and propeptide/VWF ratio in VWD type 1**

Shortage of circulating VWF may originate from abnormalities in the balance between production and clearance. A surrogate marker for VWF clearance is represented by propeptide/VWF ratios, which are indeed increased in several VWD type 1 patients who have an unusually short VWF survival.27 As for VWD type 1 patients in the present study, the average propeptide/VWF ratio was increased 2-fold compared with controls (0.38 ± 0.18 and 0.17 ± 0.03 for patients and controls, respectively; P < .001; Figure 5A), suggesting that VWF is cleared more rapidly, at least in some of the patients. When divided in quartiles with respect to propeptide/VWF ratios, patients with the lowest ratios (ranging from 0.14 to 0.23; n = 8) had mean VWF levels of 0.45 ± 0.20 U/mL and PNA binding was 145% ± 51%. For the quartile with the highest ratios (0.46-0.82; n = 8), values were 0.22 ± 0.08 U/mL and 229% ± 77% for VWF antigen levels and PNA binding, respectively. For both parameters, these values were significantly different between both quartiles (P < .05). We then plotted the extent of PNA binding versus propeptide/VWF ratios for all patients (Figure 5B). Of interest, a linear correlation between PNA binding and propeptide/VWF ratios was observed (Spearman rank = 0.50; P = .004). Apparently, an increased extent of glycosylation with O-linked T antigen is associated with high propeptide/VWF ratios. This may point to a relationship between increased numbers of sialylated Gal-(β1-3)-GalNAc structures on VWF and reduced survival of VWF.

**Dissimilar PNA binding to pre- and post-DDAVP VWF**

Part of VWF that is synthesized in endothelial cells remains stored intracellularly in Weibel-Palade bodies. These Weibel-Palade bodies release their content upon DDAVP treatment, which increases VWF levels in plasma in a temporary manner. This treatment strategy is applied not only in VWD type 1 but also in other disorders, such as mild hemophilia A and primary platelet function disorders. To investigate the effect of DDAVP treatment on the extent of glycosylation of VWF with O-linked sialylated T antigen, we analyzed pre- and post-DDAVP samples of 18 patients who had received DDAVP as part of routine patient care. These included a subset of the VWD type 1 patients presented in Figure 5 (n = 11), VWD type 2A (n = 2), VWD type 2N (n = 1), hemophilia A (n = 1), and primary platelet function disorder (n = 3). VWF levels rose in all patients treated with DDAVP (Figure 6A), with mean VWF levels at 0.6 ± 0.5 U/mL before treatment and that increased up to 1.6 ± 0.7 U/mL 60 minutes after DDAVP treatment (P < .001). In 2 of the samples tested, the extent of PNA binding to VWF after neuraminidase incubation was slightly, if at all (< 5%),
controls
0.25
0.50
1.00
50
0.75
post-DDAVP
200
250
200
post-DDAVP

Figure 5. PNA binding correlates with propeptide/VWF ratios. (A) VWF propeptide and mature VWF antigen levels were determined for healthy volunteers (n = 24) and VWD type 1 patients (n = 32) depicted in Figure 4, and the molar ratio propeptide over VWF was calculated as described. Difference between both groups was tested using the unpaired 2-tailed t test with the Welch correction applied. (B) Values for relative PNA binding obtained for VWD type 1 patients were plotted versus the corresponding propeptide/VWF ratios. The drawn line was obtained by linear regression analysis. Correlation was found to be significant (Spearman rank = 0.50; P = .004). Bars represent mean values.

Figure 6. Reduced PNA binding to post-DDAVP VWF. For a selection (n = 11) of the VWD type 1 patients (Figure 4) as well as VWD type 2A (n = 2), VWD type 2N (n = 1), hemophilia A (n = 1), and storage-pool disease (n = 3) patients, VWF antigen levels (A) and PNA binding (B) were determined in plasma samples that were taken prior or 60 minutes after DDAVP treatment. Mean VWF levels were 3.04 ± 0.46 U/mL and 1.55 ± 0.74 U/mL before and after DDAVP, respectively (P < .001). Mean PNA binding was 131% ± 45% and 85% ± 33% (P < .001). For 2 patients, changes in VWF antigen levels (C-D; right axes) and PNA binding (C-D; left axes) were followed in time.
structure that associates with VWF plasma levels. Of importance, our data do not distinguish whether this structure should be considered as a determinant, or whether it only correlates with VWF antigen levels. Additional studies are required in this respect. Other glycosylation-related elements that have been reported to influence VWF levels include ST3Gal-IV transferase (which adds sialyl groups to terminal galactose residues), α(1,2)-fucosyltransferase (which adds terminal fucose to glycans, and is encoded by the Secretor-locus), and the ABO blood group structures, which are added to N-linked carbohydrates.12,13,38

The variation in PNA binding in relation to VWF levels was explored in more detail using 2 distinct patient groups. One group consisted of a heterogeneous but well-characterized patient cohort with liver cirrhosis and elevated VWF levels (up to 18.6 U/mL; Figure 3 and Lisman et al17). In this group, high levels of VWF correlated with reduced PNA binding, suggesting a low occupation with Gal-(β1-3)-GalNAc. Moreover, this low occupation correlated with the severity of the disease (Figure 3B). It remains to be investigated whether this reduced glycosylation of VWF with sialylated T antigen results from disease-related impairments in the glycosylation pathway, or that VWF production is too high to allow full glycosylation with this carbohydrate structure. Alternatively, a prolonged survival of the VWF molecules in the circulation due to impaired hepatic clearance may result in prolonged exposure to glycosidases that remove O-linked sugars. However, this option seems less likely since (1) one would expect sialyl groups to be removed prior to removal of O-linked glycans, and no PNA binding prior to neuraminidase treatment could be detected, and (2) to our knowledge no such glycosidases have been reported to be present in plasma. Another explanation for our observations could be that the sites for O-linked glycosylation are used for linking of other non–T-antigen structures. Such changes in O-linked glycosylation occur for instance in the so-called Tn syndrome.36 In this syndrome, there is reduced polypeptide β1-3-galactosyltransferase (T-synthese) activity.36,37 Consequently, the O-linked carbohydrate structure consists predominantly of the Tn antigen (GalNAc 1-Ser/Thr) instead of the T antigen, and this Tn antigen is not recognized by PNA. Of interest, platelet VWF of patients with the Tn syndrome reacts differently with anti-VWF antibodies when assessed via crossed immunoelectrophoresis,38 underscoring the notion that O-linked glycosylation is an essential element of VWF biology. Of note, mice genetically deficient for T syntheae indeed lack T-antigen–bearing proteins, and these knock-out mice display defective angiogenesis and fatal embryonic hemorrhage.39

The second group of patients with aberrant VWF levels consisted of VWD type 1 patients in whom antigen levels are reduced (Figure 4A). In contrast to liver cirrhosis patients, we found that VWD type 1 patients were characterized by increased PNA binding in our assay (up to 361%). We considered the possibility that VWF molecules could have been hyposialylated, making them potentially more susceptible for PNA binding. However, since we could not observe any PNA binding in the absence of neuraminidase incubation in any of the samples tested, this possibility seems rather unlikely. Alternatively, increased PNA binding may indeed correlate with an increased number of O-linked sialylated Gal-(β1-3)-GalNAc structures per VWF molecule. This raises of course the question to which residues within the VWF molecule these additional carbohydrate structures are coupled. It is of importance to mention in this regard that structural requirements for O-linked glycosylation are less well defined compared with those for N-linked glycosylation. Dependent on the algorithm for prediction that is used, different amino acids may fulfill the criteria as potential targets for O-linked glycosylation. Thus, the excess of PNA binding could be explained by the presence of O-linked Gal-(β1-3)-GalNAc structures that are coupled to nonpredicted glycosylation sites. On the other hand, it cannot be excluded that certain conditions allow the addition and exposure of Gal-(β1-3)-GalNAc structures on N-linked glycans. Another option that could explain increased PNA binding is that a mutation-associated disturbance of the VWF conformation results in a better exposure of the Gal-(β1-3)-GalNAc element, allowing more efficient PNA binding.

A substantial portion of the VWD type 1 patients was characterized by increased propeptide/VWF antigen ratios (Figure 5A). From several studies, it has become evident that increased ratios in VWD type 1 patients are associated with a reduced survival of endogenous VWF.27,40 Since altered glycosylation may affect protein survival, it was of interest to analyze the relationship between PNA binding and propeptide/VWF ratio. This analysis revealed a linear correlation between both parameters (Figure 5B), pointing to the possibility that increased glycosylation of VWF with O-linked sialylated Gal-(β1-3)-GalNAc results in decreased survival of VWF in these patients. However, our data do not allow for the distinction among reduced survival originating from the molecular defect itself, increased glycosylation with the Gal-(β1-3)-GalNAc, or a combination of both. On the other hand, the contribution of increased Gal-(β1-3)-GalNAc coupling would provide an explanation for the observations that increased clearance of VWF has been found in VWD type 1 patients with unrelated genetic defects.27,29,41,42 We are currently investigating whether aberrant glycosylation of VWF with O-linked sialylated Gal-(β1-3)-GalNAc also occurs in other types of VWD or in other patients with reduced VWF levels.

An often-applied treatment protocol to accomplish a temporary rise in VWF levels involves administration of the desmopressin analog DDAVP. This treatment provokes release of VWF that is stored in the endothelial Weibel-Palade bodies. The availability of patient samples before and after DDAVP treatment allowed us to analyze for the effect of VWF storage on glycosylation. Of interest, there was a marked decrease (20%-50%) in PNA binding in 16 of 18 samples tested (Figure 6B). This was observed not only in VWD type 1 patients, but also in non-VWD patients (hemophilia A, storage pool disease), illustrating that it represents a general effect that is not restricted to VWD. Moreover, it provides an alternative explanation for reduced PNA binding to VWF from patients with liver cirrhosis. It cannot be excluded that the increase in VWF levels in these patients is related to acute endothelial damage,17 a condition that results in the release of the Weibel-Palade bodies. The differences in O-linked glycosylation of VWF in pre- and post-DDAVP samples indicate that the glycosylation pattern of VWF stored in Weibel-Palade bodies differs from VWF that circulates in plasma. Whether differences in O-linked glycosylation contribute to the intracellular sorting and/or secretion of VWF is a challenging concept, but awaits further studies.

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**Authorship**

Contribution: C.V.D., H.M.B., and P.G.G. conceived and designed the study, revised the draft, and approved the final paper; T.L., J.C.J.E., F.W.L., J.G., and E.F. contributed patient material and approved the final paper; C.J.M.S. performed experiments, analyzed data, and worked on the draft and final version of the paper; P.J.L. conceived and designed the study, analyzed the data, and worked on the draft and final version of the paper.

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**References**


Variations in glycosylation of von Willebrand factor with O-linked sialylated T antigen are associated with its plasma levels

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